

Cardiovascular Biology and Cell Signalling

The VEGF-induced transcriptional response comprises gene clusters at the crossroad of angiogenesis and inflammation

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Summary

VEGF-A is the major trigger of vasculogenesis and physiologic angiogenesis. We have investigated to which extent the gene repertoire induced by VEGF-A in endothelial cells is distinct from that of other growth factors and inflammatory cytokines. Genes upregulated in human umbilical vein endothelial cells treated with VEGF, EGF or IL-1 were compared by microarray analysis and clusters characteristic for individual or combinations of inducers were defined. VEGF-A upregulated in comparison to EGF a five-fold larger gene repertoire, which surprisingly overlapped to 60% with the inflammatory repertoire of IL-1. As shown by real-time RT-PCR for selected genes, VEGF-induction was mostly mediated by VEGF receptor-2 and the capacity of VEGF-A to induce genes in common with IL-1 largely

depended on activation of the calcineurin/NFAT pathway, since cyclosporin A inhibited this induction. Another angiogenic growth factor, bFGF, did not share a comparable induction of inflammatory genes, but partially induced a small group of genes in common with VEGF-A, which were not regulated by EGF. Thus, the data display that VEGF-A induces a distinct gene repertoire, which, contrasting with other growth factors such as EGF or bFGF, includes an inherent inflammatory component possibly contributing to the cross-regulation of angiogenesis and inflammation as further indicated by the VEGF-mediated induction of leukocyte adhesion. Furthermore, a small group of genes selectively induced by VEGF-A with potential importance for angiogenesis is defined.

Keywords

VEGF-A, endothelial cells, angiogenesis, inflammation, gene repertoire

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Introduction

Vascular endothelial growth factor-A (VEGF-A) is the primary inducer of vascular development during embryogenesis and physiological blood vessel formation in the adult. It is also a causative factor of pathologic angiogenesis associated with a multitude of diseases including cancer, chronic inflammatory diseases and retinopathy (1, 2). VEGF-A is normally produced in tissues in response to low oxygen tension and forms a chemotactic gradient sensed by endothelial cells in preexisting vessels, which respond by growing sprouts leading to the formation of new capillaries (3, 4). Several peptide products are generated by differential splicing from the VEGF-A gene such as VEGF-A₁₂₁,

VEGF-A₁₆₅ and VEGF-A₁₈₉ or the inhibitory variant VEGF-A_{165b}. However, isoform VEGF-A₁₆₅ seems to be the predominant form responsible for major angiogenic effects (5).

The responses induced by VEGF-A in endothelial cells include promotion of survival, proliferation, migration and invasion of surrounding tissue as well as the formation of the three-dimensional vascular tube. Furthermore, VEGF-A induces vascular leakage and is also known as vascular permeability factor (2). These biological effects of VEGF are thought to be mediated mainly by VEGF receptor-2 (VEGFR-2), whereas VEGF receptor-1 (VEGFR-1) transduces only a weak intracellular signal and was initially proposed to function in endothelial cells primarily as a decoy receptor and negative regulator of angiogenesis (6). In

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line with this proposal VEGF-E, a viral VEGF homologue selectively binding VEGFR-2, can reproduce many of the angiogenic effects of VEGF-A in assays *in vitro* and *in vivo* (7), whereas PlGF, which specifically binds VEGFR-1, is much less potent in this respect (8). However, additional data support a synergy of VEGFR-1 and VEGFR-2 in pathologic angiogenesis (9).

The VEGF receptors share certain regulatory mechanisms with other receptor tyrosine kinases such as the platelet-derived and epidermal growth factor receptors. These include receptor dimerization and phosphorylation of the cytoplasmic domain on tyrosine residues, which serve as docking sites for downstream signal transducers (10, 11). EGF and the HER or erbB receptors are the prototypes and probably most studied of growth factor and receptor tyrosine kinase systems (12) of wide importance for the development and proliferation of epithelial and many other cell types. In endothelial cells EGF was described to mediate proliferation (13). However, due to the more specific and distinct roles of VEGF-A in directing endothelial sprouting and tubulogenesis it is to be anticipated that VEGF transduces in part unique signals and upregulates a unique gene repertoire in endothelial cells in addition to genes involved in survival and proliferation.

In addition to VEGF-A, bFGF has been known for a long time as potent stimulator of angiogenesis *in vitro* and *in vivo*. Its exact role in physiological vessel formation has remained controversial (15), but bFGF production by tumour cells can play an important role in tumour angiogenesis (16). bFGF exerts its activity via the receptor tyrosine kinase FGF receptor-1.

Angiogenesis during wound healing and in tumours takes place in an inflammatory surrounding created by immune cells invading the damaged or malignant tissue. Inflammatory cytokines such as interleukin (IL)-1 induce a response program in endothelial cells including a large number of inflammatory genes like cytokines and adhesion molecules involved in various aspects of immune cell recruitment and tissue repair. Inflammatory cytokine receptors such as the IL-1 receptor transduce signals by recruiting adaptor molecules which link to the inhibitor of κ B (I κ B)-kinase complex leading to the release of nuclear factor- κ B (NF- κ B) subunits for nuclear transfer (17). The strong upregulation of the NF- κ B pathway seems to be the major determinant for the inflammatory transcriptional response (18, 19).

To define the genes specifically induced by VEGF-A, which might be involved in specialised functions of the factor not shared by other growth factors and inflammatory cytokines, we have investigated the gene repertoire induced by VEGF-A in endothelial cells in comparison to the repertoire of a more general growth factor, EGF, and an inflammatory cytokine, IL-1. We detect a surprisingly large overlap with the inflammatory repertoire of IL-1 and define a small group of genes specifically regulated by VEGF with the potential to be involved in processes selectively induced by VEGF-A.

Materials and methods

Cell culture and materials

Human umbilical vein endothelial cells (HUVEC) were isolated and cultured as described previously (14) and used between passage 3 and 5. In short, HUVEC were cultured in M199 medium

(Invitrogen, Carlsbad, CA, USA) supplemented with 20% SCS (HyClone, Logan, UT, USA), 50 μ g/ml ECGS (Technoclone, Vienna, Austria), 1 U/ml heparin (Roche Diagnostics GmbH, Mannheim, Germany), 2 mM glutamine, 100 U/ml penicillin and 1 mg/ml streptomycin (BioWhittaker, Verviers, Belgium). Cells were seeded into gelatine-coated six-well cell culture plates at 1×10^6 cells/well and grown to density for four days without further medium change. Then cells were induced by addition of 100 ng/ml VEGF-A₁₆₅, VEGF-E, PlGF-1, IL-1 or 50 ng/ml EGF, respectively, for time periods of 30 to 360 min. When indicated cells were preincubated with CsA at 1 μ g/ml for 30 minutes (min) before addition of the factors. Recombinant human VEGF-A₁₆₅ was obtained from PromoKine (Heidelberg, Germany) or PeproTech (Rocky Hill, NJ, USA), VEGF-E and PlGF-1 from Reliatech (Braunschweig, Germany), bFGF from PeproTech. IL-1 α was purchased from Biosource (Nivelles, Belgium) and EGF from PromoKine. Cyclosporin A (CsA) was kindly provided by the Novartis Research Institute (Vienna, Austria).

RNA preparation

After incubation with the indicated factors, cells were treated with RNAlater (Ambion, Austin, TX, USA), shortly washed with DEPC-treated water and RNA was extracted with Trizol (Invitrogen), according to the instructions of the manufacturer.

Affymetrix microarray hybridisation

Preparation of cRNA, hybridization to the human HG-U133 or HG-U133 Plus 2.0 GeneChip set (Affymetrix, Santa Clara, CA, USA), and scanning of the arrays were carried out according to the manufacturer's protocols (20) as described previously (21). Data were analysed with GeneChip software (MAS 5.0, Affymetrix), and normalised to "Selected Probe Sets" (100 housekeeping genes pre-selected by Affymetrix in a mask file) and a target signal of 2000. Gene expression changes were calculated as the ratio of induced cells to uninduced control cells. Sets of data for two independent experimental series using the HG-U133 and HG-U133 Plus 2.0 GeneChip sets, respectively, have been submitted to NCBI's Gene Expression Omnibus (GEO) (22), accession number GSE10778 and GSE15464. A list of the 59 genes most strongly upregulated by VEGF-A in both experimental series is provided as Supplementary Table 1 (available online at www.thrombosis-online.com).

Real-time RT-PCR analysis

Two μ g of total RNA were reverse transcribed into cDNA (SuperscriptTM II RT, Invitrogen) according to instruction of the manufacturer, using oligo-dT primers. Real-time PCR was used to monitor specific gene expression using SybrGreen detection and a Light Cycler instrument (Roche Diagnostics GmbH) according to the manual. As internal standard β 2-microglobulin mRNA was used for normalisation. Primers were designed using the program Primer3 (23). Sequences of the oligonucleotide primers used are listed in Supplemental Table 2 (available online at www.thrombosis-online.com). To choose sense and antisense primers annealing to different exons, the genomic organisation of the genes was obtained from the University of California, Santa Cruz Human Genome Browser (24).

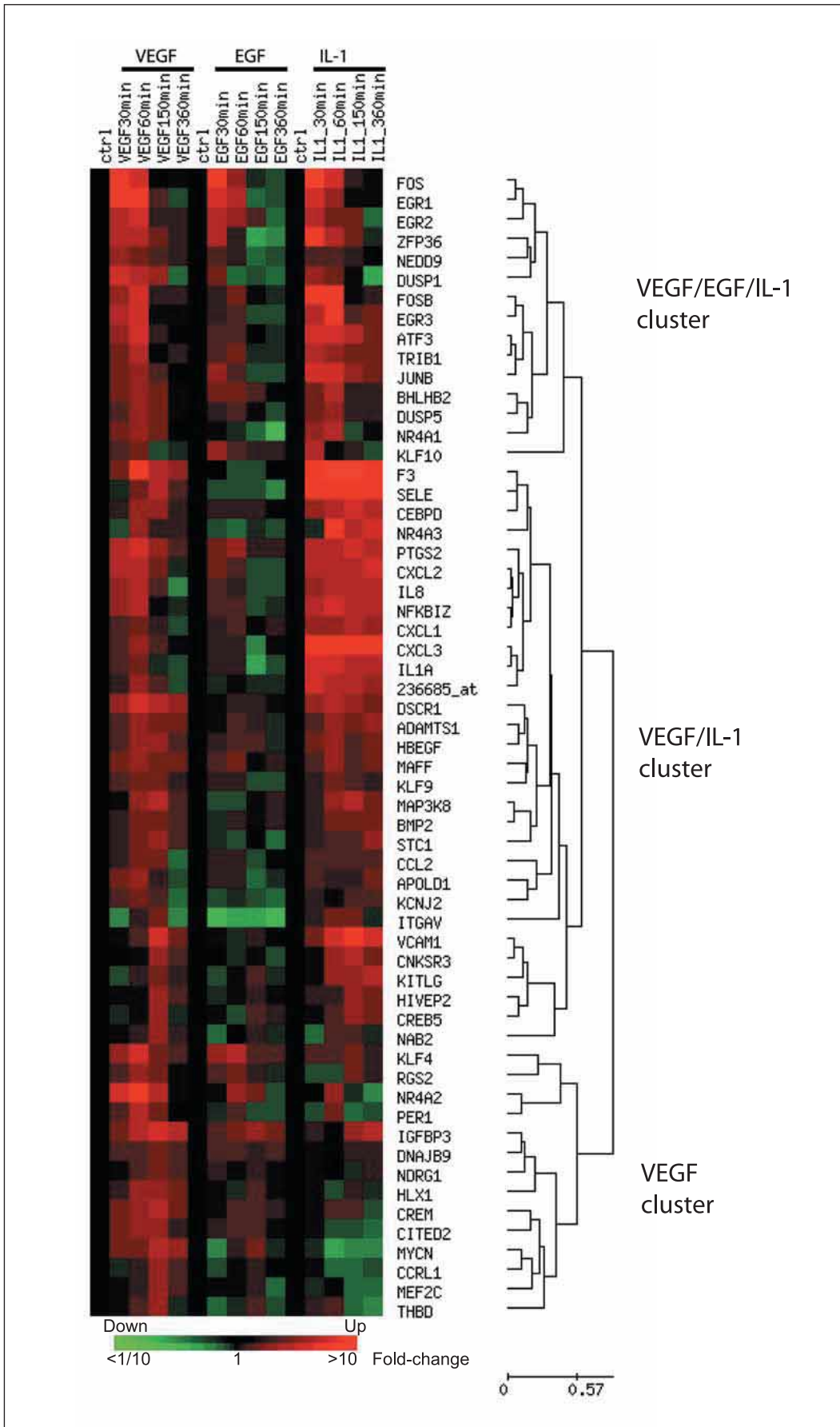


Figure 1: VEGF-A induced genes compared to IL-1 α and EGF induction patterns in HUVEC. The hierarchical clustering of all genes induced in HUVEC by VEGF-A, more than three-fold at one of the indicated time points, is displayed as a heatmap in comparison to the IL-1 α and the EGF gene expression pattern. Genes were clustered with EPCLUST (<http://www.ebi.ac.uk/microarray-srv/EP/>) using Pearson's correlation coefficient as distance measure and average linkage. Induction of expression is indicated by shades of red, repression by shades of green indicating approximately 10- to 10⁻¹-fold regulation. To visually separate the induction patterns of the individual factors, the null control value (ctrl) is shown in black before the respective induction.

Clustering analysis

First, genes as represented by Affymetrix “probe sets” showing absolute calls of „absence“, difference calls of „no change“ as determined by the Affymetrix MAS 5.0 software, less than three-fold upregulation or a signal intensity of less than 60 for all VEGF-induced data points were excluded from further analysis. Then the genes selected for more than three-fold upregulation by VEGF in two independent induction series were subjected to real-time RT-PCR analyses to confirm over three-fold upregulation for at least one time point and genes displaying lower regulation were again abandoned. For the remaining fifty-nine genes cluster analysis was performed using the Expression Profile data CLUSTERing and analysis (EPCLUST) program (25) using linear correlation based distance (Pearson, centered) and average linkage (UPGMA).

Flow cytometry

HUVEC stimulated for six hours (h) with VEGF-A₁₆₅, IL-1 or EGF were harvested following short trypsin treatment, washed and incubated with PBS containing 5% FCS for 15 min. Then 5 µg/ml mouse anti-human VCAM1 antibody (R&D systems, Minneapolis, MN, USA) was added and the cells incubated for 30 min on ice. Samples were washed and incubated for another 30 min with FITC-labeled secondary anti-mouse antibody (Dako, Denmark). After a final washing step, flow cytometry was performed using a FACSCalibur (Becton-Dickinson, San Jose, CA, USA). The number of VCAM1-positive cells was deduced in comparison to uninduced cells.

Adhesion assay

HUVEC induced for 4, 6 or 8 h with VEGF-A₁₆₅, IL-1 or EGF were incubated with 3,3'-diiododecyl-oxacarbocyanine perchlorate (DiO)-labeled (Invitrogen) HL-60 cells on a shaker for 30 min (15 min horizontal, 15 min vertical shaking) at 37°C in a 5% CO₂ atmosphere. Non-adherent HL-60 cells were removed by washing with phosphate-buffered saline and adhering HL-60 cells and HUVEC were detached by trypsin treatment. The percentage of DiO-labeled HL-60 cells in the cell mixture was determined by flow cytometry using a FACSCalibur.

Results

A VEGF-A-specific gene signature: clustering of VEGF-induced genes

To delineate genes induced by VEGF-A and involved in VEGF-specific functions during angiogenesis and/or endothelial differentiation we comparatively analysed the gene repertoires upregulated in endothelial cells by VEGF-A, EGF or IL-1. The selection of these factors was based on the assumption that VEGF-A, the main trigger of vasculogenesis and angiogenesis, would induce gene clusters in addition to the gene repertoire upregulated by EGF, a growth factor which promotes proliferation in endothelial cells and is a general component of the growth supplements used for primary endothelial cell cultures (26). The VEGF-induced genes should further be distinguished from the IL-1 response, which should primarily include genes involved in endothelial inflammation.

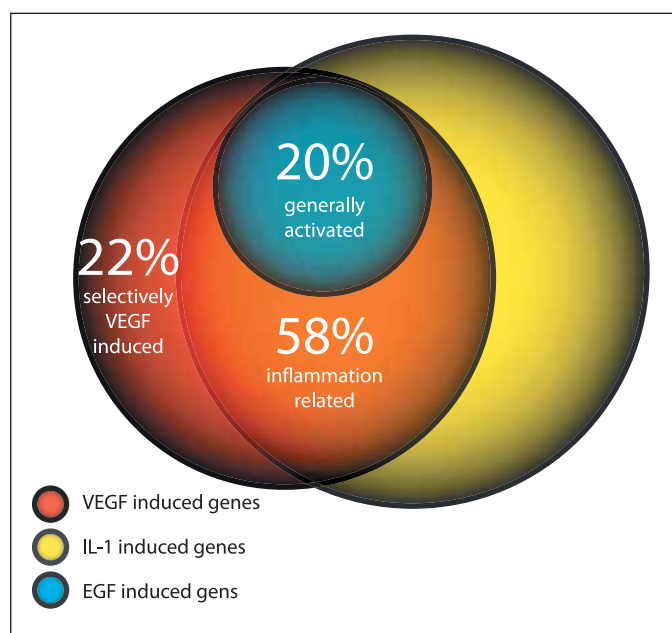


Figure 2: Venn Diagram schematically displaying the percentage of genes in each of the three clusters. Percentage numbers are calculated by comparing the number of genes in the respective groups (VEGF/EGF/IL-1 or generally activated cluster; VEGF/IL-1 or inflammation-related cluster; VEGF-specific cluster) to the total number of VEGF-induced genes. As indicated by the diagram, the number of genes induced by IL-1 is higher than the number of genes induced by VEGF-A. EGF induced a much smaller repertoire than VEGF-A.

Quiescent HUVEC were treated in parallel with VEGF-A or EGF for time periods ranging from 30 to 360 min and total RNA was isolated from the cultures and subjected to Affymetrix microarray analysis. The obtained results were analysed including a separate comparable experiment with IL-1 using identical culture conditions and the same Affymetrix chips (27). Fifty-nine genes were selected to be reproducibly upregulated by VEGF-A more than three-fold as described in *Methods* (see Supplementary Table 1 available online at www.thrombosis-online.com).

The clustering of the gene repertoire induced by VEGF-A more than three-fold is displayed in Figure 1. Two smaller clusters of genes, one regulated similarly by VEGF-A, EGF or IL-1, another preferentially by VEGF-A, and a larger cluster of genes induced by VEGF-A as well as IL-1 can be distinguished. The distribution of the genes in the VEGF-specific, the VEGF/IL-1 and the VEGF/EGF/IL-1 clusters is schematically indicated in the Venn diagram of Figure 2. The regulation of all genes of the VEGF-specific gene cluster and of about half of the genes of the other two clusters were further tested by real-time RT-PCR using RNA from three independent induction experiments and found to closely resemble the results of the microarray analysis (Table 1).

The VEGF-selective gene cluster defines a group of genes induced by VEGF-A, but not by EGF or IL1

Whereas the VEGF/EGF/IL-1 cluster contains to a large extent immediate early genes reaching peak levels between 30 and 60 min of induction such as the transcription factors FOS, JUNB

Group	Relative VEGF induction	At timepoint	Gene symbol	Induction by bFGF	VEGF inducibility in presence of CsA
SVI (VEGF)	1300	60	NR4A2	- (26)	- (260)
	120	60	EGR3	+ (25)	+ (38)
	13	60	IGFBP3	-	- (3)
	7	60	HLX1	++ (5)	+++ (7)
	2	60	CREM	+++ (2)	+
	6	150	PER1	++ (3)	n/d
	4	150	MEF2C	+ (2)	++ (3)
	2	150	CCRL1	+	-
	2	150	THBD	+	+
	36	360	NDRG1	+ (9)	++ (23)
	19	360	DNAJB9	n/d	n/d
5	360	MYCN	++ (3)	+++ (4)	
IR (VEGF + IL1)	225	30	FOSB	- (25)	++++ (1240)
	105	30	ATF3	- (2)	+++ (92)
	51	30	CXCL2	-	++ (27)
	30	30	NEDD9	- (3)	++ (17)
	11	30	KCNJ2	- (3)	+ (5)
	9	30	NFKBIZ	-	+++ (7)
	80	60	DSCR1	-	-
	40	60	IL8	- (2)	+++ (34)
	17	60	PTGS2	- (2)	- (4)
	10	60	ITGAV	-	- (2)
	100	150	F3	- (2)	++ (72)
	4	150	CNKSR3	- (2)	+ (2)
	380	360	VCAM1	-	- (60)
	60	360	SELE	-	+++ (70)
16	360	STC1	- (4)	+ (6)	
GA (VEGF+IL1+EGF)	130	30	EGR2	++ (73)	+++ (88)
	35	30	FOS	+++ (35)	++++ (192)
	25	30	EGR1	++++ (60)	++++ (39)
	8	60	BHLHB2	+ (3)	++++ (8)
	4	60	KLF4	++ (3)	n/d

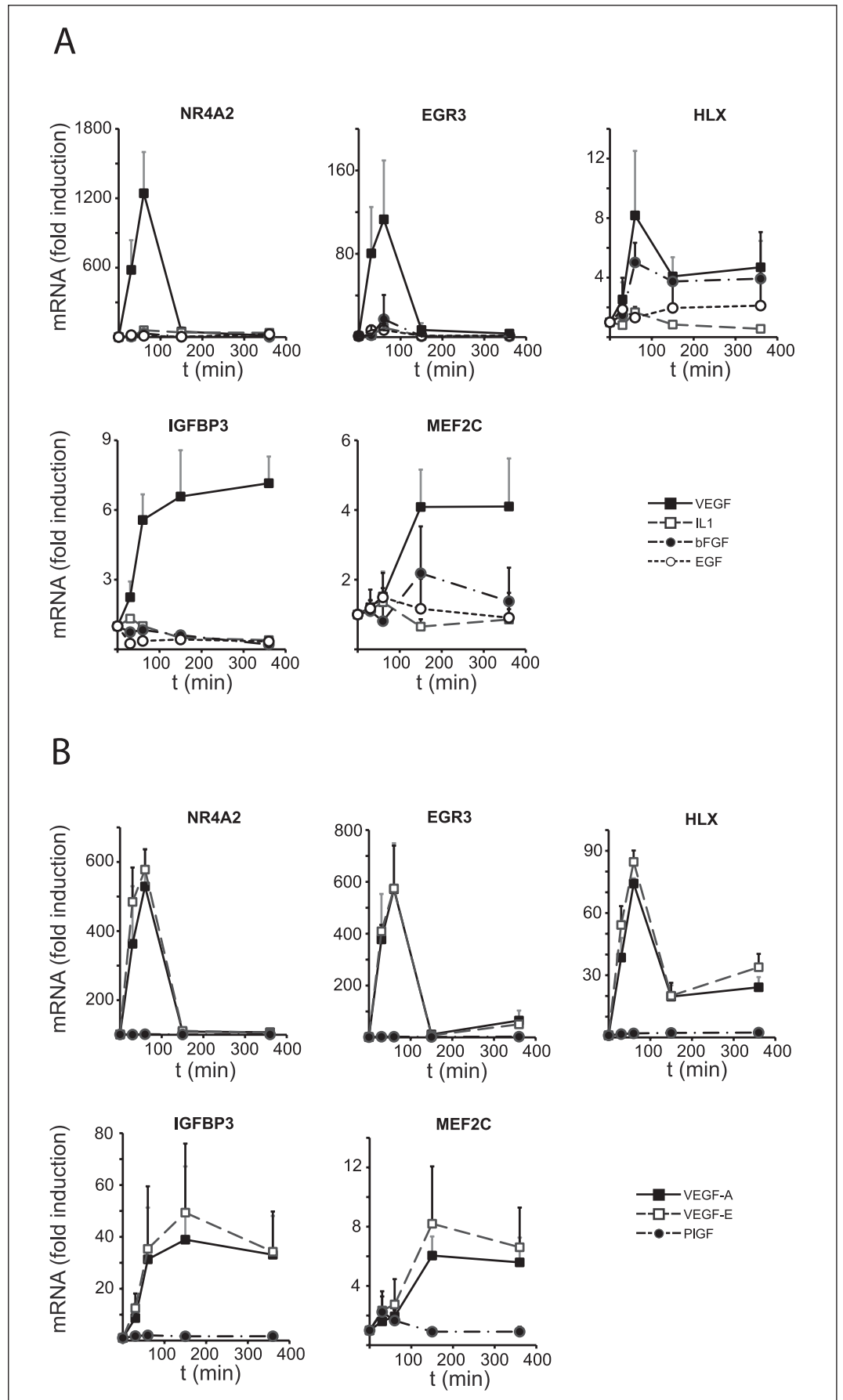
Relative mRNA induction by VEGF is given in -fold of uninduced control. Time points displayed are those when peak levels were observed for the respective mRNA and are given in minutes following induction. For bFGF induction and VEGF inducibility in presence of CsA the percentage of the comparable VEGF value is indicated by: - <25%; + 25-50%; ++ 50-75%; +++ 75-100%; ++++ >>100% of VEGF induction. The values in parentheses display the approximate -fold upregulation observed in comparison to the corresponding untreated control values. Values are given only for at least two-fold or larger changes. SVI, selectively VEGF induced; IR, inflammation related; GA, generally activated; n/d, not determined.

Table 1: Real-time RT-PCR based grouping of VEGF-induced genes displaying bFGF induction and CsA inhibition. Genes were grouped according to inducibility by VEGF-A, IL-1 and EGF. Genes representing individual groups were further sorted in relation to 1) fold induction and 2) the time point when maximum induction is observed. Furthermore, induction by bFGF is shown as percentage of the VEGF-A induction, and the capacity of VEGF-A to induce the genes in the presence of CsA is depicted. Data display the average from at least three independent experiments.

and EGR-1, the VEGF-specific gene cluster comprises rather genes upregulated with peak values between 60 and 360 min and includes transcription factors, intracellular signalling molecules as well as secreted proteins and receptors. In the VEGF-specific group the earliest and most highly VEGF-upregulated genes are the transcription factors NR4A2 (Nurr1) and EGR-3, which could function in the induction of secondary genes of importance for angiogenesis. It further includes genes with more delayed induction kinetics such as the transcription factors HLX1,

MEF2C and PER1, the secreted IGFBP3 and the chemokine receptor CCRL1 (Fig. 3A and Table 1).

To establish whether the upregulation of these genes is mediated via VEGFR-2 or VEGFR-1 we used further VEGF-E as a specific trigger of VEGFR-2 and PIGF as a factor binding specifically to VEGFR-1. As shown in Figure 3B, VEGF-E was as potent as VEGF-A in the upregulation of VEGF-cluster genes, whereas PIGF had no significant effects.



The VEGF/IL-1 gene cluster comprises the majority of VEGF-induced genes

Surprisingly the largest group is the gene cluster upregulated by VEGF-A as well as IL-1, but not by EGF. This cluster contains a heterogeneous group of genes displaying peak values between 30 to 360 min. It comprises a whole group of classical inflammatory genes such as CXCL2, IL8, PTGS2 (COX-2), tissue factor and the adhesion molecules VCAM1 and E-selectin. Whereas VEGF-A induction of IL8, PTGS2 and tissue factor is largely comparable to the induction rate observed for IL-1, induction of VCAM1, E-selectin and CXCL2 by VEGF was approximately 10-fold and 100-fold less pronounced than IL-1 induction, respectively. In addition transcription factors such as ATF3, members of cytoplasmic signalling pathways such as NEDD9 and NFKBIZ and the ion channel KCNJ2 belong to this cluster (Table 1 and Supplementary Fig. 1A available online at www.thrombosis-online.com).

To test for the involvement of VEGFR-2 in the upregulation we evaluated VEGF-E and PlGF effects on selected inflammatory genes. Again VEGF-E could completely or in part reproduce VEGF-A effects, whereas PlGF did not display detectable activity (Supplementary Fig. 1B available online at www.thrombosis-online.com).

To confirm that also the corresponding inflammatory proteins are upregulated in the cells, we have analysed VCAM1 expression by flow cytometry. As shown in Figure 4, VCAM1 expression was significantly induced by VEGF-A on the surface of the cells to about 25% of the level observed following treatment with IL-1. This correlates well with the observed VCAM1 mRNA induction (Supplementary Fig. 1A available online at www.thrombosis-online.com). In contrast, EGF did not induce any detectable VCAM1. Furthermore, we have tested whether VEGF-A induction would also mediate corresponding leukocyte adhesion in an assay using HL60 cells. Indeed, treatment of HUVEC with VEGF-A induced a significant increase in the adhesion of HL60 to the HUVEC monolayer. Adhesion was about 15% of the level obtained with IL-1. Again EGF did not induce increased adhesion.

The upregulation of the majority of the VEGF-specific and inflammatory cluster genes depends on the transcription factor NFAT

Since we have previously described that VEGF-A strongly induces a pathway via PLC- γ and calcineurin/NFAT activation (14, 28), which is in accordance with the reports of others (29–31), we have been interested to determine, whether the upregulation of the VEGF/IL-1 cluster is due to NFAT activation and distinguished from the upregulation of the VEGF-specific and the VEGF/EGF/IL-1 cluster. Alternatively, it would also be possible, that similar to the induction by inflammatory cytokines (18), VEGF-A could use NF- κ B for the upregulation of the VEGF/IL-1 cluster genes. To test the involvement of calcineurin/NFAT we have used a specific inhibitor of calcineurin, cyclosporin A (CsA) (32). The effects of CsA on the upregulation of selected VEGF cluster genes is displayed in Figure 5 and summarised in Table 1. The data clearly show that the VEGF induction of most of the genes of the VEGF-specific cluster can be inhibited by CsA, which is similar to the inhibition of VEGF/IL-1 cluster

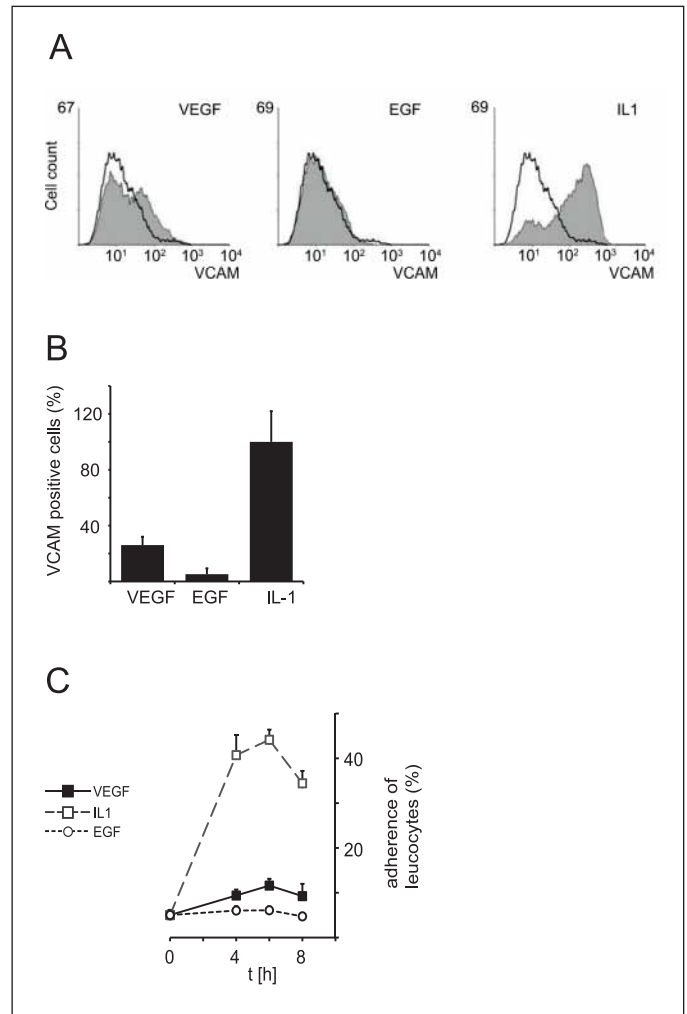


Figure 4: Expression of VCAM1 and adherence of leukocytes to monolayers of VEGF-induced HUVEC. A) Flow cytometry analysis displaying VCAM1 expression on VEGF-A- or IL-1-induced HUVEC. Cells were treated VEGF-A, IL-1 or EGF for 6 hours, harvested and stained with anti-VCAM1 antibodies and corresponding FITC-labelled secondary antibodies. Filled histograms display treated, open histogram uninduced cells. B) Quantification of VCAM1-positive cells. The number of VCAM1 expressing cells was determined from the flow cytometry data by setting the appropriate gate in comparison to uninduced cells. C) Assay displaying adhesion of HL60 cells to VEGF-A- or IL-1-induced HUVEC. HUVEC treated for 4, 6 and 8 hours with VEGF, IL-1 or EGF were incubated with DiO-labelled HL60 cells. The number of DiO-labelled adherent HL60 cells were determined following detachment of all cells by flow cytometry and displayed as percentage of total cells. Mean values and standard deviations are calculated from two experiments performed with triplicate wells.

genes (Supplementary Fig. 1B available online at www.thrombosis-online.com). In contrast, none of the genes inducible by all three factors could be inhibited, but rather displayed in most cases an enhancement of their VEGF-induction in the presence of CsA (Table 1). This shows that calcineurin/NFAT does not contribute to, but rather negatively regulates the induction of the immediate early genes upregulated by all three factors. However, NFAT is an important component in the induction of the major-

ity of the genes of the VEGF/IL-1 cluster as well as the VEGF-specific cluster. To exclude a significant contribution of NF- κ B we in parallel tested a potential cytoplasmic/nuclear translocation of the p65/RelA subunit of NF- κ B in response to VEGF-A (Supplementary Fig. 2 available online at www.thrombosis-online.com). In line with our previous results displaying absence of I κ B degradation following VEGF-A treatment (28), no significant translocation of NF- κ B was observed.

bFGF does not upregulate inflammatory VEGF/IL-1 cluster genes, but part of the VEGF cluster genes

In addition to VEGF-A, bFGF is a growth factor with strong capacity to induce angiogenesis *in vitro* and *in vivo*. Although the extent of its participation in physiological angiogenesis is not clarified, it has been described as a major contributor to pathologic tumour angiogenesis. Therefore we have evaluated to which extent bFGF would be able to induce genes of the selected VEGF repertoire by real-time RT-PCR. As summarised in Table 1, bFGF, as expected, upregulated all generally induced genes. It could also induce in part VEGF-specific cluster genes (Fig. 3A); however, it was unable to significantly induce any of the inflammatory VEGF/IL-1 cluster genes (Supplementary Fig. 1 available online at www.thrombosis-online.com). This is in accordance with the possibility of a functional overlap of VEGF-A and bFGF in the induction of specific angiogenesis-related responses.

Discussion

In comparison to other growth factors there are unique responses mediated by VEGF-A signalling, which comprise the triggering of progenitor cell differentiation towards the endothelial cell lineage, directing tip cell filopodia extension (3), formation of a three-dimensional vascular tube, and regulation of vascular permeability (2). Although several forms of VEGF-A have been described to be produced by differential splicing, which may act in concert, it is generally assumed that isoform VEGF-A₁₆₅ is the major form, which seems to be to a large degree responsible for gene regulation important for vasculogenesis and angiogenesis of blood vessels (5). The functions exerted by VEGF-A appear not to be inducible to the same extent and quality by other growth factors and cytokines. It has therefore to be anticipated that the unique properties of VEGF-A are mediated via a distinct capacity to signal and to induce a unique gene signature in comparison to other factors. VEGF-A has been shown to bind to VEGFR-1 as well as VEGFR-2, both receptors being expressed on HUVEC (33). However, whereas binding to VEGFR-2 results in the strong induction of downstream signalling pathways such as PLC- γ and PI3-kinase, VEGFR-1 can only trigger much weaker intracellular signals (8) and little gene regulation (see below). Given the fact that the VEGFR-2 contains 19 tyrosine residues in its cytoplasmic domain, which in part seem to be phosphorylated in a dynamic way dependent on the activity state of the cell (11),

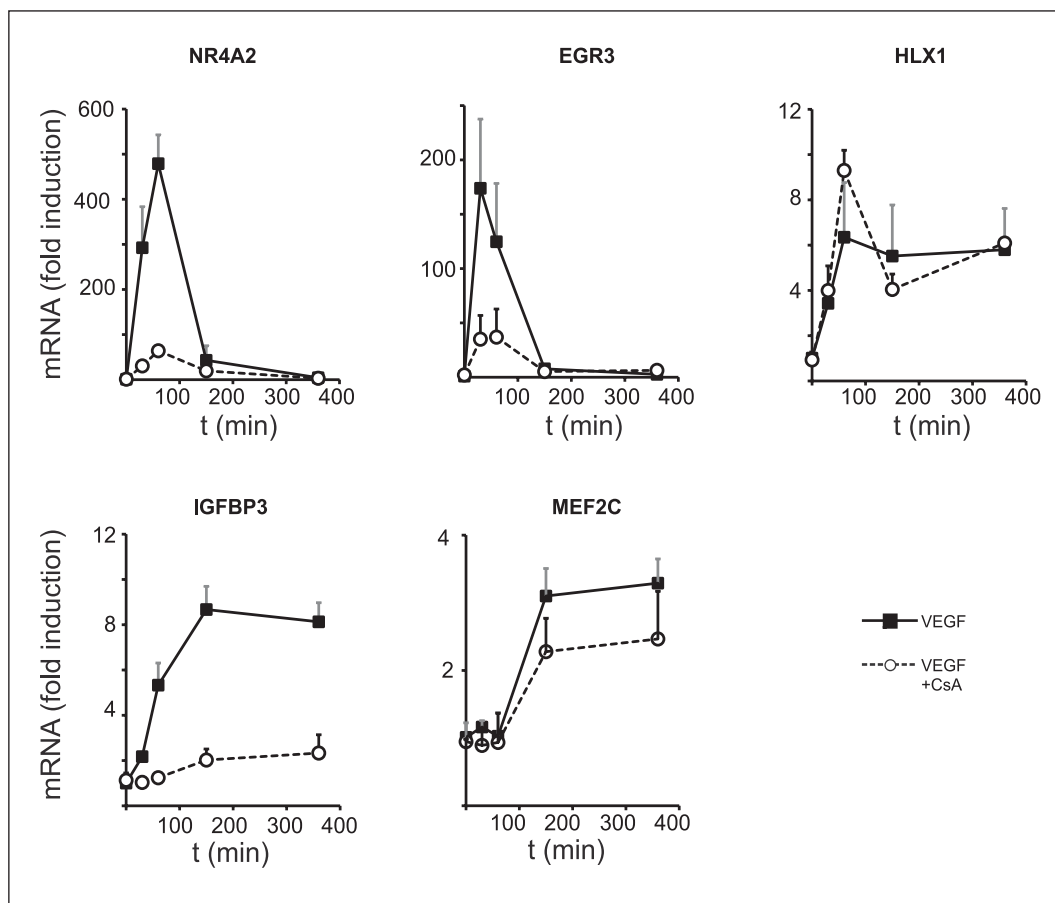


Figure 5: Effect of the calcineurin inhibitor CsA on the VEGF-mediated induction of selected genes of the VEGF-specific cluster. HUVEC were seeded in six-well plates and grown to confluence. Following preincubation with CsA (1 μ g/ml) for 30 minutes the cells were induced with VEGF-A (100 ng/ml) for 0.5, 1, 2.5 and 6 hours. Real-time PCR data are obtained and displayed as described for Figure 3.

it is likely the qualitatively and quantitatively differential docking of signal transducers which mediates a unique gene signature and responses important for specific VEGF-A₁₆₅ functions.

Indeed, a group of about 20% of the VEGF-induced genes were selectively induced by VEGF-A and not at all or to a much lower extent by EGF or IL-1. Addressing the question whether the upregulation of these genes was mediated by VEGFR-2 and/or VEGFR-1 we have tested by real-time RT-PCR the regulation of selected genes in response to VEGF-E and PlGF, two factors binding selectively to VEGFR-2 or VEGFR-1, respectively. Clearly, VEGF-E resulted in an upregulation of these genes comparable to VEGF-A, whereas PlGF had little if any effect on gene expression suggesting that VEGFR-2 triggering by itself can mediate the majority of the observed transcriptional response. We propose that these genes regulated by VEGF-A via VEGFR-2 are induced to fulfill a function exerted predominantly by VEGF-A, but not by EGF or IL-1. The most strongly upregulated genes in this group are NR4A2 (Nurr1) and EGR-3. NR4A2 is a member of the NR4A subfamily of orphan nuclear receptors and has,

among others, been previously implicated in nerve cell development (35). It could be involved in processes of axon guidance as well as angiogenesis via regulation of neuropilin levels (36), EGR-3 is unique among the EGR family, in that it is preferentially upregulated by VEGF and partially sensitive to NFAT inhibition, whereas EGR-1 and EGR-2 seem to be more generally activated and induced without NFAT contribution. EGR-3 has been previously described as an important regulatory factor in T lymphocytes (37) and certain nerve cells, e.g. to be essential for adaptation to stress and novelty (38). It should be interesting to define the secondary response genes regulated by NR4A2 and EGR-3, as, while this work was in progress, it has been reported that NR4A2 as well as EGR-3 induction are a prerequisite for VEGF-mediated endothelial proliferation, migration and tubulogenesis *in vitro* (39).

Three additional transcription factors selectively induced by VEGF-A are the homeobox gene HLX1, the MADS box factor MEF2C and the period family member PER1. In accordance with the proposed importance of the VEGF cluster genes for angiogenic processes, our recent results support that HLX1 is a specific regulator of the expression of guidance receptors involved in endothelial sprouting (Testori et al., in preparation). MEF2C has been previously shown to be required for vascular development (40) and PER1 extends the list of VEGF-regulated genes, which reported functions in nerve cells (41). Another gene of interest from the VEGF-specific group encoding a secreted protein is IGFBP-3. Although several available reports are conflicting in regard of pro- or antiangiogenic effects of IGFBP-3, two recent reports suggest a role for IGFBP3 in endothelial progenitor cell migration, differentiation and capillary formation (42).

The contribution of inflammatory mediators to angiogenic processes is a debated issue. There is considerable evidence of cross-regulation of inflammation and angiogenesis in many pathologies (43). Increased angiogenesis is frequently observed in chronic inflammatory diseases and excessive angiogenesis and inflammation is the hallmark of malignant tumours. Inflammatory cytokines such as IL-1 as well as VEGF-A may therefore play an important role in the complex link between inflammation and angiogenesis. It was therefore of interest to investigate to which extent the inflammatory signalling cascades and gene signatures overlap with or are distinct from angiogenic VEGF-mediated signalling and function.

Surprisingly, 60% of the VEGF cluster genes are IL-1-regulated genes and are not induced by EGF, indicating that the VEGF-A response in contrast to other growth factors contains a significant inflammatory component. It appears that mainly VEGFR-2 is mediating this property of VEGF-A as VEGF-E, but not PlGF, could mediate induction of selected genes from this group. The inflammatory gene cluster encodes proteins with a typical inflammatory function such as PTGS2/COX-2, which via synthesis of prostaglandins plays a prominent role in inflammatory processes (44), or VCAM1, which mediates leukocyte adhesion (45). As we have previously shown it also includes as one of the most strongly VEGF-upregulated genes the tissue factor gene (14, 28), which as a primary initiator of the extrinsic coagulation pathway is an important component of the repair mechanisms of vascular cells after inflammatory or mechanical injury. Whereas some genes such as PTGS2/COX-2 and tissue

What is known about this topic?

- VEGF-A is the major trigger of angiogenesis and vasculogenesis and VEGF-regulated genes have been described by several authors.
- However, it remains so far undefined which part of the gene repertoire induced by VEGF is specifically induced by VEGF-A₁₆₅, necessary for angiogenesis and cannot be induced by other growth factors or cytokines and to what degree the VEGF repertoire overlaps with that of the other factors.
- Furthermore, the major key regulatory transcription factors mediating the VEGF-response and angiogenesis remain largely undefined.

What does this paper add?

- We have directly compared in parallel experiments the gene repertoires induced by the "angiogenesis-inducing" growth factor VEGF-A, the "proliferation-inducing" growth factor EGF and the "inflammation-inducing" cytokine IL-1.
- The obtained data define for the first time
 - i) a group of genes selectively regulated by VEGF-A which we propose to fulfill specific roles for angiogenesis which can not be contributed by the other factors,
 - ii) the data display an unexpected large overlap of the VEGF repertoire with that of inflammatory cytokines (which is in contrast to the EGF-repertoire) suggesting that VEGF has itself some inflammatory capacity,
 - iii) define the transcription factor NFAT as a mediator not only of a major part of the inflammatory component of VEGF induction, but also as an important component of the "VEGF-specific" gene induction and
 - iv) suggest several additional transcription factors as key mediators of specific VEGF effects such as HLX1 and MEF2C.

factor are upregulated to a similar extent by VEGF-A and IL-1, others such as VCAM1 are much stronger induced by IL-1 than by VEGF-A.

To minimise the possibility that culture conditions using sparse cells would induce the upregulation of inflammatory genes, we have used for induction densely grown HUVEC monolayers for which cell to cell contact should mediate contact inhibition via VE-cadherin (46). Although we can not completely exclude that the cell culture conditions would facilitate the induction of inflammatory genes, nevertheless we believe that the data clearly show the principal capacity of VEGF to induce genes with inflammatory functions. In the case of VCAM1, which is involved in immune cell adhesion during extravasation (47), we have demonstrated that the protein is expressed on the cell surface of endothelial cells following VEGF-A induction. Furthermore, significantly increased leukocyte adhesion can be observed as to be expected for cells with upregulated adhesion molecules such as VCAM1.

Taken together it appears that VEGF-A preferentially induces a significant subfraction of inflammatory genes, each to a specific extent, which may vary from IL-1 induction. This may indicate a role of these inflammatory genes for neovascularisation occurring during repair processes. Given the intrinsic inflammatory component in VEGF-A signalling, it is possible, that VEGF-A by itself is capable to promote inflammation, as it may be necessary for efficient tissue repair and angiogenesis. Furthermore, it may be that inflammatory cytokine signalling may considerably lower the threshold for angiogenic stimulation and vice versa, which could facilitate the initiation and progression of chronic inflammatory diseases.

It needs to be mentioned that whereas many of the genes of the VEGF-specific and of the inflammatory cluster are upregulated with peak values between 30 and 60 min after addition of the factors, some genes such as MEF2C and PER1 of the VEGF-specific cluster or VCAM1 and E-selectin of the inflammatory cluster display more delayed induction kinetics with mRNA peak values between 2 and 6 h. It is likely that the induction of these genes involve secondary gene regulatory events presumably requiring some of the transcription factors induced with immediate early kinetics.

We have previously analysed signalling cascades triggered by VEGF-A and EGF in endothelial cells and have shown that a major difference is that VEGF preferentially activates pathways leading via Ca^{++} /calcineurin and PKC/MEK/ERK to transcription factors such as NFAT and EGR-1, respectively. In contrast, EGF was not capable to induce NFAT at all and upregulated EGR-1 in a PKC-independent way, probably via RAS-mediated MEK/ERK activation (14, 28). Given the strong induction of NFAT by VEGF-A and the inability of EGF to induce NFAT, it is likely that NFAT is a predominant factor endowing VEGF-A with the potency to induce a several fold larger gene repertoire when compared to EGF. The sensitivity of the majority of the VEGF- and VEGF/IL-1 cluster genes to the specific calcineurin inhibitor CsA (32) strongly supports this possibility. Given the short preincubation time of 30 min and the rapid VEGF-mediated induction of a major part of the genes within 1–2 h the CsA-mediated inhibition should be due to the direct inhibition of

calcineurin and NFAT activation and the influence of secondary CsA effects as reported after longer incubation periods of 24 h (48) is less likely.

It is well established that inflammatory cytokines such as IL-1 strongly induce the NF- κ B pathway, which is essential for the upregulation of most of the inflammatory response genes (18, 19). It is therefore conceivable that the VEGF/IL-1 cluster genes comprise a group of genes with NFAT as well as NF- κ B binding sites in their promoters. VEGF-A would then preferentially use NFAT and IL-1 NF- κ B for the induction of these genes. Results of a TOUCAN promoter analysis (49) of the genes of the VEGF/IL-1 cluster supported this possibility (B. Schweighofer and E. Hofer, unpublished results).

Another growth factor long known to have a role in repair associated angiogenesis and pathologic tumour angiogenesis is bFGF (15), which in endothelial cells induces signals mainly via the receptor tyrosine kinase FGF receptor-1. Whereas part of the experimental evidence suggests that bFGF induces vascularisation indirectly via upregulation of the VEGF/VEGFR system, other data have shown a direct dependence of tube formation in embryonic explants on bFGF and a synergistic activity of bFGF with VEGF in tumour angiogenesis (15). In our study bFGF was competent to upregulate in part genes from the VEGF cluster, which we propose to fulfill specific roles in angiogenesis.

As shown in Figure 2, about 20% of the VEGF-induced genes were similarly induced by VEGF-A, EGF and IL-1. This group of generally induced genes, which includes for example members of the Fos, Jun and EGR families, are presumably necessary for general responses triggered by all three factors and induced by a wide variety of different growth factors and cytokines. The promoters of these genes are characterized by the presence of a high number of serum response factor (SRF) binding sites (B. Schweighofer and E. Hofer, unpublished observation) suggesting their predominant activation by the MEK/ERK MAP-kinase pathway (50). These immediate early response genes encode mainly transcription factors involved in survival and proliferation responses and/or necessary in part as co-factors to activate secondary transcriptional responses together with the more inducer-specific transcription factors such as NFAT or NF- κ B. For example, it has been shown that AP-1 functions by interacting with NFAT as a necessary factor in the upregulation of NFAT-controlled genes such as IL-2 (51) and we have recently shown that EGR-1 similarly can functionally interact with NFAT to induce tissue factor expression by VEGF-A (14).

From the multitude of genes regulated by VEGF-A (30) this work has defined a small group selectively upregulated by VEGF-A, but not by EGF or IL-1. It is intriguing that this group contains several genes previously implicated in nerve cell differentiation and axon guidance which could provide additional examples for the proposed analogy of endothelial with neuron sprouting and guidance mechanisms (52). Based on the hypothesis that the VEGF cluster genes may have a role in specific functions induced by VEGF-A and essential for endothelial differentiation, angiogenic sprouting and/or tubulogenesis, it is possible that these genes will constitute preferential targets to interfere with angiogenesis as it has already been indicated for some examples by the reports of others (39).

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