Fluid shear stress-induced transcriptional activation of the vascular endothelial growth factor receptor-2 gene requires Sp1-dependent DNA binding

Carmen Urbich[a], Monika Stein[b], Kerstin Reisinger[b], Roland Kaufmann[b], Stefanie Dimmeler[a], Jens Gille[b,c,*]

[a] *Molecular Cardiology, Department of Internal Medicine IV, Theodor-Stern-Kai 7, D-60590 Frankfurt am Main, Germany*
[b] *Department of Dermatology, Klinikum der J. W. Goethe-Universität, Theodor-Stern-Kai 7, D-60590 Frankfurt am Main, Germany*
[c] *Department of Molecular Biology, Max-Planck-Institut für Physiologische und Klinische Forschung, Parkstr. 1, D-61231 Bad Nauheim, Germany*

Received 10 October 2002; revised 16 December 2002; accepted 16 December 2002

First published online 2 January 2003

Edited by Robert Barouki

Abstract Hemodynamic forces play a fundamental role in the regulation of endothelial cell survival. As signaling via the vascular endothelial growth factor (VEGF) receptor-2 pathway has been previously demonstrated to impact endothelial cell survival, we hypothesized that laminar shear stress may facilitate survival in part by inducing VEGF receptor-2 expression. This study shows a time- and dose-dependent upregulation of endothelial VEGF receptor-2 expression by fluid shear stress in microvascular and large-vessel derived endothelial cells. A functional analysis of the 5′-regulatory region of the VEGF receptor-2 promoter localized the shear stress-response element to a sequence between bp −60 and −37 that encompasses two adjacent consensus Sp1 transcription factor binding sites. Constitutive and shear stress-inducible Sp1-dependent complexes are bound to this element, indicating that fluid shear stress-induced transcriptional activation of the VEGF receptor-2 gene requires Sp1-dependent DNA binding. Together, these results suggest that biomechanical stimulation may lead to endothelial cell survival by upregulating VEGF receptor-2 expression.

Key words: Vascular endothelium; Endothelial growth factor receptor; Mechanical stress; Transcription factor; Promoter region

1. Introduction

Physiological and pathological vessel formation is regulated by complementary and coordinated signaling between different growth factors and their receptors [1]. Within the family of endothelium-specific polypeptide growth factors, vascular endothelial growth factor (VEGF, currently also referred to as VEGF-A) is viewed as a prime regulator of blood vessel growth by vasculogenesis and angiogenic sprouting [2].

VEGF is known for its ability to induce vascular permeability, to promote endothelial proliferation and migration, and to act as a critical survival factor for endothelial cells [3,4]. Two structurally related receptor tyrosine kinases, VEGF receptor-1 (VEGFR1, originally known as Flt-1) and VEGF receptor-2 (VEGFR2, formerly termed KDR/Flk-1), are predominantly involved in initiating signaling cascades in response to VEGF [5]. Whereas VEGFR1 may mainly act in a negative fashion by inhibiting VEGFR2 signaling or by functioning as a decoy receptor [6,7], VEGFR2 is thought to mediate the major permeability and growth effects of VEGF [8,9].

While VEGF is secreted by various different cell types, VEGFR2 expression is largely restricted to vascular endothelial cells [10]. In the adult, VEGFR2 is only detectable at relatively low amounts in the vasculature [9,11]. However, VEGFR2 may be markedly upregulated by blood vessels during tumor growth, wound repair, and in inflammatory diseases [12,3]. At the same time, VEGFR2 expression closely correlates with VEGF expression in angiogenic responses. Therefore, suppression of the VEGF/VEGFR2 signaling pathway is being intensely explored as attractive therapeutic avenue to interfere with new blood vessel formation [13].

The coordinated regulation of VEGFR expression is critical for blood vessel formation and angiogenic remodeling [2]. Yet, the stimuli and mechanisms of VEGFR regulation are only partially understood. Whereas hypoxia appears to induce both VEGFR1 and VEGFR2 in vivo [14,15], VEGFR2 expression is not affected by hypoxia in vitro [16,17]. Thus, a paracrine mode via VEGF expression has been proposed, as culture media from hypoxic cells may induce VEGFR2 protein [16], and since VEGF treatment has been shown to induce VEGFR2 expression in cerebral slide cultures [18]. However, this assumption remains controversial, as different in vitro studies fail to show upregulation of VEGFR2 expression in response to VEGF [19,20]. In addition, basic fibroblast growth factor appears to increase VEGFR2 expression [20,21], whereas tumor necrosis factor-α [22] and transforming growth factor-β1 [23] seem to downregulate VEGFR2 expression.

Increased recognition of biomechanical forces as critical stimuli for maintenance of vascular integrity and microvascular remodeling has created great interest in cellular mechanisms that link changes in gene expression to shear stress

*Corresponding author. Fax: (49)-69-6301 3804.
E-mail address: gille@em.uni-frankfurt.de (J. Gille).

Abbreviations: EMSA, electrophoretic mobility shift assay; HDMEC, human dermal microvascular endothelial cells; HUVEC, human umbilical vein endothelial cells; Luc, luciferase; VEGF, vascular endothelial growth factor; VEGFR, vascular endothelial growth factor receptor

0014-5793/02/$22.00
doi:10.1016/S0014-5793(02)03879-6

stimulation [24,25]. We hypothesized that laminar shear stress may facilitate endothelial cell survival by inducing VEGFR2 expression, as fluid shear stress may provide a critical survival stimulus and has been implicated in microvascular remodeling previously [26,27].

The present study reveals that exposure of endothelial monolayers to fluid shear stress induces VEGFR2 expression in a time- and dose-dependent fashion. To determine molecular mechanisms responsible for shear stress-mediated VEGFR2 gene transcription, we utilized 5′-deletional and mutational VEGFR2 promoter-based constructs in transcriptional activation studies. A shear stress-response element was localized to a bp −60/−37 region that contains two adjacent consensus Sp1 transcription factor binding sites. Subsequent electrophoretic mobility shift assays (EMSAs) showed constitutive Sp1-site-dependent complex formation which was inducible upon shear stress exposure. Thus, fluid shear stress-induced transcriptional activation of the VEGFR2 gene is shown to require Sp1-dependent DNA binding.

2. Materials and methods

2.1. Cell culture

Pooled human umbilical vein endothelial cells (HUVECs) and human dermal microvascular endothelial cells (HDMECs) were purchased from PromoCell (Heidelberg, Germany) and cultured at 37°C and 5% CO_2 in Endothelial Basal Medium MV (PromoCell), supplemented with hydrocortisone (1 μg/ml), gentamicin (50 μg/ml), amphotericin B (50 ng/ml), epidermal growth factor (10 ng/ml), and 5% fetal calf serum (FCS) until the fifth passage. After detachment with trypsin, 3.0×10^5 cells were seeded in 60-mm cell culture dishes for 24 h, and were then rendered quiescent by changing to medium containing 1% FCS. HUVECs or HDMECs were exposed to laminar fluid flow in a cone-and-plate apparatus as previously described [28,29]. A constant shear stress of 5, 15, or 45 dynes/cm^2 was used to simulate physiological levels of shear stress.

2.2. Western blot analysis

After exposure to shear stress, endothelial cells were washed twice with cold phosphate-buffered saline, lysed in cold buffer containing 20 mM HEPES, 150 mM NaCl, 0.2% Triton X (v/v), 10% glycerol (v/v), supplemented with proteinase inhibitors antipain (2 μg/ml), aprotinin (2.2 μg/ml), pepstatin A (1 μg/ml), leupeptin (2 μg/ml) and phenylmethylsulfonyl fluoride (1 mM, all from Sigma Chemicals, Deisenhofen, Germany), for 30 min on ice. Protein concentrations were determined with the DC Protein Standard Assay (Bio-Rad, Munich, Germany). Samples containing equal amounts of protein (~10 μg/lane) were boiled at 95°C for 3 min, were then subjected to 8% SDS–polyacrylamide gel electrophoresis (PAGE) and transferred to polyvinylidene fluoride membranes (Millipore, Eschborn, Germany) at 50 V, and subsequently blocked in Tris-buffered saline–Tween 20 containing 5% non-fat milk. The membranes were incubated with the indicated primary antibodies (VEGFR1, clone H-225, VEGFR2, clone A-3, from Santa Cruz Biotechnology, Heidelberg, Germany; Tubulin Ab from LabVision Ltd., Newmarket Suffolk, UK), followed by incubation with horseradish peroxidase-conjugated secondary antibodies (anti-mouse or anti-rabbit IgG, Amersham Biosciences, Freiburg, Germany). The blots were developed using the enhanced chemiluminescence (ECL) detection system according to the instructions of the manufacturer (Amersham).

2.3. Quantitative real-time RT-PCR

Total cellular RNA was isolated from cell cultures grown in 6-well plates by the RNeasy Mini Procedure (Qiagen, Hilden, Germany) after homogenization of cell lysates using the QIAshredder (Qiagen). The LightCycler®-RNA Master SYBR Green I kit was employed for one-step RT-PCR using the LightCycler® Instrument (Roche Molecular Biochemicals, Mannheim, Germany). The reaction mixture contained 100 ng of RNA, oligo primer at 0.4 μM each, and $Mn(OAc)_2$ at 3.25 mM in a total volume of 20 μl. The experimental protocol was adapted to use with the LightCycler®-Control Kit RNA (Roche) as outlined by the supplier. The primers (all from TIB Molbiol, Berlin, Germany) spanning specific exon boundaries were as follows: VEGFR2 as target gene, 5′-ATGCTCAGCAGGATGGAA-3′ (GenBank accession number AF035121; corresponding to 3803–3821) and 5′-TTTGGTTCTGTCTTCCAAAGTT-3′ (4134–4113); human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as reference gene, 5′-GAAGGTGAAGGTCGGAGTC-3′ (GenBank accession number BC025925, corresponding to 63–81) and 5′-GAAGATGGTGATGGGATTTC-3′ (269–288). As external standard method, a common standard curve was obtained by the LightCycler®-Control Kit RNA (Roche) that was included in each LightCycler® run. The standard curve was used to determine the concentration of the VEGFR2 and GAPDH gene. The similar calculated melting temperatures for all primers enabled the use of the same cycling program for all samples.

2.4. Plasmids

Reporter constructs containing 5′-regulatory sequences of the human VEGFR2 gene have been described previously [30]. Briefly, the sequences were inserted into pGL2-Basic vector (Promega, Madison, WI, USA) and named according to the length of the fragment (from the transcription start site) in the 5′ and 3′ directions (−4kb/+296, −164/+268, −77/+268). The encompassing sequence for the −60/+268 construct has been amplified by PCR technique with flanking 5′-*Hin*dIII and 3′-*Xho*I enzyme restriction sites to facilitate directional cloning into the pGL2-Basic vector. The construct carrying two-nucleotide (2-nt) mutations (CC→AA) within the Sp1 consensus sites (−60 *m*/+268) was generated identically, except that a primer was used which included the respective 2-nt mutations. All constructs were sequenced from the 5′- and 3′-ends to confirm orientation and sequence correctness.

2.5. Transient transfection and analysis of reporter gene expression

HUVECs (3.5×10^5, seeded in 60-mm dishes) were transfected with 3 μg of appropriate *firefly* luciferase (Luc) construct and 0.6 μg pRL-TK vector (as internal control *Renilla* Luc vector to normalize for transfection efficiency; Promega) using SuperFect Transfection Reagent (Qiagen, Hilden, Germany). Fourty-eight hours after transfection, control transfectants were left untreated (static controls) and test transfectants were exposed to shear stress (15 dynes/cm^2) for 24 h. The activities of *firefly* and *Renilla* Lucs were measured sequentially from a single sample by the Lumat LB 9507 (Berthold Technologies, Bad Wildbad, Germany), utilizing the Dual-Luciferase® Reporter Assay System from Promega.

2.6. Preparation of nuclear extracts and gel mobility shift analysis

HUVECs were left untreated or were subjected to shear stress for (15 dynes/cm^2) for 24 h. Nuclear proteins were extracted as described previously [31]. The oligonucleotides were synthesized to span the region between −63 bp and −31 bp of the human VEGFR2 promoter: 5′-CGGCCCCGCCCCGCATGGCCCCGCCTCCGCGCT-3′. The mutated probe carried 2-nt mutations (CC→AA) within the Sp1 consensus sites: 5′-CGGCC**AA**GCCCCGCATGGCC**AA**GCCTCCGCGCT-3′. The underlined sequences served as a template for synthesis of the second strand. Radiolabeled double-stranded DNA was synthesized by annealing an oligonucleotide complementary to the underlined sequence listed above (5′-AGCGCGGAGGC-3′), and by extension of the second strand with Klenow fragment, 50 μCi of [α-^{32}P]dCTP, unlabeled dATP, dTTP and dGTP. Unincorporated nucleotides were removed by column chromatography. Cold 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, 10^5 cpm α-^{32}P-labeled probe (~0.5–1.0 ng), 0.1 mg/ml poly[dI:dC] (Sigma), 5 μl of 4×binding buffer (1×buffer: 10 mM Tris–HCl, pH 7.8, 100 mM KCl, 5 mM $MgCl_2$, 1 mM EDTA, 10% [v/v] glycerol, 1 mM DTT) with or without excess of unlabeled competitor, Sp1 consensus-oligonucleotide (Promega), Sp1 or RelA/NF-κB antibody (Santa Cruz). Samples were subjected to electrophoresis on a native 4% PAGE for 2.5 h at 120 V.

2.7. Statistical analysis

Data are expressed as mean ± S.D./S.E.M. from at least three independent experiments. Statistical analysis was performed by Student's *t*-test.

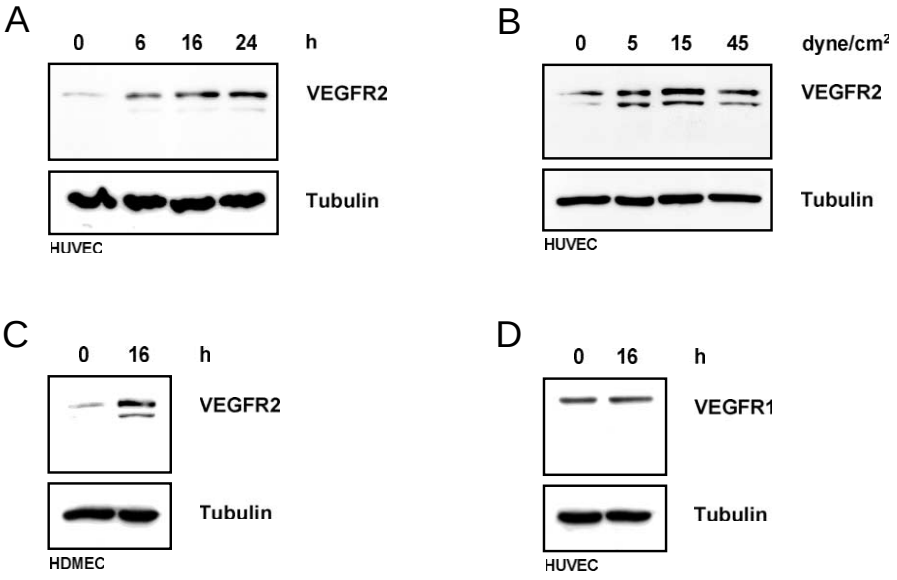

Fig. 1. VEGFR2 expression is regulated by shear stress in large vessel-derived and microvascular endothelial cells. A: Representative Western blot analyses of HUVECs that were grown under static conditions or were exposed to laminar shear stress for 6, 16 or 24 h (15 dyne/cm^2) or (B) were exposed to different shear strengths (5, 15, 45 dyne/cm^2) for 16 h. C: VEGFR2 protein expression by HDMECs either left untreated or subjected to shear stress for 16 h (15 dyne/cm^2). D: VEGFR1 protein expression by HUVECs that were grown under static conditions or were exposed to laminar shear stress 16 h (15 dyne/cm^2). Total cellular protein was separated by 8% SDS–PAGE. VEGFR1, VEGFR2 and tubulin protein were detected by ECL. Comparable results were obtained from at least three independent experiments.

3. Results

3.1. Time- and dose-dependent upregulation of VEGFR2 expression by fluid shear stress in human endothelial cells

To examine the effect of fluid shear stress on VEGFR2 regulation, HUVECs were exposed to shear stress for different time intervals (Fig. 1A). Western blot analyses revealed a substantial increase in VEGFR2 protein expression by 6 h, which was further increased by 16 and 24 h. To explore dose-dependent VEGFR2 induction in HUVEC, different doses of shear stress were applied for 16 h (Fig. 1B). Marked induction was seen in HUVECs exposed to shear stress of 5 dynes/cm^2. Further upregulation of VEGFR2 protein expression was noticed after exposure up to 15 dynes/cm^2. Shear stress-mediated VEGFR2 induction was also demonstrable in dermal microvascular endothelial cells (HDMECs, Fig. 1C). Interestingly, VEGFR1 expression levels were essentially unaffected by shear stress exposure (Fig. 1D). In addition, VEGFR2 transcript levels are increased in a time-dependent fashion (Fig. 2). These results are in line with a recent report, revealing VEGFR2 upregulation in response to 24 h shear stress by DNA microarray analysis [32]. Hence, shear stress-induced VEGFR2 expression may be mediated at the transcriptional level. Together, our data demonstrate that fluid shear stress represents a robust stimulus for VEGFR2 induction in endothelial cells from different origins.

3.2. Functional analysis of the 5′-regulatory region of the VEGFR2 promoter in response to fluid shear stress

To determine molecular mechanisms responsible for shear stress-regulated VEGFR2 gene transcription, a series of 5′-deletional VEGFR2 promoter-based reporter gene constructs was transiently transfected into HUVECs (Fig. 3). Analyses of the respective Luc expressions in control and shear-stressed cells revealed significant basal activity of the −4 kb/+296 bp and −164/+268 bp VEGFR2 Luc constructs that was increased in response to shear stress by a factor of 2 to 2.5. Shorter constructs, including the −77/+268 and the −60/+268 bp VEGFR2 construct, showed less constitutive and induced expression compared to the longer reporter plasmids, however, considerable basal activity was retained, including the ability to significantly enhance reporter gene activity (2.0 ± 0.5; $P < 0.04$). These findings suggest that key gene-regulatory elements necessary for both constitutive and shear stress-inducible expression are located 3′ of the −60 bp. The significance of a Sp1 cluster in close proximity to transcription start site has been demonstrated previously [30,33]. As two adjacent Sp1 consensus binding sites are located at position

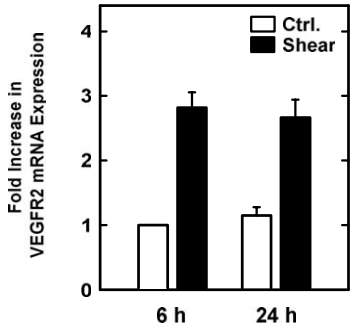

Fig. 2. VEGFR2 mRNA expression is induced by laminar shear stress. HUVECs were grown under static conditions or exposed to laminar shear stress for 6 or 24 h (15 dyne/cm^2). Total cellular RNA was isolated by the RNeasy Mini Procedure (Qiagen). Quantification of mRNA expression was accomplished by real-time LightCycler® RT-PCR using GAPDH expression as a reference against which VEGFR2 transcript levels were normalized. An external standard curve obtained by the LightCycler®-Control Kit RNA (Roche) was used to determine the concentration of VEGFR2 and GAPDH gene transcripts. Normalized VEGFR2 mRNA level in HUVECs grown under static conditions for 6 h was arbitrarily set at '1'. The data are displayed as fold increase and represent the mean ± S.E.M. of three independent duplicate experiments (Student's *t*-test; $P < 0.02$).

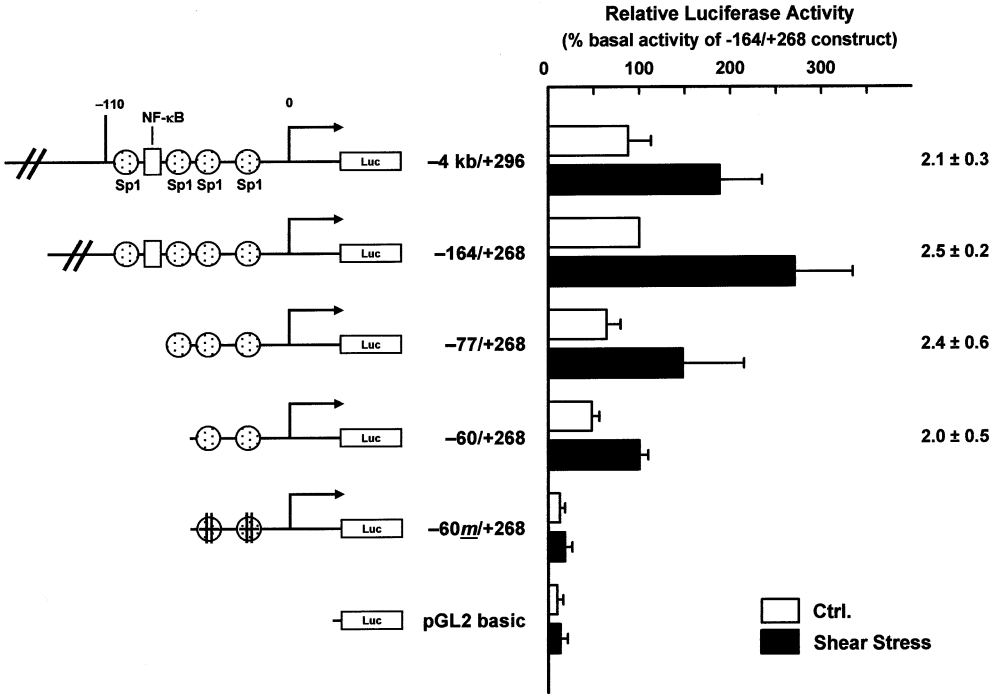

Fig. 3. The shear stress-responsive region is localized between bp −60 and −37 of the VEGFR2 gene promoter. Analyses of 5′-deletional and mutational VEGFR2 promoter-based Luc constructs in HUVECs. Schematic representation of the respective reporter gene constructs on the left, coordinates with respect to the transcription start site in the center, and the relative Luc activities (expressed as % basal activity of the −164/+268 construct) in graphic format on the right: white bars, static controls (Ctrl.); black bars, shear stress-subjected cells (mean ± S.D. of three independent duplicate assays); the fold increase in Luc activity after shear stress exposure is depicted to the right (mean ± S.D. of three independent duplicate transfections and assays).

−58 bp and −44 bp (Fig. 4), we next examined the potential impact on transcriptional activation of a mutant −60/+268 bp Luc construct, in which critical 2-nt mutations [34] were incorporated within the Sp1 sites (Fig. 3). Analyses of the mutant −60/+268 bp construct showed loss of both basal and shear stress-induced reporter gene expression. By comparison, expression in control and shear-stressed HUVECs was equivalent to that of the backbone vector alone. These data indicate that basal and shear-mediated VEGFR2 transcription is Sp1-site-dependent.

3.3. *Constitutive and shear stress-inducible Sp1-dependent binding activity to the −63/−31 bp VEGFR2 promoter sequence*

We next explored whether specific protein complexes could be identified that interacted with the VEGFR2 promoter region which had been demonstrated to be important in our transcriptional activation studies. We utilized a double-stranded DNA probe corresponding to the −63/−31 bp VEGFR2 promoter sequence in electrophoretic mobility shift assays (Fig. 4). When incubated with nuclear extracts of untreated HUVECs, constitutive DNA binding activity of two distinct complexes was observed (lane 1). In lysates of cells that had been exposed to fluid shear stress for 1 h, a significant increase in DNA binding activity was detected (lane 2). These data indicate that shear stress augments constitutively expressed protein complexes without inducing additional DNA binding activity. The binding specificity of the complexes was determined by competition through excess of unlabeled identical DNA (lanes 5 and 6), but not Sp1-mutated DNA (lanes 7 and 8). The complexes were also competed with excess of unlabeled double-stranded Sp1 consensus oligonucleotides (lanes 3 and 4), further supporting the assumption that nuclear proteins bind to the −63/−31 bp VEGFR2 promoter sequence in a Sp1-site-exclusive manner. Addition of Sp1 antibody led to formation of more slowly migrating complexes (lane 10). An irrelevant antibody directed against RelA/NF-κB, however, did neither affect the complex formation nor it induced a supershift (lane 9). Together, these findings provide strong evidence that shear stress-induced transcriptional activation of the VEGFR2 gene is conferred by increased Sp1-like binding activity to the GC-rich core promoter.

4. Discussion

New blood vessel formation and angiogenic remodeling during inflammation and tumor growth are associated with a number of structural and hemodynamic changes. Besides the action of different established humoral factors [2], there is increasing evidence that biochemical stimulation of endothelial cells plays a critical role in maintenance of vascular integrity and microvascular remodeling [25]. However, information on molecular mechanisms by which mechanical forces impact pathological vascular remodeling is still limited.

The vasculature in inflammatory processes and tumors is functionally and structurally abnormal, presenting in part with excessive shunts and disproportionate branches [35]. Uneven diameters as a result of dilated and tortuous vessel architecture lead to variable and disordered blood flow [36,37]. Since fluid shear stress may act as a critical survival stimulus and has been implicated in microvascular remodeling previously [26,27], we hypothesized that laminar shear stress may facilitate endothelial cell survival by inducing VEGFR2 expression. This assumption is bolstered by accumulating data,

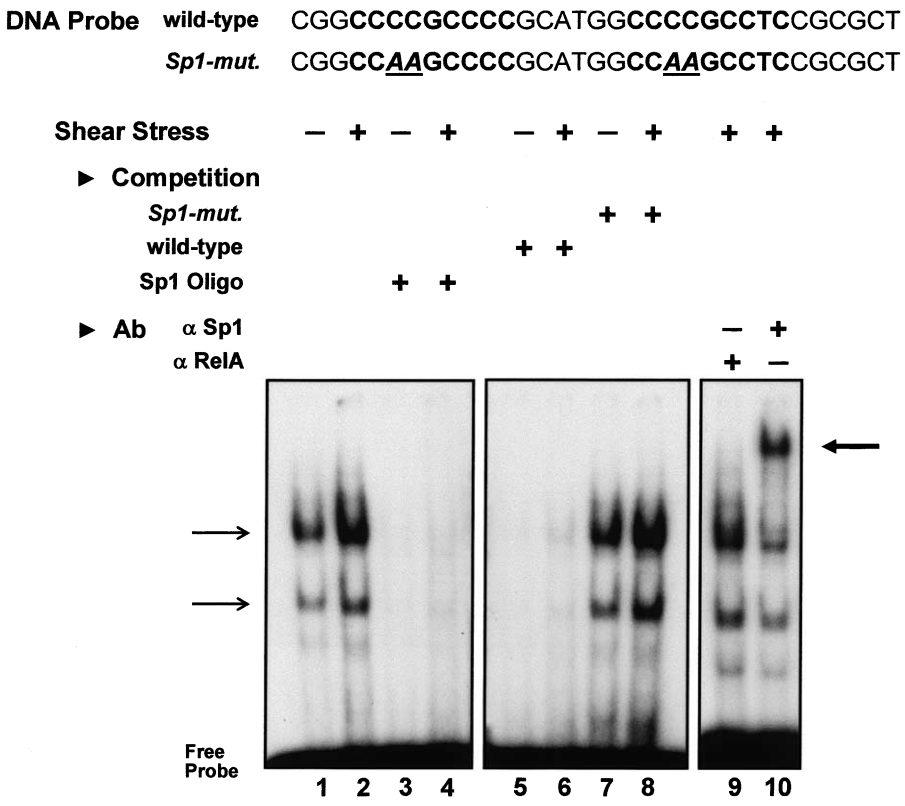

Fig. 4. Constitutive and shear stress-inducible Sp1-site-dependent DNA binding activity of HUVEC nuclear protein extracts to the −63/−31 bp VEGFR2 promoter sequence. Representative EMSAs using nuclear extracts of untreated (lanes 1, 3, 5 and 7) and shear-stressed HUVECs (lanes 2, 4, 6, 8, 9 and 10); competition with unlabeled excess double-stranded Sp1 consensus oligonucleotides (lanes 3 and 4; at a final concentration of 0.35 μM), or with unlabeled −63/−31 bp wild-type DNA (lanes 5 and 6; at 100 M excess), or with a mutated unlabeled −63/−31 bp DNA oligo (lanes 7 and 8; at 100 M excess) in which critical 2-nt changes were incorporated within the Sp1 sites. Supershift analyses were performed by addition of specific RelA/NF-κB or Sp1 antibody (Santa Cruz) at a final concentration of 100 ng/μl (lanes 9 and 10). The DNA sequence of the utilized probe is shown at the top (Sp1 sites in bold), the formation of Sp1-dependent binding complexes is indicated by arrows to the left, supershifted complexes are marked by a bold arrow to the right.

closely linking the VEGF/VEGFR2 signaling pathway to endothelial cell survival both in vitro and in vivo [5].

The present study demonstrates that shear stress acts as a powerful stimulus for VEGFR2 expression by microvascular (HDMEC) and large vessel-derived endothelial cells (HUVEC). While our manuscript was in preparation, Abumiya and co-workers [38] reported on shear stress effects being comparable to ours with regard to VEGFR2 protein induction. In line with their data, we observed a strength- and time-dependent VEGFR2 regulation in HUVECs. Induction of VEGFR2 mRNA expression peaks at 6–8 h, whereas maximal protein expression is seen after 16–24 h.

As a major control point of gene expression, transcriptional activation has been previously identified as a key regulatory mechanism of VEGFR2 expression [39,40,41]. We thus examined VEGFR2 gene transcriptional regulation in response to laminar shear stress within HUVECs. In our analyses of 5′-deletional and mutational reporter gene constructs, a core promoter region was identified that confers shear stress-mediated transactivation of the VEGFR2 gene. This GC-rich region between bp −60 and −37 contains two adjacent consensus Sp1 transcription factor binding sites and was subsequently utilized as a DNA probe in EMSAs. These experiments showed constitutive Sp1-site-dependent complex formation which was significantly induced in response to laminar shear stress.

In 5′-deletional analyses, Abumiya et al. localized the shear stress-responsive element to a VEGFR2 promoter region between bp −94 and −31 [38]. Previous site-specific mutation studies in bovine retinal endothelial cells found an atypical Sp1-like binding site (bp −74/−70) to be important for basilar VEGFR2 promoter activity [42]. Guided by these findings, Abumiya and co-workers evaluated the impact of a 3-nt mutation (TCC→AAG at bp −73/−71) on shear stress-induced transcriptional activity. While baseline activity was modestly reduced, shear stress-mediated induction of a −94/+265 bp VEGFR2 promoter construct was seen to be lost through incorporation of the 3-nt mutation [38]. In our studies, however, deletion from bp −77 to −60 retained the capacity to induce shear stress-driven VEGFR2 promoter activity (Fig. 3). Only when critical 2 nt-mutations were introduced into the consensus Sp1 sites, both basal and shear stress-induced reporter gene expression of the −60/+268 bp VEGFR2 construct were lost. Thus, our data clearly differ from the work by Abumiya et al. as to the precise localization of the shear stress-response element.

Contrary to our EMSA analyses, Abumiya and co-workers detected shear stress-induced Sp1-like binding activity merely to a CT-rich −85/−64 bp DNA probe, but not to double-stranded Sp1 consensus oligonucleotides [38]. However, our EMSA studies revealed constitutive and shear stress-inducible Sp1-site-dependent complex formation to the −63/−31 bp

VEGFR2 promoter sequence (Fig. 4). This region, which has been shown to be functionally important in our transcriptional activation assays (Fig. 3), contains two adjacent Sp1 binding sites. Basal and shear stress-induced DNA binding activity was entirely competed with excess unlabeled Sp1 consensus oligonucleotides, but not with a mutated unlabeled −63/−31 bp DNA probe in which the critical 2-nt changes were incorporated in the Sp1 sites. Our findings are in line with previous observations on nuclear protein interactions with the VEGFR2 promoter by in vitro DNase I footprint analyses, in which the protection pattern produced by HUVEC nuclear extracts could be fully mimicked through recombinant Sp1 protein alone [33]. Together, the data presented herein firmly support the assumption that essential *cis*-acting elements for shear stress-mediated VEGFR2 transcription reside within the −60/−37 bp sequence.

The VEGFR2 promoter lacks a TATA sequence motif [30]. Thus mechanisms other than direct recruitment of TATA-binding proteins are required for positioning of the basal transcription complex and initiation of transcription from a definite site. In particular, Sp1 transcription factors have been shown to function as key proteins in accurate transcription initiation from TATA-less promoters [43]. These mechanisms appear to apply for the VEGFR2 promoter as well, as our studies show that mutation of the two most 3′ Sp1 binding sites decreased both basal and induced Luc expression to levels comparable to those obtained by parent vector only.

Several diverse effects of hemodynamic forces on vascular cells have been identified in recent years [25], including rapid protein phosphorylation and cytoskeletal rearrangement [29,44]. In addition, endothelial cells have been shown to adapt to fluid biochemical stress by changes in gene expression [24,45]. Characterization of shear stress-response elements in promoters of reactive genes provided an enhanced understanding of cellular mechanisms that link gene regulatory events to shear stress stimulation. Pertinent to these findings, transcription factor Sp1 has been previously implicated in shear stress induction of the tissue factor gene [46]. In the absence of shear stress-induced binding activity to a Sp1-site cluster within the tissue factor promoter, phosphorylation of Sp1 was proposed as a mean to increase transcriptional activity. While our study differs from the recent report by Abumiya et al. as to the precise localization of the shear stress-responsive region in the VEGFR2 promoter [38], both studies showed increased Sp1-like activity in nuclear extracts from shear-stressed HUVECs. Hence, Sp1-like binding sites may serve as shear stress-response elements that are also transactivated via increased DNA binding activity.

Together, the present study suggest that shear stress, an important driving force in vascular remodeling and restructuring of blood vessels, contributes to endothelial cell survival in part by inducing VEGFR2 expression. Our findings implicate Sp1-site-dependent DNA binding in fluid shear stress-induced transcriptional activation of the VEGFR2 gene. The involvement of hemodynamic forces in endothelial cell survival may potentially explain as to why adjacent vessel sections differ in their extent to respond to soluble angiogenic factors.

Acknowledgements: We are thankful to Dr. Cam Patterson (University of North Carolina, Chapel Hill, NC, USA) for providing the VEGFR2 promoter-based Luc reporter gene constructs. The expert technical assistance by Melanie Näher is gratefully acknowledged. This work was supported by Deutsche Forschungsgemeinschaft grants Di 600/2-4 (S.D.), Gi 229/5-1 (J.G.), Gi 229/6-1 (J.G.), and by a Heinrich und Erna Schaufler-Stiftung and Dr. Paul und Cilli Weill-Stiftung grant (J.G.).

References

[1] Carmeliet, P. and Jain, R.K. (2000) Nature 407, 249–257.
[2] Yancopoulos, G.D., Davis, S., Gale, N.W., Rudge, J.S., Wiegand, S.J. and Holash, J. (2000) Nature 407, 242–248.
[3] Ferrara, N. (1999) Curr. Top. Microbiol. Immunol. 237, 1–30.
[4] Dvorak, H.F., Nagy, J.A., Feng, D., Brown, L.F. and Dvorak, A.M. (1999) Curr. Top. Microbiol. Immunol. 237, 97–132.
[5] Matsumoto, T. and Claesson-Welsh, L. (2001) Sci. STKE. 112, RE21.
[6] Rahimi, N., Dayanir, V. and Lashkari, K. (2000) J. Biol. Chem. 275, 16986–16992.
[7] Hiratsuka, S., Minowa, O., Kuno, J., Noda, T. and Shibuya, M. (1998) Proc. Natl. Acad. Sci. USA 95, 9349–9354.
[8] Waltenberger, J., Claesson-Welsh, L., Siegbahn, A., Shibuya, M. and Heldin, C.H. (1994) J. Biol. Chem. 269, 26988–26995.
[9] Millauer, B., Wizigmann-Voos, S., Schnurch, H., Martinez, R., Moller, N.P., Risau, W. and Ullrich, A. (1993) Cell 72, 835–846.
[10] Petrova, T.V., Makinen, T. and Alitalo, K. (1999) Exp. Cell Res. 253, 117–130.
[11] Detmar, M. (1996) J. Invest. Dermatol. 106, 207–208.
[12] Shibuya, M., Ito, N. and Claesson-Welsh, L. (1999) Curr. Top. Microbiol. Immunol. 237, 59–83.
[13] Schlaeppi, J.M. and Wood, J.M. (1999) Cancer Metastasis Rev. 18, 473–481.
[14] Tuder, R.M., Flook, B.E. and Voelkel, N.F. (1995) J. Clin. Invest. 95, 1798–1807.
[15] Marti, H.H. and Risau, W. (1998) Proc. Natl. Acad. Sci. USA 95, 15809–15814.
[16] Brogi, E., Schatteman, G., Wu, T., Kim, E.A., Varticovski, L., Keyt, B. and Isner, J.M. (1996) J. Clin. Invest. 97, 469–476.
[17] Gerber, H.P., Condorelli, F., Park, J. and Ferrara, N. (1997) J. Biol. Chem. 272, 23659–23667.
[18] Kremer, C., Breier, G., Risau, W. and Plate, K.H. (1997) Cancer Res. 57, 3852–3859.
[19] Barleon, B., Siemeister, G., Martiny-Baron, G., Weindel, K., Herzog, C. and Marme, D. (1997) Cancer Res. 57, 5421–5425.
[20] Pepper, M.S. and Mandriota, S.J. (1998) Exp. Cell Res. 241, 414–425.
[21] Flamme, I., Breier, G. and Risau, W. (1995) Dev. Biol. 169, 699–712.
[22] Patterson, C., Perrella, M.A., Endege, W.O., Yoshizumi, M., Lee, M.E. and Haber, E. (1996) J. Clin. Invest. 98, 490–496.
[23] Mandriota, S.J., Menoud, P.A. and Pepper, M.S. (1996) J. Biol. Chem. 271, 11500–11505.
[24] Resnick, N. and Gimbrone Jr., M.A. (1995) FASEB J. 9, 874–882.
[25] Skalak, T.C. and Price, R.J. (1996) Microcirculation 3, 143–165.
[26] Dimmeler, S., Assmus, B., Hermann, C., Haendeler, J. and Zeiher, A.M. (1998) Circ. Res. 83, 334–341.
[27] Benjamin, L.E. (2000) Cancer Metastasis Rev. 19, 75–81.
[28] Ayajiki, K., Kindermann, M., Hecker, M., Fleming, I. and Busse, R. (1996) Circ. Res. 78, 750–758.
[29] Dimmeler, S., Fleming, I., Fisslthaler, B., Hermann, C., Busse, R. and Zeiher, A.M. (1999) Nature 399, 601–605.
[30] Patterson, C., Perrella, M.A., Hsieh, C.M., Yoshizumi, M., Lee, M.E. and Haber, E. (1995) J. Biol. Chem. 270, 23111–23118.
[31] Dignam, J.D., Lebovitz, R.M. and Roeder, R.G. (1983) Nucleic Acids Res. 11, 1475–1489.
[32] Chen, B.P., Li, Y.S., Zhao, Y., Chen, K.D., Li, S., Lao, J., Yuan, S., Shyy, J.Y. and Chien, S. (2001) Physiol. Genomics 7, 55–63.
[33] Patterson, C., Wu, Y., Lee, M.E., DeVault, J.D., Runge, M.S. and Haber, E. (1997) J. Biol. Chem. 272, 8410–8416.
[34] Kriwacki, R.W., Schultz, S.C., Steitz, T.A. and Caradonna, J.P. (1992) Proc. Natl. Acad. Sci. USA 89, 9759–9763.
[35] Gazit, Y., Baish, J.W., Safabakhsh, N., Leunig, M., Baxter, L.T. and Jain, R.K. (1997) Microcirculation 4, 395–402.
[36] Jain, R.K. (1988) Cancer Res. 48, 2641–2658.
[37] Thurston, G., Baluk, P. and McDonald, D.M. (2000) Microcirculation 7, 67–80.

[38] Abumiya, T., Sasaguri, T., Taba, Y., Miwa, Y. and Miyagi, M. (2002) Arterioscler. Thromb. Vasc. Biol. 22, 907–913.
[39] Kappel, A., Ronicke, V., Damert, A., Flamme, I., Risau, W. and Breier, G. (1999) Blood 93, 4284–4292.
[40] Shen, B.Q., Lee, D.Y., Gerber, H.P., Keyt, B.A., Ferrara, N. and Zioncheck, T.F. (1998) J. Biol. Chem. 273, 29979–29985.
[41] Minami, T., Rosenberg, R.D. and Aird, W.C. (2001) J. Biol. Chem. 276, 5395–5402.
[42] Hata, Y., Duh, E., Zhang, K., Robinson, G.S. and Aiello, L.P. (1998) J. Biol. Chem. 273, 19294–19303.
[43] Azizkhan, J.C., Jensen, D.E., Pierce, A.J. and Wade, M. (1993) Crit. Rev. Eukaryot. Gene Expr. 3, 229–254.
[44] Franke, R.P., Grafe, M., Schnittler, H., Seiffge, D., Mittermayer, C. and Drenckhahn, D. (1984) Nature 307, 648–649.
[45] Urbich, C., Mallat, Z., Tedgui, A., Clauss, M., Zeiher, A.M. and Dimmeler, S. (2001) J. Clin. Invest. 108, 1451–1458.
[46] Lin, M.C., Almus-Jacobs, F., Chen, H.H., Parry, G.C., Mackman, N., Shyy, J.Y. and Chien, S. (1997) J. Clin. Invest. 99, 737–744.