

NAB2, a Corepressor of EGR-1, Inhibits Vascular Endothelial Growth Factor-mediated Gene Induction and Angiogenic Responses of Endothelial Cells*

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In this study we have investigated the role of a specific corepressor of EGR-1, NAB2, to down-regulate vascular endothelial growth factor (VEGF)-induced gene expression in endothelial cells and to inhibit angiogenesis. Firstly, we show a reciprocal regulation of EGR-1 and NAB2 following VEGF treatment. During the initial phase EGR-1 is rapidly induced and NAB2 levels are down-regulated. This is followed by a reduction of EGR-1 and a concomitant increase of NAB2. Secondly, using the tissue factor gene as a readout for VEGF-induced and EGR-1-regulated gene expression we demonstrate that NAB2 can completely block VEGF-induced tissue factor reporter gene activity. Thirdly, by adenovirus-mediated expression we show that NAB2 inhibits up-regulation of tissue factor, VEGF receptor-1, and urokinase plasminogen activator mRNAs even when a combination of VEGF and bFGF is used for induction. In addition, NAB2 overexpression significantly reduced tubule and sprout formation in two different *in vitro* angiogenesis assays and largely prevented the invasion of cells and formation of vessel-like structures in the murine Matrigel model. These data suggest that NAB2 regulation represents a mechanism to guarantee transient EGR-1 activity following exposure of endothelial cells to VEGF and that NAB2 overexpression could be used to inhibit signals involved in the early phase of angiogenesis.

Vascular endothelial growth factor, VEGF,¹ has a predominant role in vasculogenesis as well as in physiological and

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¹ The abbreviations used are: VEGF, vascular endothelial growth factor; PKB, protein kinase B; PKC, protein kinase C; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase; ERK, extracellular signal-regulated kinase; MAP, mitogen-activated protein; TF, tissue factor; uPA, urokinase-type plasminogen activator; EGR, early growth response protein; HUVEC, human umbilical vein endothelial cells; HMEC, human uterine microvascular endothelial cells; SCS, supplemented calf serum; HLMEC, human lung microvascular endothelial cells; GFP, green fluorescent protein; PBS, phosphate-

pathological angiogenesis (1–3). Major signals induced by VEGF via VEGFR-2 in endothelial cells include activation of the phosphoinositol 3-kinase/PKB and phospholipase C- γ /PKC pathways (4, 5). Whereas activation of PKB has been primarily implicated in cell survival (6), recent *in vitro* studies have shown that VEGF treatment of endothelial cells leads to a PKC-dependent activation of the MEK/ERK module of MAP kinases resulting in a rapid up-regulation of the transcription factor EGR-1 (7, 8), which has been associated with growth and differentiation of various cell types (9, 10). Furthermore, EGR-1 is critically involved in the up-regulation of genes such as tissue factor (TF) (7, 8), VEGF receptor-1 (VEGFR-1) (11, 12), and urokinase-type plasminogen activator (uPA) (13). These genes have been proposed to fulfill important functions for different aspects of vasculogenesis and angiogenesis (1, 2, 14).

Comparable to some other transactivators, EGR-1 associates with corepressor proteins that can modulate transcription of EGR-dependent genes. Two corepressors of EGR-1, NAB1 and NAB2, have been identified using yeast two-hybrid screening (15, 16). These factors bind to EGR-1 by direct protein-protein interactions with a conserved R1 region found in several members of the EGR family (EGR-1, -2 and -3), thus inhibiting the transactivating potential of EGR-1. Whereas NAB1 is constitutively expressed in most tissues and appears to be a general transcriptional regulator (15), NAB2 may function as an important inducible regulator of gene expression (16). Initially, the physiologic activities of NAB2 have been analyzed in nerve cells where the EGR-1-mediated differentiation process stimulated by nerve growth factor was blocked by the corepressor NAB2 (9). Furthermore, an inhibition of EGR-1-dependent transcription and growth factor production in smooth muscle cells with implications for tissue repair and angiogenesis was recently reported (17, 18). In general, gene regulation mediated by the interplay of EGR-1 and NAB2 might be a unifying principle in different invasive processes such as neurite outgrowth, wound healing, angiogenesis, and tumor invasion (19).

Based on our previous finding that the transcription factor EGR-1 is decisively involved in the up-regulation of the TF gene by VEGF in endothelial cells (7, 8), we have here analyzed to what extent NAB2 can down-modulate expression of several different genes induced by angiogenic growth factors and thus plays a direct role in the control of angiogenesis-related responses of endothelial cells. In this respect NAB2 gene transfer

buffered saline; CMV, cytomegalovirus; KIU, kallikrein-inactivating unit; pfu, plaque-forming unit; CCD, charge-coupled device; m.o.i., multiplicity of infection; DAPI, 4',6-diamidino-2-phenylindole.

to endothelial cells by recombinant adenoviruses was further evaluated as a potential approach to inhibit angiogenesis. Our results show that NAB2 can strongly inhibit VEGF- and bFGF-induced expression of the TF, VEGFR-1, and uPA genes. Furthermore, the adenovirus-mediated overexpression of NAB2 led to significant inhibition of migration, sprouting, and tubule formation in angiogenesis models *in vitro* and *in vivo* without obvious cytotoxic side effects.

MATERIALS AND METHODS

Cell Culture and Materials—Human umbilical vein endothelial cells (HUVEC) and human uterine microvascular endothelial cells (HUMEC) were isolated and cultured in medium 199 with 20% SCS or a 1:1 mixture of SCS and fetal calf serum (HyClone, Logan, UT), respectively, supplemented with 1 unit/ml heparin, 50 μ g/ml endothelial cell growth supplement (Technoclone, Vienna, Austria), 2 mM glutamine, 100 units/ml penicillin, and 0.1 mg/ml streptomycin as described in more detail in Refs. 8 and 20. Human lung microvascular cells (HLMEC) were obtained from Bio-Whittaker (Walkersville, MD) and cultured as described in the protocol provided. Short-starved cells were obtained by starving with 1% serum for 5 h. Recombinant human VEGF₁₆₅ and bFGF was obtained from PromoCell (Heidelberg, Germany). Polyclonal anti-EGR-1 and anti-Sp1 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA) and polyclonal anti-GFP antibodies were from New England Biolabs (Beverly, MA). Monoclonal anti-NAB2 antibodies 1C4 (21) were a gift of Dr. Judith Johnson (Institute of Immunology, University of Munich, Munich, Germany). Peroxidase-conjugated donkey anti-rabbit immunoglobulin G (IgG) and sheep anti-mouse IgG were purchased from Amersham Biosciences, and goat anti-rat IgG was from Serotec (Oxford, UK).

Real-time PCR—RNA was extracted from endothelial cells with TRIzol Reagent (Invitrogen). 2 μ g of total RNA was reverse-transcribed using SuperScript™ II enzyme using oligo-dT primers as specified by Invitrogen. Real-time PCR including SYBR Green PCR reagent was performed on a Light Cycler™ instrument (Hoffmann-La Roche) according to instructions provided by the manufacturer (22). Oligonucleotides used were TF-forward: ccgaacagttaccggaaga, TF-reverse: tcagtggggagttctctctc; EGR-1-forward: cagcacttcaacctcag, EGR-1-reverse: cacaaggtgtgacctgtt; NAB2-forward: acatctgcagcagactg, NAB2-reverse: ctccactttaccgtctc; VEGFR-1-forward: tgctcagctgtctctctc, VEGFR-1-reverse: ccatttcagcaaacacat; and uPA-forward: tgaggtagaaacctctacc, uPA-reverse: ggaggcagatggtctgtat.

Western Blot Analysis—Cells were washed twice with PBS, lysed in 100 μ l of Laemmli buffer, scraped, and heated for 5 min at 95 °C. Total cell lysates were separated by SDS-PAGE and transferred to Immobilon-P membrane (Millipore, Bedford, MA). The membrane was blocked for 30 min with PBS containing 0.1% Tween 20 and 3% skim milk and incubated for 1 h at room temperature with the primary antibody diluted in blocking buffer. Then the membrane was washed three times for 5 min with PBS containing 0.1% Tween 20 and incubated with peroxidase-conjugated secondary antibodies for 1 h at room temperature. After a washing step, the membrane was incubated for 1 min with ECL reagent (Amersham Biosciences) and exposed to film. For reprobing with another antibody, the membrane was washed twice in PBS, stripped for 30 min at 55 °C with stripping buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 100 mM 2-mercaptoethanol), and washed three times for 5 min with PBS at room temperature.

Transient Transfections—The TF reporter gene construct containing the TF promoter from -330 to +118 bp in a luciferase expression vector was previously described (7, 23). The coding region of the human EGR-1 gene including a single intron was obtained from the PAC clone E13873Q3 (library number 704, RZPD, Berlin, Germany) by PCR amplification. The resulting 2.3-kb DNA product was subcloned into *Hind*III-*Eco*RI-digested pACCMVpASR+ vector (24). The expression constructs for the human full-length NAB2 (pCMVNAB2) and the alternatively spliced NAB2.AS (pCMVNAB2.AS) (16) were kindly provided by J. Milbrandt and J. Svaren (Departments of Pathology and Internal Medicine, Washington University, St. Louis, MO). Transient transfections of HUVEC were carried out by using the LipofectAMINE PLUS™ reagent (Invitrogen) as previously described (8). 24 h prior to transfection, HUVEC were seeded in six-well tissue culture plates to reach 70–90% confluency the next morning. Cells were incubated with transfection mixtures containing a total of 1.5 μ g of DNA (0.5 μ g of TF promoter/luciferase reporter, 0.5 μ g of a CMV- β -galactosidase construct as internal control and various amounts of NAB2, NAB2.AS, and EGR-1 expression plasmids or empty control vector), 6 μ l of PLUS

reagent, and 4 μ l of LipofectAMINE in a total volume of 1 ml of medium 199 per well for 2 h. All experimental values were determined from triplicate wells.

Recombinant Adenoviral Constructs and Infection—Construction of recombinant adenoviruses was done as previously described (25, 26). NAB2 and NAB2.AS cDNAs (16) were first subcloned into the *Xma*I site of the pBluescript II SK +/- vector and then transferred to the *Bam*HI site of the vector pACCMVpLpASR+ (24). The obtained constructs were verified by sequencing and cotransfected with pJM17, a plasmid containing the adenoviral genome with a deletion in the E1 region (27), into 293 cells. Clones were tested for protein expression by Western blots. Purification of large batches of the recombinant adenoviruses was done by two consecutive cesium chloride centrifugations (28). Adenoviruses without cDNA inserts and viruses expressing GFP (26) were grown in parallel and used as controls.

For infection HUVEC were incubated in PBS complete for 30 min at a m.o.i. of 100. Thereafter, cells were washed and cultured in normal medium 199.

In Vitro Angiogenesis Assays—The formation of capillary tube-like structures by HUVEC was analyzed on tumor-derived extracellular membrane matrix (Matrigel; Becton Dickinson, Franklin Lakes, NJ). 48-well culture dishes (Costar, Cambridge, MA) were coated with 100 μ l/well Matrigel, and the gel was allowed to solidify. Cells were starved for 4 h in medium 199 with 1% SCS, seeded on the polymerized Matrigel (3×10^4 cells/well), further incubated for 16 h, and then fixed in PBS containing 3% formaldehyde and 2% sucrose (29). Images of the network formed were taken on a phase contrast microscope (Nikon Diaphot TMD) using a cooled charge-coupled device camera (Kappa DX30, Kappa GmbH, Gleichen, Germany). The total length of the tube-like structures formed was then determined with the help of the Analysis Software (Softimaging System, Munster, Germany).

An endothelial sprouting angiogenesis assay was performed using HLMEC or HUMEC according to a modification of the method used by Nehls *et al.* (30). Briefly, microcarrier beads coated with denatured collagen (Cytodex 3; Sigma) were seeded with the infected cells, the cells were grown overnight on the beads in medium 199, and the beads then embedded in fibrin gels in 12-well plates (Costar). To prepare the fibrin gel, human fibrinogen (Sigma) was dissolved in PBS complete at a concentration of 2 mg/ml and aprotinin (Bayer, Leverkusen, Germany) was added at a concentration of 200 KIU/ml. The fibrinogen solution was then supplemented with 50 ng/ml VEGF. The solutions were transferred to 12-well plates together with the beads covered by cells at a density of about 200 beads/well, and fibrin formation was induced by addition of 1.2 units/ml thrombin (Sigma). Fibrin gels were equilibrated with serum-free medium containing aprotinin (200 KIU/ml) for 1 h and then incubated with M199 medium supplemented with 20% FCS and growth factors as indicated. After 1 to 2 days the number of capillary-like sprouts formed was counted in the microscope. Only sprouts with a minimal length of ~150 μ m were counted. To visualize cell nuclei, cells were fixed with 3.7% formaldehyde and 2% sucrose in PBS and permeabilized with 0.5% Triton X-100 in PBS. Then Hoechst 333258 was added to the cells at 500 ng/ml for 30 min. The cytoskeleton was stained with rhodamine-phalloidin (Molecular Probes, Eugene, OR) for 1 h in the dark. Cells were analyzed by phase contrast microscopy and images taken using a CCD camera.

In Vivo Matrigel Assay—Matrigel solution (BD Biosciences) was supplemented with 1.5×10^8 pfu/ml of recombinant adenoviruses and 300 ng/ml VEGF and injected subcutaneously into the flank of C57BL/6 mice (31). On day 6 post injection the mice were sacrificed, and the Matrigel plug was removed and embedded in paraffin. Freeze sections of the plug were prepared and stained with hematoxylin (Merck), DAPI (Vector Laboratories, Burlingame, CA), or rat anti-mouse CD31 antibodies (Oxford, UK) as described in Ref. 31. Pictures were taken on an AX-70 Olympus microscope (Olympus Optical Co.) using an Optronics DEI-750D CCD camera (Optronics, Muskogee, OK). The number of cells in the Matrigel plugs were quantitated on pictures displaying largely complete sections of the plugs, which were established from serial images taken from the individual sections. The circular images of the sections were divided into 10-degree segments, and the number of the cells within six segments were counted for each section.

RESULTS

Regulation of EGR-1 and NAB2 Expression by VEGF—The transcription factor EGR-1 has been previously shown by us to be induced by VEGF in endothelial cells with the kinetics typical for an immediate-early gene product showing maximal levels ~60 min after stimulation (7). Here we tested the regu-

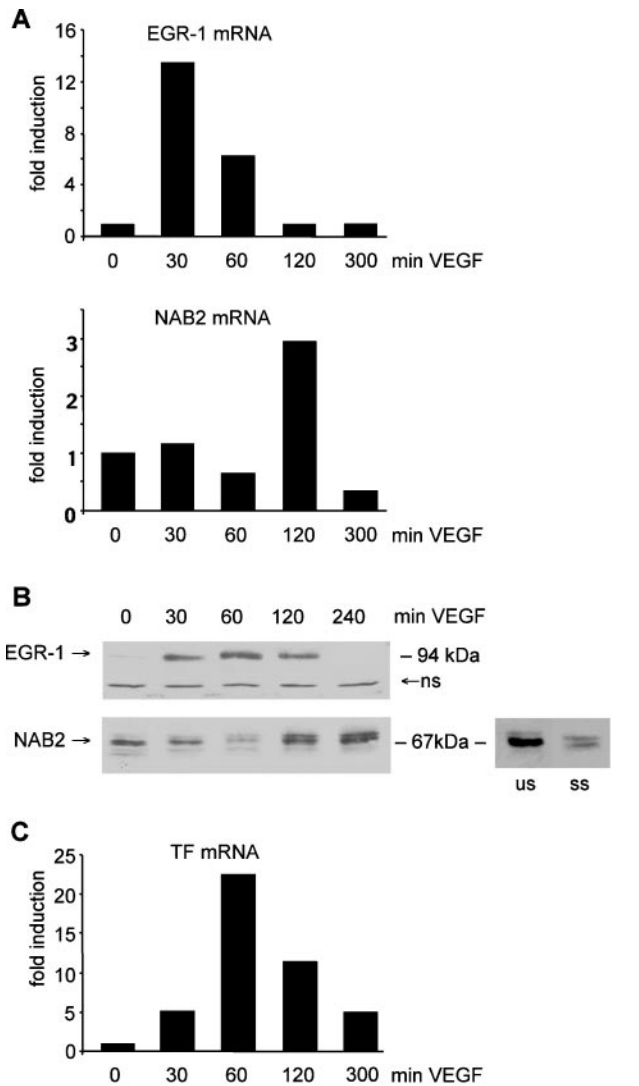


FIG. 1. EGR-1 and NAB2 expression is regulated by VEGF in endothelial cells. *A*, real-time PCR analysis of EGR-1 and NAB2 mRNA. RNA was isolated from 5-h short-starved unstimulated cells (“0” time point) and cells exposed for 30–300 min to VEGF (1.25 nM). Real-time PCR was performed as described under “Materials and Methods.” Data are displayed as fold induction of the value obtained with RNA from unstimulated cells. *B*, Western blot analysis of total cell extracts from short-starved unstimulated cells and short-starved cells exposed to VEGF (1.25 nM). Samples were harvested, and the proteins in the lysates separated by SDS-PAGE and subjected to Western blot analysis. Membranes were probed with anti-NAB2 antibodies followed by reprobing with anti-EGR-1 antibodies. An arrow indicates a nonspecific (*ns*) band displaying equal loading of the samples. NAB2 protein levels in unstarved cells (*us*) are compared with short-starved cells (*ss*) in the right part of the panel. *C*, real-time PCR analysis of TF mRNA. The RNA samples described under *A* were tested for TF mRNA. Results representative for two (*A* and *C*) or three separate experiments (*B*) are shown.

lation of NAB2 mRNA and protein expression in endothelial cells following VEGF treatment in comparison to EGR-1. HUVEC were short-starved for 5 h in medium containing 1% serum and then treated with VEGF (1.25 nM) for various time periods up to 6 h, and the amount of EGR-1 and NAB2 mRNA and protein was determined by real-time reverse transcription-PCR and Western blotting, respectively. A transient induction of EGR-1 mRNA was observed with maximal expression at about 30 min (Fig. 1*A*), which was followed by a transient increase in NAB2 mRNA displaying highest values at 120 min. EGR-1 protein levels decreased during starvation (data not

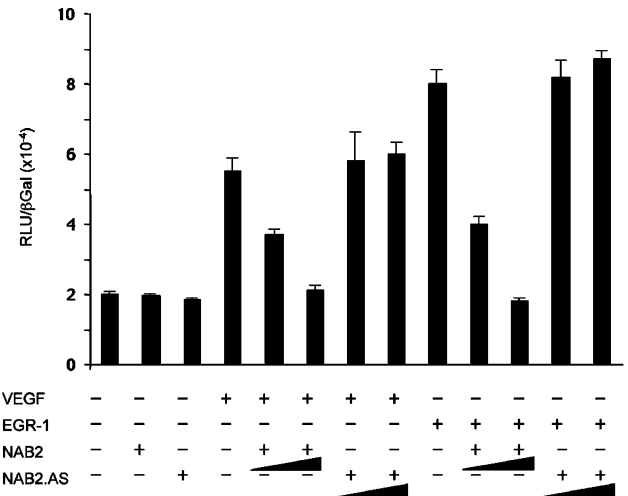


FIG. 2. Activation of the TF promoter by VEGF and EGR-1 is equally inhibited by NAB2. HUVEC were cotransfected with a TF promoter/luciferase reporter gene, a CMV promoter/β-galactosidase construct and increasing amounts of NAB2 or NAB2.AS (1.6 and 3.2 μg, respectively) expression vectors as indicated. 20-h post transfection cells were treated with VEGF for an additional 6 h, harvested, and analyzed. When indicated, an EGR-1 expression plasmid (1.6 μg) was cotransfected instead of VEGF induction. Results are displayed as relative light units (*RLU*) luciferase normalized to β-galactosidase concentration. Results are shown as mean values ± S.D. Data are representative of three independent experiments performed with triplicate wells.

shown), were very low in 5 h serum-starved endothelial cells, and reached maximal values 60 min after VEGF treatment (Fig. 1*B*). NAB2 levels also decreased during starvation reaching a constant basal level within 5 h. In contrast to EGR-1, VEGF treatment resulted first in a further 4- to 5-fold decrease of NAB2 within 60 min, the time period when EGR-1 levels reached highest values. Thereafter, concomitant with a decrease in EGR-1, NAB2 protein increased again about 10-fold reaching levels at least 2- to 3-fold over initial values. Thus, NAB2 and EGR-1 expression are regulated in a reciprocal way by VEGF. In accordance with important roles of EGR-1 and NAB2 for TF gene transcription, TF mRNA levels reached highest levels at 60 min (Fig. 1*C*) at a time period when maximal EGR-1 and lowest NAB2 levels were observed.

VEGF-mediated Induction of the TF Promoter Is Blocked by NAB2—We have previously shown (7, 8) that EGR-1 plays an essential role in the activation of the TF gene by VEGF. Therefore, overexpression of NAB2 was tested for its ability to repress VEGF-induced TF promoter activity in reporter gene assays in comparison to EGR-1-triggered activation. Indeed, overexpression of NAB2 resulted in a dose-dependent complete inhibition of VEGF-induced TF promoter activity (Fig. 2), which was comparable to the inhibition of EGR-1-triggered promoter activity. NAB2.AS, a truncated variant of NAB2, which lacks the C-terminal part interacting with EGR-1 (16) and does not localize to the nucleus,² did not block VEGF- and EGR-1-mediated transactivation. These results demonstrate that the transcriptional corepressor NAB2 is able to block VEGF-induced gene regulation mediated by EGR-1 in endothelial cells.

NAB2 Overexpression by Recombinant Adenoviruses Inhibits Inducible Expression of TF, VEGFR-1, and uPA mRNAs—To test the effect of overexpression of NAB2 on VEGF-inducible responses of endothelial cells that are linked to and regulated

² D. Mechtcheriakova, M. Lucerna, and E. Hofer, unpublished observation.

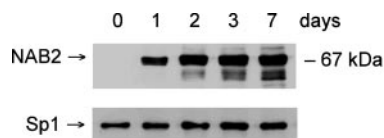


FIG. 3. Kinetics of adenovirus-mediated overexpression of NAB2. A recombinant adenovirus expressing NAB2 cDNA under the control of the CMV promoter was produced and used to infect HUVEC at a m.o.i. of 100. Cells were harvested 1–3 days post infection, and cellular lysates subjected to Western blotting using anti-NAB2 antibodies. The same blot was subsequently reprobred with anti-Sp1 antibodies to ensure equal loading of samples.

by EGR-1, we constructed a recombinant adenovirus expressing NAB2 and infected human endothelial cells with 10^7 pfu/ 10^5 cells. Infected HUVEC showed increasing levels of NAB2 expression from day 1 to day 3 following infection that persisted for over 7 days (Fig. 3). These expression levels were significantly higher than physiological levels in endothelial cells as indicated by the fact that the endogenous NAB2 is not visible on images of short Western blot exposures displaying strong bands of adenovirus-expressed NAB2.

We first evaluated the effect of adenovirus-mediated NAB2 expression on the induction of mRNAs for TF, uPA, and VEGFR-1 by VEGF (data not shown) and a combination of VEGF and bFGF (Fig. 4). uPA and VEGFR-1 were chosen in addition to TF since EGR-1 has been reported to be involved in the up-regulation of the respective genes (12, 13). Furthermore, several lines of evidence support an important role of uPA for migration and invasion and of VEGFR-1 for pathological angiogenesis, respectively (2, 14). A combination of VEGF and bFGF was tested because both factors induce EGR-1, have been described to be present in the Matrigel preparations used for the angiogenesis assays described below, and contribute to tumor angiogenesis *in vivo* (2, 32). When endothelial cells were infected with the NAB2-expressing virus and induced with the growth factors 2 days thereafter, the normally observed induction of all three mRNAs was inhibited to a large degree (Fig. 4). This shows that by blocking EGR-1 activity NAB2 can inhibit the expression of several genes induced by angiogenic growth factors and involved in angiogenesis.

Effects of NAB2 Overexpression in the *in Vitro* Angiogenesis Models—Next we have tested whether this inhibition of gene up-regulation by NAB2 would have consequences for the cellular angiogenic responses of endothelial cells in two different *in vitro* angiogenesis assays. In one assay we have evaluated the potential of Ad.NAB2 to inhibit migration and tubular network formation after plating of endothelial cells on Matrigel. In the second assay the capacity of endothelial cells to form sprouts and migrate into fibrin gels was investigated. In both cases cells infected with Ad.NAB2 were compared with cells infected with control virus for 24 h. Infection with Ad.NAB2 reduced by $52 \pm 14\%$ the tubular network established by HUVEC 16 h after seeding on Matrigel (Fig. 5). Parallel cultures seeded on gelatin-coated plates did not display any cytotoxicity caused by the virus infection (data not shown). For the sprouting assay HLMEC or HUVEC were seeded on microcarrier beads, and the beads incorporated into fibrin gels. In this assay the sprouting of the microvascular endothelial cells from the beads into the fibrin gel was dependent to a large degree on the presence of VEGF in the medium (Fig. 6). Individual sprouts contained usually between 1 to 3 cells in a string and displayed thin protrusions into the fibrin gel. Also in this assay the capacity of the VEGF-induced cells to form sprouts and to migrate into the fibrin gel was significantly reduced ($45 \pm 18\%$, Fig. 6B).

Effects of NAB2 Overexpression in the *in Vivo* Matrigel Model—Finally, we have evaluated to which degree Ad.NAB2

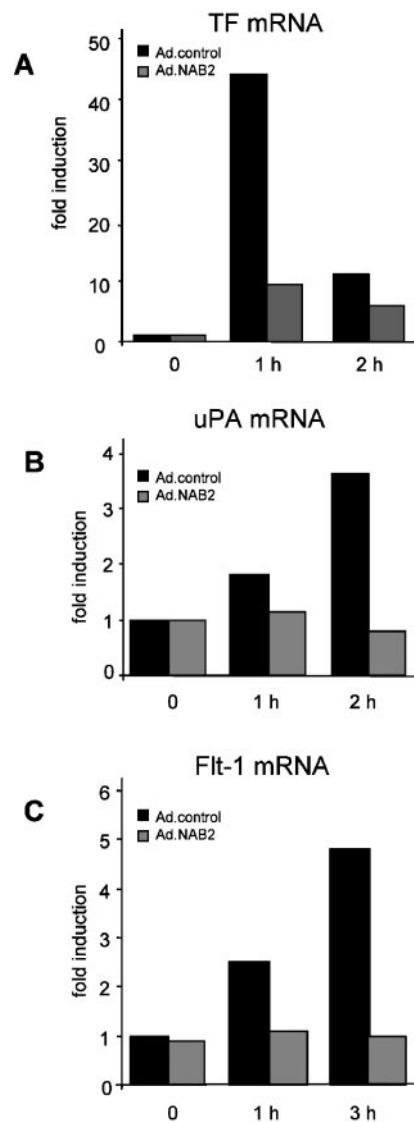


FIG. 4. Adenovirus-mediated overexpression of NAB2 inhibits induction of TF, VEGFR-1, and uPA mRNAs. HUVEC cells were infected with control virus or the NAB2 expressing adenovirus at a m.o.i. of 100. Two days following infection cells were treated with a combination of VEGF (50 ng/ml) and bFGF (50 ng/ml) for 1–3 h, and total RNA was isolated and used for real-time PCR analysis. Oligonucleotides specific for TF (A), VEGFR-1 (B) and uPA mRNA (C) as given under “Materials and Methods” were used. Results are displayed as fold induction of the corresponding values obtained with uninduced samples. The figure displays an experiment representative of two performed with duplicate values.

would inhibit the invasion of cells and the formation of vessel-like structures in the murine Matrigel model *in vivo* (31). For this purpose GFP- or NAB2-expressing adenoviruses were mixed into Matrigel solution without or supplemented with VEGF. These mixtures were injected subcutaneously into mice and analyzed 6 days thereafter. We have first tested whether adenoviruses would efficiently infect cells invading the Matrigel plug by staining sections of Ad.GFP- and VEGF-containing plugs with anti-GFP antibodies and DAPI. By comparing the number of GFP-expressing cells inside the plug (Fig. 7, picture 1) with the number of nuclei stained with DAPI (Fig. 7, picture 2), it was evident that the cells inside the Matrigel plug were almost completely infected. This is best displayed in the overlay of both stainings in Fig. 7, picture 3. Even the multiple cell layers that had formed around the Matrigel plug were infected

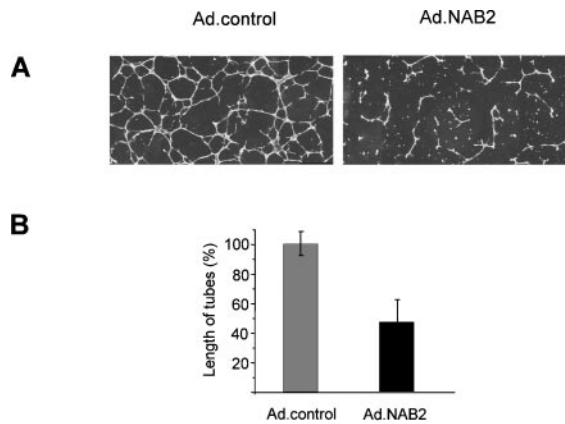


FIG. 5. NAB2 overexpression inhibits tubule formation on Matrigel. HUVEC cultures were infected with Ad.NAB2 or control virus. 24 h following infection cells were short-starved for 5 h in 4% serum-containing medium and then trypsinized, and samples were seeded in parallel on Matrigel in 48-well tissue culture plates to analyze tubule formation. Cells were photographed using a CCD camera 16 h after seeding, and the total length of the tubule-like network formed in the well was established as described under "Materials and Methods." *A*, displays the strong inhibition of tubule formation by Ad.NAB2 in a representative experiment. *B*, shows a quantitation of the reduction of tube-length obtained with Ad.NAB2 in comparison to cells infected with control virus. Results are displayed as mean values \pm S.D. obtained from three independent experiments performed with duplicate wells.

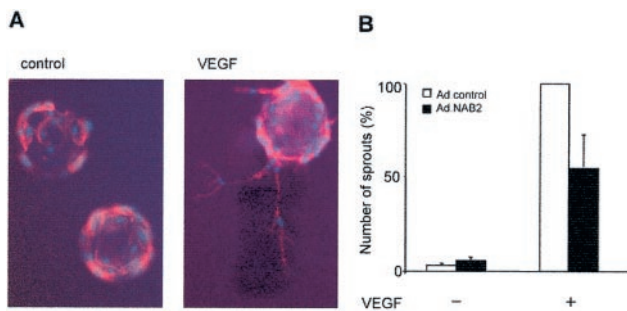


FIG. 6. Overexpression of NAB2 reduces sprouting in fibrin gels. Cultures of HLMEC or HUMEC were infected with Ad.NAB2 or control virus. After 24 h cells were trypsinized, seeded onto microcarrier beads and further cultivated overnight. On the following day the microcarrier beads covered by a dense monolayer of cells were incorporated into fibrin gels in 24-well tissue culture plates and incubated with growth medium without added growth factors or supplemented with VEGF. 24 to 48 h after incorporation into fibrin gels and incubation with the growth factor cells migrated and formed sprouts into the fibrin gel. *A*, shows an example of sprouts formed by HLMEC in the presence of VEGF. The cytoskeleton of the cells and the nuclei were stained with rhodamine-phalloidin and Hoechst 333258, respectively. An overlay of the stained cytoskeleton (red) and nuclei (blue) is displayed. Typically between 0.5 and 3 sprouts per bead were observed for growth factor-treated cells. *B*, shows the analysis of the inhibition in the number of sprouts observed in VEGF-containing culture when Ad.NAB2 infected cells were used in comparison to control virus-infected cells. The mean value \pm S.D. calculated from three independent experiments performed with duplicate wells is given.

to a large degree. Then we analyzed the total number of cells in the sections by hematoxylin staining and displayed endothelial cells by staining with anti-CD31. As seen in the hematoxylin staining of sections (Fig. 8A), a high number of cells invaded the Matrigel plug when VEGF was present, whereas only few cells could be detected in the Matrigel without added VEGF. In both cases, without and in the presence of added VEGF, multiple cell layers had formed on the outside of the Matrigel plug. A fraction of the invading cells were endothelial cells as shown by staining with anti-CD31 antibodies (Fig. 8B). Some of these endothelial cells formed strings, and several vessel-like struc-

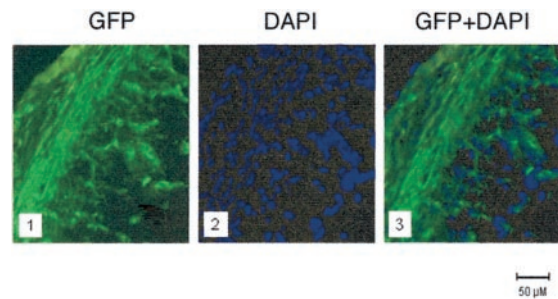


FIG. 7. Efficient infection of cells in the murine Matrigel assay. GFP adenoviruses (1.5×10^8 pfu/ml) and VEGF (300 ng/ml) were added to the Matrigel solution and injected into the abdominal subcutaneous tissue of C57BL/6 mice. On day 6 plugs were excised and freeze sections prepared. Staining of the sections was performed with GFP antibodies (picture 1) or DAPI to reveal cell nuclei (picture 2). Picture 3 displays an overlay of the staining with GFP antibodies and DAPI confirming that a majority of the cells was infected. The left upper parts of the pictures correspond to the cell layers formed around the Matrigel plug from which cells invade toward the center of the plug located in the direction of the right lower corner.

tures were observed. Significant numbers of invading endothelial cells in the plug were again only observed in Matrigel supplemented with VEGF. A quantification of cells in the Matrigel plugs demonstrated that the presence of Ad.NAB2 in the Matrigel reduced the number of total cells in sections of plugs supplemented with VEGF to the low level seen in sections of plugs without added VEGF (Fig. 8C). Furthermore, no vessel-like structures formed by endothelial cells could be observed inside the Matrigel in Ad.NAB2-containing plugs. These inhibitory effects observed for Ad.NAB2 support an important role of EGR-1-mediated gene regulation for the *in vivo* processes.

DISCUSSION

VEGF is the most critical driver of vascular formation since it is required to initiate the formation of vessels by vasculogenesis and angiogenic sprouting (1, 2, 33, 34). It can trigger several different cellular responses involved in angiogenesis such as proliferation, survival, sprouting, migration, and increased vascular permeability. These responses are likely linked to distinct, although partially overlapping, signaling pathways connected to various gene expression programs. VEGF can bind to two different receptors on endothelial cells, VEGFR-1/Flt-1 and VEGFR-2/Flk-1, but primarily VEGFR-2 seems to mediate the initial responses leading to angiogenesis and increased permeability (35). Over the last years, signal transduction pathways activated by VEGF via VEGFR-2 have been studied intensively. Important signals induced by VEGF include phospholipase C- γ , MAP kinases, phosphoinositol 3-kinase, Akt/protein kinase B, and focal adhesion kinase (4, 36–38). However, the key transcription factors and regulatory genes activated by these signals have not yet been unequivocally identified. In this context, we evaluated the physiological role of EGR-1 and of NAB2, a specific corepressor of EGR-1, in VEGF-triggered responses in endothelial cells.

An important association with growth and development has been suggested by several reports for EGR-1 (9, 10). Furthermore, a number of data support a major role of EGR-1 in the acute response to various kinds of stress such as physical and ischemic injury (39). EGR-1 was suggested to contribute to restenosis and atherosclerotic disease through the up-regulation of a number of pathophysiologically relevant genes including PDGF A/B chains, TNF- α , TGF- β , and TF and was found to be itself induced by factors such as bFGF (32, 40).

According to our previous data, EGR-1 is prominently in-

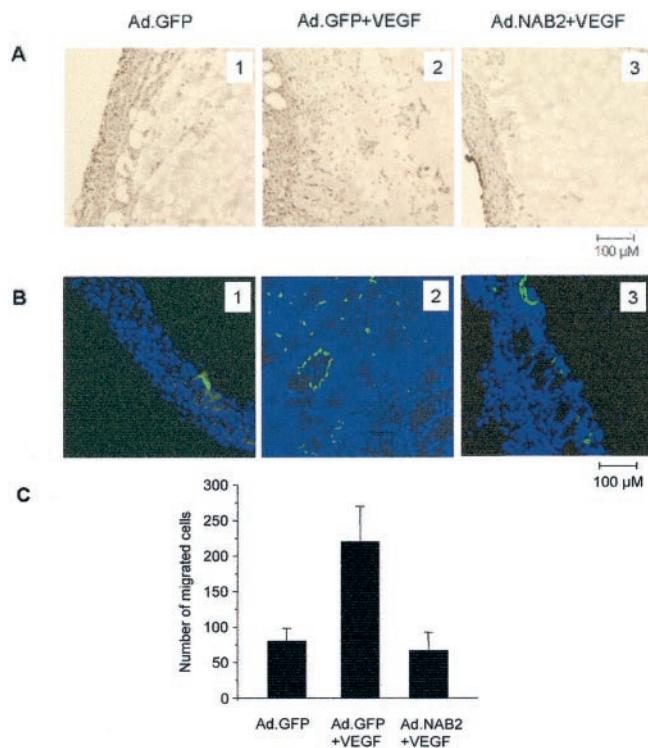


FIG. 8. The NAB2-expressing adenovirus inhibits invasion and tubule formation by endothelial cells in the murine Matrigel assay. GFP- and NAB2-expressing adenoviruses (1.5×10^8 pfu/ml) were added to Matrigel solution containing VEGF (300 ng/ml) or without added growth factor and injected subcutaneously into mice. After 6 days plugs were removed and processed to prepare freeze sections as described under "Materials and Methods." Individual sections were either stained with hematoxylin (A) to detect total invading cells or with anti-CD31 and DAPI (B) to visualize endothelial cells and total number of cell nuclei, respectively. Overlays of the CD31 staining (green) and DAPI staining (blue) are shown. Pictures 1 of A and B display sections of a Matrigel plug containing Ad.GFP without VEGF, pictures 2 of A and B containing Ad.GFP and VEGF and pictures 3 containing Ad.NAB2 and VEGF. The cell layers formed around the plug are on the left side, the center of the plug is located toward the right or right upper side of the pictures. Significant invasion of endothelial cells is only seen in VEGF and Ad.GFP containing Matrigel. Picture 2 of B displays an area with a high number of invaded cells close to the outer cell layer on the left border. C, total cells in sections of the Matrigel plugs were quantitated as described under "Materials and Methods." Results obtained from two experiments with duplicate plugs are displayed as mean values \pm S.D.

duced in endothelial cells by VEGF and this induction is mediated by the PKC/MEK/ERK cascade and followed by the up-regulation of the TF gene (7, 8). Since EGR-1-mediated transcription is negatively controlled by a corepressor, NAB2 (16), we analyzed NAB2 expression in endothelial cells. The obtained data support an inhibitory role of NAB2 for VEGF-induced transcription. However, whereas in nerve PC12 cells stimulated by nerve growth factor (9) or in bovine aortic smooth muscle cells induced by phorbol 12-myristate 13-acetate (17) NAB2 protein levels were initially very low and peaked at 2 h after exposure to stimuli; in endothelial cells significant basal NAB2 levels were present and declined rapidly following serum starvation. Furthermore, VEGF-regulation included a further down-regulation phase that was followed by an up-regulation beginning 60 min after VEGF exposure. These kinetics suggest that the down-modulation of NAB2 allows full EGR-1 activity and that the following up-regulation phase ensures that EGR-1 activity will be transient and turned off again.

We have previously shown that TF is an example of a protein that is induced by VEGF mainly via EGR-1 (7). TF has been proposed to possess, in addition to its role as initiator of the

coagulation cascade, important functions for cell adhesion and signaling (41) and has been reported to be expressed on tumor endothelium (42). Although the potential contribution of endothelial TF expression to angiogenesis has so far not been clarified, induced TF expression can be used as a readout for modulations on the level of transcriptional activity mediated by EGR-1. Therefore we have tested whether overexpression of NAB2 can modulate VEGF-triggered TF promoter activity. The results show that NAB2 is able to completely inhibit TF reporter gene activity triggered by VEGF. Since the used -330-bp TF promoter fragment includes binding sites for NF κ B, NFAT, AP-1, and Sp1 transcription factors (23, 43, 44), these data underline the importance of the EGR-1/NAB2 balance for VEGF-mediated TF reporter gene induction.

This is in line with findings indicating an important function of EGR-1 in the regulation of growth and differentiation by growth factors. PKC-dependent EGR-1 activation has been implicated in some other cell types in differentiation processes. Examples are the nerve growth factor-induced differentiation of nerve cells and the IL-3 and GM-CSF-triggered differentiation of hematopoietic progenitor cells (9, 10). In regard to angiogenesis, it is possible that sprouting and tube formation starting from mature endothelial cells include regulatory mechanisms similar to angioblast differentiation (2), and there is further evidence that the recruitment of angioblasts from the circulation and their differentiation plays a role for vessel formation in the adult. VEGF-induced and EGR-1-mediated gene transcription could be one of the mechanisms important for both processes.

However, the absence of an abnormal vascular phenotype in unchallenged EGR-1 null mice (45) suggests that efficient back-up mechanisms are available or get activated in knockout animals. In this respect a role for EGR-2 and EGR-3 appears possible. Since the regulatory R1 domain, which is involved in the binding of NAB2, is also present in EGR-2 and EGR-3, we have tested expression of EGR-2 in HUVEC, but it was almost undetectable by Western blotting and not inducible by VEGF. In the case of EGR-3 significant levels were detected but did not change following VEGF treatment.² Therefore it appears that at least in cell culture assays using normal endothelial cells mainly EGR-1 can be considered as a mediator of VEGF-induced gene transcription.

To further substantiate the role of EGR-1 and NAB2 for angiogenic responses of endothelial cells, we have prepared adenoviruses overexpressing NAB2. In line with a more general role of EGR-1 for VEGF-regulated genes Ad.NAB2 strongly inhibited, in addition to the induction of TF mRNA, also the up-regulation of mRNAs for VEGFR-1 and uPA. Both genes have previously been described to contain functional EGR-1 binding sites in their promoters (12, 13) and to fulfill important roles during angiogenesis. Although VEGFR-1 has been proposed initially to function mainly as an inert decoy by regulating availability of VEGF, more recent data demonstrate an important contribution to angiogenesis (46). The responsiveness of endothelial cells to VEGF is amplified by up-regulating PlGF and VEGFR-1 in many pathological disorders. The uPA/uPAR system contributes to cell migration through the activation of signaling pathways, extracellular proteolysis, cell adhesion, and chemotaxis (14). Pericellular proteolysis and plasmin generation is an important component of the ability of the endothelial cell to cleave linkages to the extracellular matrix and other cells, to degrade basement membrane barriers, and thus to invade surrounding tissue and fibrin clots. As such Flt-1 and uPA are likely contributors to the migration and invasion of endothelial cells in the angiogenesis models used in this study. By inhibiting the up-regulation of at least three

genes with importance for processes essential for pathological angiogenesis, NAB2 seems to fulfill the criteria for a key negative regulator of angiogenesis.

The inhibition of VEGFR-1 and uPA expression was observed even when a combination of VEGF and bFGF was used. Similar to VEGF, bFGF has previously been described to be a strong inducer of EGR-1 (32). We have used bFGF in addition to VEGF in certain experiments since this factor is a known potent inducer of endothelial cell proliferation, migration, and angiogenesis *in vitro* and *in vivo* (47) and contributes to tumor angiogenesis (48). Inhibition of VEGF and bFGF-triggered induction indicates a broad activity of NAB2 irrespective of the angiogenic inducer.

In line with an inhibition of genes and processes important for angiogenesis, infection with Ad.NAB2 inhibited tubule formation on Matrigel and sprouting in fibrin gels. Whereas tubule formation on Matrigel is observed without added growth factors, since several growth factors including bFGF are present in the Matrigel, sprouting of endothelial cells in fibrin gels was dependent on the addition of growth factors. Our data show that AdNAB2 inhibited the number of sprouts specifically induced by VEGF. These observations suggest a significant contribution of EGR-1 to the angiogenic response of endothelial cells.

Finally, we have evaluated the NAB2 adenovirus in the murine Matrigel model, which is a widely used model to evaluate migration, invasion, and formation of vessel-like structures *in vivo* (31, 49). This model evaluates the more complex effects of NAB2 expression in several cell types. In accordance with an important role of transcriptional processes regulated by EGR-1 and NAB2 for invasion and angiogenesis *in vivo*, we observed that the invasion of cells was strongly inhibited when Ad.NAB2 was included in the Matrigel, and the occurrence of vessel-like structures, as observed in control VEGF-containing plugs, was largely prevented. Generally the system is characterized by the formation of a fibroblast cell-like wall around the plug. A massive invasion of cells into the plug is observed only when the Matrigel is supplemented with growth factors. Part of the invading cells are endothelial cells, which build rod-like and unordered vessel-like structures as has been previously described (31). The cell invasion is likely triggered indirectly by VEGF-stimulating endothelial and possibly monocytic cells to produce additional factors with activities on several cell types and by direct stimulation of migration of endothelial cells. In addition, VEGF diffusing out of the gel may induce endothelial cells of the vessels in the outer cell wall to become leaky and to produce uPA and proteases facilitating cellular invasion. The inhibition observed *in vivo* was more strongly pronounced than the effects in the *in vitro* models supporting that NAB2 exerts its effects on several mechanisms in different cell types. Although we can not exclude that NAB2 might interact with and also inhibit other transcription factors, these data support the view that the EGR-1 pathway contributes at multiple steps to invasion and angiogenesis.

One of these steps could be, as previously reported, the EGR-1-dependent production of angiogenic growth factors by smooth muscle cells, which can be inhibited by NAB2 (18). Whereas our work is the first report to show a direct inhibition of early angiogenic mechanisms of endothelial cells *in vitro*, it is likely that interference at several levels contribute to inhibition *in vivo*. In summary, our results suggest that modulation of EGR-1 activity by NAB2 is important for physiological and pathological angiogenesis and, based on the preliminary observation that NAB2 may have little pathological consequences, could be a promising modality for gene therapies of diseases with excess angiogenesis.

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