Inhibition of Vascular Permeability Factor/Vascular Endothelial Growth Factor-mediated Angiogenesis by the Kruppel-like Factor KLF2*§

Received for publication, May 13, 2005, and in revised form, June 14, 2005 Published, JBC Papers in Press, June 26, 2005, DOI 10.1074/jbc.C500200200

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The Kruppel-like factor KLF2 was recently identified as a novel regulator of endothelial pro-inflammatory and pro-thrombotic function. Here it is shown that overexpression of KLF2 potently inhibits vascular permeability factor/vascular endothelial growth factor (VEGF-A)-mediated angiogenesis and tissue edema in the nude ear mouse model of angiogenesis. In vitro, KLF2 expression retards VEGF-mediated calcium flux, proliferation and induction of pro-inflammatory factors in endothelial cells. This effect is due to a potent inhibition of VEGFR2/KDR expression and promoter activity. These observations identify KLF2 as a regulator of VEGFR2/KDR and provide a foundation for novel approaches to regulate angiogenesis.

Angiogenesis, the outgrowth of new vessels from preexisting blood vessels, is an important feature of both normal physiology and pathologic states including chronic inflammatory diseases and tumor development (1–3). The growth factor VEGF-A† is a key regulator of physiologic and pathologic angiogenesis (4). Originally identified on the basis of its ability to induce vascular permeability (5), VEGF-A is now recognized as a potent inducer of endothelial proliferation, migration, and survival. Furthermore, VEGF-A also acts as a proinflammatory cytokine and induces the expression of a number of molecules implicated in regulating angiogenesis such specific enzymes (e.g. cyclooxygenase-2 (COX-2)), adhesion molecules (e.g. E-selectin, VCAM-1) (6), and pro-coagulant factors (e.g. tissue factor) (7).

The effects of VEGF-A and its family members are mediated by three structurally related receptor tyrosine kinases termed VEGFR1/Flt-1, VEGFR2/KDR/Flk-1, and VEGFR3/Flt4 (8–13). Among these three receptors, VEGFR2 has emerged as the main receptor mediating VEGF-A effects related to angiogenesis such as endothelial cell proliferation, migration, and pro-inflammatory activation. In contrast, VEGFR1 is thought to mediate inhibitory and/or decoy effects (14) (15) in vascular endothelial cells. Finally, VEGFR3 is mainly expressed in lymphatics and regulates aspects of lymphatic endothelial cell biology (13). The importance of VEGF-A/VEGFR2 axis is further underscored by the fact that both ligand and receptor levels is increased in pathologic states such as tumor beds (16–20). It follows that identification of mechanisms that may reduce the expression of either ligand or receptor may serve as the basis for inhibiting angiogenesis in pathologic states.

The Sp/Kruppel-like factor (KLF) family of transcription factors is a subclass of the zinc-finger family of transcriptional regulators implicated in the regulation of cellular growth and differentiation (21). To date 20 members have been identified that include 4 Sp factors (Sp1–4) and 16 KLF factors (KLF1–16) (22). Members of this family can bind with varying affinities to the same DNA sequences (termed GC-box or CACCC element) and varying transcriptional activities. Furthermore, members of this family can modulate each other’s function through a number of distinct mechanisms such as regulating each other’s expression or through direct interaction (22–24). One member of this family, KLF2 (25), is strongly expressed in endothelial cells and required for normal vessel formation (26). More recently, our group has provided evidence that KLF2 regulates endothelial gene expression and pro-inflammatory activation (27, 28). These studies demonstrate that overexpression of KLF2 can strongly attenuate the cytokine-mediated induction of pro-inflammatory targets such as VCAM-1, E-selectin, and tissue factor (27, 28). As such, we reasoned that KLF2 may regulate aspects of angiogenesis. In this study we provide evidence that KLF2 strongly inhibits VEGF-A-mediated angiogenesis via a reduction in VEGFR2 expression and promoter activity.

EXPERIMENTAL PROCEDURES

Reagents—VEGF165 and bFGF were obtained from R&D systems, Minneapolis, MN. [3H]Thymidine was from Amersham Biosciences. The antibodies were obtained from the following sources: anti-VEGFR2 (Santa Cruz Biotechnology), anti-FLT1 (Santa Cruz Biotechnology), anti-CD34 (Abcam), and anti-GFP (CLONTECH).

Cell Proliferation Assay—HUVECs (2 × 10⁴) were seeded in 24-well plates cultured for 24 h in EGM. Ad-KLF2 or empty vector (10 m.o.i.) was added, and after 24 h the cells were serum-starved (0.1% serum) overnight and then treated with VEGF165 (10 ng/ml). After culture for 20 h, 1 mCi of [3H]Thymidine was added to each well, and 4 h later, cells were washed with chilled in phosphate-buffered saline, fixed with 100% cold methanol, and collected for the measurement of trichloroacetic acid-precipitable radioactivity (29, 30). Experiments were repeated at least three times each in triplicate.
Western Blot Analysis—HUVECs were cultured in EGM overnight and Ad-KLF2 or empty vector was added (10 m.o.i.). Cell lysates in radioimmune precipitation assay buffer along with protease inhibitors were collected after 24 and 48 h by centrifugation at 14,000 × g for 10 min at 4 °C. Supernatant was collected and suspended in 4X sample buffer, boiled for 5 min, and run on a Tris-glycine SDS gel. Experiments were repeated at least three times.

Intracellular Ca²⁺ Release—HUVECs were cultured in EGM overnight, and Ad-KLF2 or empty vector (Ad-GFP) was added (10 m.o.i.). The cells were serum-starved overnight, loaded with Fura-2 AM and then stimulated with 10 ng/ml VEGF165 (15). Intracellular Ca²⁺ concentrations were measured with the DeltaScan illumination system (Photon Technology International) using Felix software. Experiments were repeated at least three times. For VEGFR1 blocking studies, the antibody MF-1 was used at final concentration of 2 μg/ml. In the case of studies using the chimeric EGFR/VEGFR2 receptor (termed EGDR), HUVECs were retrovirally infected 1 day prior to infection with KLF2.

Nude Mouse Ear Experiments—6–8-Week-old nude mice were purchased from the NCI, National Institutes of Health. A non-replicating adenoviral vector was engineered to express the predominant (164 amino acid) murine isoform of VEGF-A as described previously (31). The KLF2 adenovirus was constructed as described previously (27). Immediately prior to use, the adenovirus was desalted using Quick Spin High Capacity G-50 Sephadex columns (Roche Applied Science) and immediately prior to use, the adenovirus was desalted using Quick Spin High Capacity G-50 Sephadex columns (Roche Applied Science) and diluted in phosphate-buffered saline, 3% glycerol, 0.5 × 10⁻⁶ plaque-forming units of Ad-VEGF virus or empty vector (5) in a volume of 10 μl was injected intradermally into the mouse ears. These mice also received two injections of Ad-KLF2 and empty vector 2 days prior and 2 days post-Ad-VEGF injection. Animals were sacrificed on day 7 post-Ad-VEGF administration, and ears were collected and processed for 1-μm Epon sections and paraffin embedding. All experiments were performed under protocols approved by the Beth Israel Deaconess Medical Center Institutional Animal Care and Use Committee. Ad-VEGF was kindly provided by Dr. Hal Dvorak and Dr. Janice Nagy.

Transfection Studies—Transient transfection studies were performed using FuGENETM 6 reagent (Roche Applied Science) according to instructions by the manufacturer in bovine aortic endothelial cells. A total of 1 μg of plasmid DNA was used in transfections, and total DNA was always kept constant. Cells were harvested 48 h after transfection at which time they were nearly confluent. Then assays were performed for luciferase activity and normalized to β-galactosidase activity in each sample.

Gel-shift Assays—Nuclear extracts were harvested form HUVECs infected with control or KLF2 virus and gel-shift studies performed as described previously (27, 32). The supershift antibody for Sp1 was obtained from Santa Cruz Biotechnology and the anti-KLF2 antibody from J. Leiden (Abbott Laboratories).

RESULTS

Adenoviral KLF2 Overexpression Inhibits VEGF-mediated Angiogenesis—To assess the effect of KLF2 on VEGF-A-mediated angiogenesis we employed the nude ear mouse model (33). As shown in the protocol in Fig. 1A, we injected control (Ad-GFP) or KLF2 adenovirus (Ad-K2) 2 days before and after injection of Ad-VEGF-A in nude mice. Animals were sacrificed at 1 week after injection of VEGF-A. In the absence of VEGF-A, injection of Ad-GFP and Ad-K2 did not reveal any significant effect on the vasculature. As expected a robust increase in vascularity and edema in the ears of mice treated with Ad-GFP in response to VEGF-A (Fig. 1B, right upper three panels labeled Mouse 1–3). In contrast, we noted a marked attenuation of the angiogenic response (Fig. 1B, right lower three panels labeled Mouse 1–3) and tissue edema in Ad-K2-treated ears. Histopathologic examination verified that adenoviral overexpression successfully infected endothelial cells in vivo (Fig. 1, C, middle panel, and D, right panels). Consistent with the data in Fig. 1B, comparison of Ad-GFP versus Ad-K2 ears revealed a marked reduction in CD34⁺ staining, a marker of endothelial cells (Fig. 1, C and D, first panel) and ear thickness apparent (compare Fig. 1C, compare upper and lower panels). These observations regarding vessel density and tissue edema were also verified by microscopy of Giemsa-stained 1-μm Epon sections of the ear (Fig. 1C, far right panels).

KLF2 Inhibits VEGF-mediated Activation of Endothelial Cells—VEGF-A is known to confer pro-proliferative and pro-inflammatory effects to endothelial cells (4, 6). To gain greater insight into how KLF2 inhibits VEGF-A effects in endothelial cells we first assessed for effect on calcium flux, a sign of cellular activation. As shown in Fig. 2A, adenoviral overexpression of KLF2 in endothelial cells strongly attenuated intracellular calcium release, a sign of VEGFR2/KDR activation. In contrast, KLF2 was unable to inhibit signaling via a chimeric receptor consisting of the extracellular domain of the EGFR receptor fused to the transmembrane/intracellular domain of VEGFR2 (supplemental Fig. 1). We next assessed effects on cellular proliferation by assessing thymidine uptake. As shown in Fig. 2B, in the presence of control adenovirus (Ad-GFP), VEGF induced thymidine incorporation ~2.3-fold. In contrast, overexpression of KLF2 strongly attenuated VEGF-A-mediated thymidine uptake (Fig. 2B).

As discussed above, VEGF-A is also known to induce pro-inflammatory genes in endothelial cells that, in turn, have been implicated in promoting the angiogenic response. To determine whether KLF2 can alter this effect of VEGF-A, we assessed the effect of KLF2 overexpression on VEGF-A-mediated induction of...
VCAM-1, tissue factor, and COX-2. As shown in Fig. 2C, the KLF2 strongly attenuated the VEGF-A-mediated mRNA induction of these factors in endothelial cells (Fig. 2C).

**KLF2 Inhibits Expression of the VEGFR2 Receptor**—To determine the molecular basis of this effect we next assessed for effects on VEGF receptor expression. As shown in Fig. 3, A and B, KLF2 overexpression inhibited VEGFR2 mRNA and protein expression. This effect was specific as, in contrast, a mild induction of VEGFR1 expression was observed. However, this induction was not responsible for the inhibitory effect of KLF2 on endothelial activation because a blocking antibody to VEGFR1 did not alter KLF2-mediated inhibition of calcium flux in response to VEGF (supplemental Fig. 2).

KLF2 is a transcription factor that can regulate gene expression via both DNA binding-dependent and -independent means (27, 28). As a first step toward understanding the molecular basis for KLF2 ability to inhibit VEGFR2 expression, we assessed its effect on the VEGFR2 promoter (34). As shown in Fig. 4A, KLF2 strongly inhibited VEGFR2 promoter activity. A combination of 5′ and 3′ promoter deletion studies demonstrated that this inhibitory effect was mediated by a region between −95 and +5 bp. Previous studies have implicated several Sp1 sites contained within this region as critical for basal promoter activity (35). As shown in Fig. 4B, KLF2 (but not the zinc finger region alone) inhibited the −95 to +5 bp VEGFR2 promoter as well as an Sp1 concatamer (Fig. 4B, right panel). These data suggested that KLF2 might inhibit VEGFR2 promoter activity via effects on Sp1. In principle this may occur through effects on Sp1 expression or function (i.e. binding to DNA). Overexpression of KLF2 did not significantly affect Sp1 protein expression (data not shown). To test the effect of KLF2 on Sp1 directly, gel-shift studies were undertaken using nuclear extracts from HUVECs infected with Ad-GFP or Ad-KLF2. As shown in Fig. 4C, in Ad-GFP-infected cells robust Sp1 binding is observed. The authenticity of this band was verified by competition and antibody experiments resulting in immunodepletion of this complex. In the presence of KLF2, the Sp1 band is markedly attenuated and the emergence of a faster migrating complex is observed. This new complex was immunodepleted by an anti-KLF2 antibody suggesting that KLF2 was contained within this faster migrating complex. These data suggest that KLF2 can bind to the Sp1 site and thereby attenuate VEGFR2 promoter activity.

In this study, we identify the transcription factor KLF2 as a potent inhibitor of VEGF-mediated angiogenesis. Our studies also suggest that this effect is mediated through inhibition of VEGFR2 and, as a consequence, the pro-proliferative and pro-inflammatory effects of VEGF. Finally, we provide evidence that the molecular basis for inhibition of VEGFR2 expression is through competition for binding between KLF2 and a known transcriptional activator of the VEGFR2 promoter, Sp1.

The VEGFR2 is the principal receptor implicated in transducing many of the pro-angiogenic effects of VEGF-A. Although it is expressed at low levels in adulthood (36), VEGFR2 expression may be induced during pathologic states such as chronic inflammation, tumor growth, and wound repair (14). For example, pro-inflammatory cytokines can induce VEGFR2 expression. Subsequently, binding to VEGF-A can enhance endothelial cell proliferation, survival, and pro-inflammatory gene expression. We provide evidence that KLF2 can inhibit endothelial cell activation and proliferation (Fig. 2, A and B). Furthermore, with respect to pro-inflammatory gene activation, we find that KLF2 can largely abrogate the VEGF-A-mediated induction of VCAM-1, tissue factor, and COX-2. Previous studies suggest that the proinflammatory effects of VEGF occur, at least in part, through activation of NF-κB (37, 38). In this regard, our previous studies demonstrating that KLF2 can prevent cytokine-mediated activation of the endothelium via inhibition of NF-κB function are particularly relevant (27). Indeed, it is quite likely that the ability of KLF2 to attenuate the proinflammatory effects of VEGF is due to inhibition of NF-κB activity.

Our studies also identify KLF2 as a novel negative regulator of VEGFR2 through competition with Sp1 for binding to the VEGFR2 promoter. The importance of Sp1 in the regulation of VEGFR2 is well established. Elegant studies by Patterson et al. (34, 35) were the first to implicate Sp1 as important in the basal expression and cell type-specific expression of the VEGFR2 promoter. In addition, pharmacologic inhibition of Sp1 binding to DNA has been shown to prevent the cytokine-mediated induction of VEGFR2 (37). Multiple previous reports support a cooperative and antagonistic relationship between KLFs and Sp1 factors. For example, KLF4 and Sp1 can synergistically activate the laminin γ1 chain promoter (39). Conversely, competition for DNA binding between Sp1 and KLFs has also been reported in the control or the human pre-α inhibitor gene transcription (40). Our deletion studies of the VEGFR2 promoter suggested that KLF2 repressive effects were mediated between −95 and +5 bp, a region densely packed with several Sp1 sites (Fig. 4B). Consistent with this
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Fig. 4. KLF2 inhibits the VEGFR2 promoter. A, effect of KLF2 on VEGFR2 promoter activity using various deletion constructs. B, effect of KLF2 on VEGFR2 (−95 to +5 bp) promoter activity as well as Sp1 concatemer. ZnF, zinc finger. C, effect of KLF2 on Sp1 binding to the VEGFR2 promoter by gel-shift analysis. The Sp1 and KLF2 bands (Fig. 4C). These gel-shift studies were also notable for the emergence of a new faster migrating DNA-protein complex that contained KLF2 (Fig. 4C). Taken together our observations support the possibility that KLF2 displaces Sp1 from the VEGFR2 promoter resulting in a reduction in transcriptional activity.

In sum, this study identifies KLF2 as a novel and potent inhibitor of VEGF-A-mediated angiogenesis. These observations coupled with a recent report implicating KLF2 as a regulator of tumor cell proliferation (41) raise the possibility that KLF2 may be an attractive target for therapies aimed at regulating angiogenesis in disease states.

Acknowledgment—We thank Dr. Harold F. Dvorak for his encouragement and support.

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