Response Gene to Complement 32, a Novel Hypoxia-Regulated Angiogenic Inhibitor

Xiaojin An, BS; Yi Jin, BS; Hongnian Guo, PhD; Shi-Yin Foo, MD, PhD; Brittany L. Cully, BA; Jiaping Wu, MD; Huiyan Zeng, PhD; Anthony Rosenzweig, MD; Jian Li, MD, PhD

Background—Response gene to complement 32 (RGC-32) is induced by activation of complement and regulates cell proliferation. To determine the mechanism of RGC-32 in angiogenesis, we examined the role of RGC-32 in hypoxia-related endothelial cell function.

Methods and Results—Hypoxia/ischemia is able to stimulate both angiogenesis and apoptosis. Hypoxia-inducible factor-1/vascular endothelial growth factor is a key transcriptional regulatory pathway for angiogenesis during hypoxia. We demonstrated that the increased RGC-32 expression by hypoxia was via hypoxia-inducible factor-1/vascular endothelial growth factor induction in cultured endothelial cells. However, overexpression of RGC-32 reduced the proliferation and migration and destabilized vascular structure formation in vitro and inhibited angiogenesis in Matrigel assays in vivo. Silencing RGC-32 had an opposing, stimulatory effect. RGC-32 also stimulated apoptosis as shown by the increased apoptotic cells and caspase-3 cleavage. Mechanistic studies revealed that the effect of RGC-32 on the angiogenic response was via attenuating fibroblast growth factor 2 expression and further inhibiting expression of cyclin E without affecting vascular endothelial growth factor and fibroblast growth factor 2 signaling in endothelial cells. In the mouse hind-limb ischemia model, RGC-32 inhibited capillary density with a significant attenuation in blood flow. Additionally, treatment with RGC-32 in the xenograft tumor model resulted in reduced growth of blood vessels that is consistent with reduced colon tumor size.

Conclusions—We provide the first direct evidence for RGC-32 as a hypoxia-inducible gene and antiangiogenic factor in endothelial cells. These data suggest that RGC-32 plays an important homeostatic role in that it contributes to differentiating the pathways for vascular endothelial growth factor and fibroblast growth factor 2 in angiogenesis and provides a new target for ischemic disorder and tumor therapies. (Circulation. 2009;120:617-627.)

Key Words: angiogenesis • apoptosis • endothelium • gene therapy • hypoxia • ischemia

Hypoxia/ischemia leading to cellular dysfunction is a complex process that involves numerous factors. In endothelial cells, it has been suggested that hypoxia induces angiogenesis via upregulation of hypoxia-inducible factor-1α (HIF-1α) protein that in turn activates the transcription of several angiogenic genes, including vascular endothelial growth factor (VEGF), VEGF receptors flt-1 and neuropilin-1, and angiopointin-2.1 In contrast, hypoxia also directs endothelial cells toward apoptosis, which is caused by changes in p53 protein levels.2 Although functional genomic analyses have revealed specific genes that are involved in hypoxic signaling,3 gene regulation for maintaining endothelial cell homeostasis between angiogenesis and apoptosis under hypoxic conditions is still not fully understood.

Clinical Perspective on p 627

The response gene to complement 32 (RGC-32) protein is localized in the cytoplasm and physically associates with cyclin-dependent kinase p34(CDC2), which increases the kinase activity to induce quiescent aortic smooth muscle cells to enter the S phase4 and plays an important role in cell proliferation by downregulating cell cycle inhibitors.5 However, studies of RGC-32 in tumor cell growth have yielded different results. Another group found that RGC-32 showed p53-dependent transcriptional activity that suppressed tumor cell line growth via the arrest of mitotic progression.6 The disparities between these reports may be due to different RGC-32 functions in different cell types.7 However, nothing is known about the regulation of RGC-32 activity in hypoxia and angiogenesis.
We report here that RGC-32 is a novel hypoxia-inducible gene. We determined that HIF-1 and VEGF significantly increased RGC-32 expression in hypoxia and ischemia. Our results suggest that HIF-1/VEGF-induced RGC-32 expression did not follow the canonical VEGF pathway to promote angiogenesis. Rather, overexpression of RGC-32 in endothelial cells inhibited cell proliferation and migration via downregulation of another major angiogenic protein, fibroblast growth factor 2 (FGF2), to further affect cyclin E. In addition, RGC-32 promoted unstable vascular structure by increasing the number of apoptotic cells. This work reveals a novel function for RGC-32 as a potential hypoxia-inducible inhibitor of angiogenesis and a mediator between VEGF and FGF2 pathways.

Methods

Cell Culture and Hypoxia
Human umbilical vein endothelial cells (HUVECs) (Lonza, Walkersville, Md) were cultured in EBM2 containing 2% FBS with growth supplements. Hypoxia (<1% O2) was induced with a modular incubator chamber (Billups-Rothenberg, San Diego, Calif). CoCl2, dimethyloxalylglycine (DMOG), and 3,4-dihydroxybenzoate (3,4-DHB) (Sigma, St Louis, Mo) were used to mimic hypoxia.

Retroviral Construction

The RGC-32 (NM_014059.1) coding sequence was cloned into the pBABE-Puro vector (Clontech, Palo Alto, Calif) for retrovirus packaging. Total RNA from HUVECs was extracted with TRIzol Reagent (Invitrogen, Carlsbad, Calif), and cDNA was synthesized by reverse transcription. A 420-bp RGC-32 cDNA was cloned with primers (forward, 5'-CCCCTC-GCGCTGTGCGAGTTTGAC-3' and reverse, 5'-CCCCCTG-GGCAGCAGATT-3') for probing. Northern blots were performed (forward primer, 5'-CCCCTC-GCGCTGTGCGAGTTTGAC-3' and 5'-AGGCAA-3', respectively. RGC-32 primers were designed to the human sequence at GeneBank: 28984) encompassing basal elements of the human RGC-32 promoter. The RGC-32 promoter construct cotransfected with HIF-1α or β and 5'-AGCCAGTTTCATCGCTGGAGGGAAGAGCT-3' and 5'-TTCCGCTGGGAGGCTGGTGTGAGC-3' were confirmed to have knockdown efficiency by Northern blotting. Another duplex of siRNA that is not targeted to any human genes was used as negative control. HUVECs were transfected with siRNA at a final concentration of 50 nmol/L with Lipofectamine 2000 (Invitrogen).

Growth Factor and Inhibitor Studies

Selective growth factors and cytokines, including VEGF, FGF2, transforming growth factor (TGF-β), interleukin 1β, tumor necrosis factor-α, and platelet-derived growth factor-BB (PDGF-BB; Sigma), were added to the HUVECs. To specifically block secreted VEGF, the monoclonal anti-human VEGF-neutralizing antibody, murine isotype control IgG (R&D Systems, Minneapolis, Minn), or SU4132 (Sigma), a suppressor of VEGFR2 activation that inhibits VEGFR2 phosphorylation, was preincubated with HUVECs at 37°C for 1 hour before hypoxia.

Western Blot Analysis

HUVECs were lysed in radioimmunoprecipitation buffer and blotted with antibodies as described. Detailed information on antibodies and materials is given in the online-only Data Supplement.

Proliferation and Migration Assays

HUVECs were seeded in 12-well plates (3×10^4 cells per well) and incubated for 5 hours counting from day 1. At the indicated time point, culture was stopped by freezing with 4% paraformaldehyde for 15 minutes and subsequently stained with 0.1% crystal violet dissolved in 10% ethanol for 20 minutes. After washing, 10% acetic acid was added to the cells, and absorbance was measured at 590-nm wavelength. HUVEC migration was measured by “wounding” assay. Cells were grown to subconfluence in 12-well plates (10^5 cells per well) and then starved for 24 hours in 0.5% serum. The cell layer was scratched with a pipette tip, producing a gap ~2 mm wide. The gap width was measured at marked locations from images taken by an inverted microscope (TMS-F; Nikon, Tokyo, Japan) immediately after the scratching and again 6 hours later at the same marked locations as described.

Apoptosis Assay

Apoptotic cells were analyzed with the Vybrant Apoptosis Kit 13 for flow cytometry and an annexin V kit for Matrigel (Invitrogen). The cells were incubated with PO-PRO-1 and 7-AAD on ice for 30 minutes. Stained HUVECs were analyzed by FACScan flow cytometry using BD FACSDiva software (BD PharMingen, San Diego, Calif). Cells that were PO-PRO-1 positive and 7-AAD negative were counted for apoptosis to exclude necrotic cells. Caspase inhibitor Z-VAD-FMK was from Calbiochem (San Diego, Calif).

Matrigel Analysis

BD Matrigel Matrix Growth Factor Reduced (BD Biosciences, San Jose, Calif) was coated on a prechilled 24-well culture plate on ice. After Matrigel solidification for 30 minutes, HUVECs were plated 10^4 cells per well with EBM2. Twenty-four to 48 hours later, the extent of network formation was observed and photographed. In vivo Matrigel angiogenesis assays were carried out as described. In total, 1×10^6 PT67 cells were infected with retroviruses expressing full-length RGC-32 or GFP cDNAs. The infected PT67 alone or GFP cDNA was used as negative control. HUVECs were transfected with siRNA at a final concentration of 50 nmol/L with Lipofectamine 2000 (Invitrogen).
**Hind-Limb Ischemia Model**

Hind-limb ischemia in 12-week-old male FVB mice was induced by ligation of the femoral artery as described.11 Immediately after surgery, animals received in the right hind limb an intramuscular injection consisting of 50 μL of either Retrovirus pBMN-GFP (1×10^6 plaque-forming units per 1 mL) or pBMN-GFP-RGC-32 (1×10^6 plaque-forming units per 1 mL) at the quadriceps. Tissue section was performed to assess the expression of retrovirus-encoded GFP from injected ischemic muscle specimens 7 and 28 days after surgery. Blood flow was measured with a MoorLDI2 infrared scanner.

**Tumor Analysis**

We implanted 1×10^6 SW480 cells subcutaneously into BALB/c nude mice. Animals were inspected daily for tumor development. Tumors were measured with a digital caliper, and volume was calculated by length×width^2×0.52, which approximates the volume of an elliptical solid. After 2 weeks, mice harboring subcutaneous tumors (~100 mm^3) were randomly divided into 2 groups (n=4) and treated with an intraneoplastic inoculation of 1×10^6 plaque-forming units of retrovirus of either pBMN or RGC-32 every 3 days. Mice were killed on postinjection day 13. All animal studies were approved by the Institutional Animal Care and Use Committee of Beth Israel Deaconess Medical Center in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care guidelines.

**Immunostaining**

Sections of in vivo Matrigel blocks, hind-limb ischemia quadriceps, and colon tumor tissues were fixed in 4% paraformaldehyde for capillary density studies. Immunohistochemistry and immunofluorescence were performed with the CD31 antibody (BD PharMingen) as described previously.10

**Statistics**

Results are expressed as mean±SEM or SD on the basis of triplicate experiments. Statistical analysis used ANOVA and Student t test (2 tailed). A value of P<0.05 was considered statistically significant. The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

**Results**

**RGC-32 Expression in Hypoxia**

To investigate the role of RGC-32 in angiogenesis, we initially examined RGC-32 expression under hypoxia compared with normoxia in endothelial cells. A significant increase in RGC-32 gene expression occurred from 3 hours (2.21±0.12-fold) to 36 hours (3.47±0.57-fold) and returned to baseline after 48 hours in hypoxia (Figure 1A), which also was confirmed in the quantitative polymerase chain reaction assay (Figure 1A of the online-only Data Supplement). Immunoblot analysis shows that RGC-32 protein response to hypoxia began at 6 hours and remained at a high level until 48 hours (Figure 1B). mRNA half-life assay indicated that the RGC-32 mRNA half-life was prolonged under hypoxia (6.05 versus 1.96 hours; Figure IB of the online-only Data Supplement). These results demonstrate that the increased expression of RGC-32 in response to hypoxia is mediated, at least partially, at the posttranscriptional level, which could reasonably account for its steady elevated protein level.

**HIF-1 and VEGF Mediate the Induction of RGC-32 Expression in Hypoxia**

To determine whether hypoxia-induced RGC-32 mRNA was mediated by HIF-1, HUVECs were exposed to CoCl_2, 3,4-

**Figure 1. Effect of hypoxia on RGC-32 expression.** RGC-32 expression in HUVECs was assessed after various durations of hypoxia, from 1 to 48 hours, with Northern (A) and Western (B) blotting. Both mRNA and protein were upregulated by hypoxia. Results are mean±SEM based on 3 experiments quantified by Image J. *P<0.05.

DHb, or DMOG, which stabilize HIF-1α expression via hydroxylase inhibition.13 RGC-32 mRNA was significantly increased when the HIF-1α protein was protected from degradation (Figure 2A). In addition, cotransfection with the RGC-32 promoter and HIF-1α cDNA led to a significant increase in RGC-32 promoter activity (Figure 2B). Chromatin immunoprecipitation assay revealed direct interaction between HIF-1 and hypoxia response element sites on the RGC-32 promoter (Figure 2C). Thus, hypoxia-induced RGC-32 expression was regulated at both the transcriptional and posttranscriptional levels via HIF-1 or HIF-1 target genes.

Compared with other reported hypoxia responsive genes, RGC-32 showed a steady, prolonged induction, suggesting that some other indirect induction also may be involved in this response. A panel of growth factors and cytokines, VEGF, FGF-2, TGF-β, interleukin-1β, tumor necrosis factor-α, and PDGF, were incubated with HUVECs for 12 hours, and RGC-32 mRNA levels were examined. As Figure 2D shows, VEGF, but not TGF-β or PDGF, significantly induced RGC-32 mRNA expression, whereas tumor necrosis factor-α, FGF2, and interleukin-1β decreased the expression of RGC-32 mRNA. VEGF was markedly induced by hypoxia in HUVECs (Figure 2E), and VEGF-induced RGC-32 expression showed a time and dose dependence (Figure 2F), indicating that VEGF might play an important role in hypoxia-related RGC-32 stimulation. To test this hypothesis, SU4312, a VEGF receptor 2 activation suppressor, was incubated with HUVECs for 1 hour before hypoxia. Hypoxia-induced RGC-32 expression was significantly blocked (Figure 2G, left). In addition, treating HUVECs with anti-VEGF-neutralizing antibody before hypoxia decreased the hypoxia-induced RGC-32 mRNA (Figure 2G, right). Thus, VEGF appears to be the predominant factor for hypoxia induction of RGC-32 in endothelial cells.
RGC-32 Attenuates Angiogenesis In Vitro and In Vivo

To test the hypothesis that RGC-32, as a downstream gene of VEGF, might enhance angiogenesis, RGC-32 cDNA carried by a retrovirus was generated in endothelial cells. RGC-32 proteins were successfully expressed in HUVECs (Figure 3A), and angiogenic activities of RGC-32 (o/e) were examined. Surprisingly, a growth curve assay showed that the RGC-32 (o/e) cells grew significantly more slowly than those infected by retrovirus vector only (Figure 3B). The migration rate of the RGC-32 (o/e) cells was dramatically decreased compared with control cells shown in Figure 3C. To further validate that RGC-32 does not act as an angiogenic enhancer within the VEGF pathways, 3 sequences of siRNA that targeted RGC-32 were synthesized, and 2 of them showed superior knockdown of RGC-32 expression (Figure 3D, left). Figure 3D (middle) demonstrates that the HUVECs decreased the velocity of growth under hypoxic conditions. However, knockdown of RGC-32 by siRNA indicated that the HUVEC growth rate was accelerated in normoxia and significantly enhanced in hypoxic conditions compared with the control siRNA. The migration rate also was accelerated in RGC-32 knockdown HUVECs compared with the control cells (Figure 3D, right).

To further verify the mechanism of action of RGC-32, we used the Matrigel assay to introduce genes of interest into the vascular endothelium in vivo.13 On day 3 after implantation of Matrigel plugs containing various cell mixtures, angiogenic responses were evaluated macroscopically (Figure 3E, top) and by histology and immunohistochemistry for the
endothelial cell marker CD31 (Figure 3E, bottom). Plugs containing only PT67 cells did not significantly induce angiogenesis (Figure 3Ea and 3Ee). However, strong angiogenesis was induced in plugs containing SK-MEL/VEGF cells (Figure 3Ec and 3Eg). When PT67/RGC-32 cells were included in the Matrigel, the angiogenic response induced by SKMEL/VEGF cells was strikingly inhibited (Figure 3Ed and 3Eh). Taken together, these results suggest that RGC-32 does not have proangiogenesis capability; instead, it can inhibit endothelial cell proliferation and migration and VEGF-induced angiogenesis.14

**Figure 3.** Effect of RGC-32 on angiogenesis. A, RGC-32 overexpression in HUVECs was determined by Western blot. B, Growth curve analysis indicates that RGC-32 (o/e) attenuated 51% of endothelial cell proliferation. C, Migration assay for RGC-32 (o/e) shows ~45% lower migrating rates compared with controls. D, Two siRNA sequences of RGC-32 showed knockdown efficiency (left). RGC-32 knockdown increased HUVEC proliferation (middle) and migration (right) compared with negative siRNA controls. E, Macroscopic (top) and CD-31–stained microscopic (bottom) images of the angiogenic response induced 3 days after implantation of Matrigels with VEGF-A165–secreting SKMEL/VEGF cells and PT67 cells packaging pBMN-GFP or pBMN–RGC-32. Note that VEGF-stimulated blood vessels (arrows in c and g) can be blocked by RGC-32 treatment (d and h).

RGC-32 Induces Apoptosis
When the effect of RGC-32 in Matrigel capillary structure formation in vitro was examined, it did not show significant differences at an early stage (10 to 24 hours) between RGC-32 (o/e) and control cells. However, at a late stage (40 hours), the vascular structures formed by RGC-32 (o/e) cells were less stable, but no significant difference was seen when the cells were incubated with the caspase inhibitor Z-VAD-FMK compared with control cells. This indicated that endothelial cell apoptosis took place in RGC-32 (o/e) cells, which was confirmed by the increase of annexin V–positive cells.
PO-PRO-1/7-aminoactinomycin D staining measured the number of apoptotic cells (Figure 4B). The obvious changes were observed after cells had undergone hypoxia for 48 hours. The number of apoptotic cells increased from 7.7% to 14.2% for controls but increased from 8.5% to 22.1% for RGC-32 (o/e) HUVECs. In agreement with the increased apoptotic cell number, there was also a significant increase in cleaved caspase 3 in RGC-32 (o/e) HUVECs after 48 hours of hypoxia (Figure 4C). These results suggest that RGC-32 accelerates hypoxia-induced endothelial apoptosis.

RGC-32 Attenuates FGF2-Related Cyclin E Expression

One possible mechanism that might explain the noncanonical VEGF angiogenic pathway is that RGC-32 could be a VEGF-negative feedback regulator. However, no significant differences were found between RGC-32 (o/e) cells and control cells in terms of phosphorylation of endothelial nitric oxide synthase, Akt, and mitogen-activated protein kinases (ERK and p38) by VEGF stimulation (Figure IIA of the online-only Data Supplement). Examination of 3 major angiogenic pathways in RGC-32 (o/e) cells showed marked decreases in the expressions of the FGF2 23- and 17-kd isoforms (Figure 5A) and RGC-32–downregulated FGF2 expression enhanced by hypoxia (Figure 5B). However, no effect on the FGF2-stimulated signaling pathway was observed (Figure IIB of the online-only Data Supplement). Meanwhile, we examined the protein levels of antiangiogenic genes, including angiostatin and endostatin, but no significant differences were found between RGC-32 (o/e) and control cells (data not shown). These results suggest that RGC-32 had no effect on the VEGF downstream pathway but did influence expression of FGF2.

In light of RGC-32 perturbations of the cell cycle described for malignant transformation, we postulated that RGC-32 might act in conjunction with cyclin in response to the downregulation of FGF2. Figure 5C indicates that RGC-32 inhibited FGF2-stimulated cyclin E but not cyclin B, D, and E2F. VEGF-induced cyclin E also was attenuated in RGC-32 (o/e) cells compared with controls. Collectively, these data suggested that RGC-32 affects the proproliferating pathway of FGF2 through inhibiting the upregulation of cyclin E by...
FGF2 in endothelial cells, which explains the ability of RGC-32 to reduce proliferation.

RGC-32 Impaired Perfusion Recovery After Hind-Limb Ischemia

In mouse hind-limb ischemia, the expression of RGC-32 was markedly induced in all ischemic muscle beds (Figure 6A). To assess whether RGC-32 can affect the normal vascular response to ischemia, RGC-32 and control retroviruses were delivered to quadriceps immediately after the procedure. The expression of the delivered gene was confirmed by GFP (Figure III of the online-only Data Supplement). Blood perfusion in ischemic hind limbs was measured by Doppler analysis on days 0, 7, 14, 21, and 28. The blood flow in the quadriceps area treated with pBMN vector was improved 88% of the nonischemic control at day 14. However, treatment with RGC-32 decreased the blood flow recovery, showing 64% of the nonischemic control (Figure 6B and 6C). The angiographic analysis at days 7 and 28 after ligation of the femoral artery revealed a decreased number of vessels in the RGC-32–treated group (Figure 6D).

RGC-32 Reduced Tumor Angiogenesis

To determine whether RGC-32 has the ability to regulate tumor angiogenesis, an intraneoplastic inoculation of either pBMN or RGC-32 retrovirus started when tumors were 100 mm³. Growth of RGC-32–expressed tumors was greatly inhibited. Compared with the control, the average tumor size in the RGC-32 group recessed by 35% on day 9 and 45% on day 13 (Figure 7A). Additionally, we found that suppression of tumor growth by RGC-32 (Figure 7B) was associated with reduced angiogenesis, particularly with a reduction in number of vessels, which was assessed by CD31 staining (Figure 7C and 7D).

RGC-32–regulated FGF2 and cyclin E also was found in RGC-32–treated tumor tissues. Figure 7E indicates that RGC-32–treated tumor tissue significantly reduced the expression of FGF2, cyclin E, and the endothelial cell marker CD31.

Discussion

The principal finding of this study is that RGC-32 is a hypoxia-induced antiangiogenesis factor in endothelial cells. We demonstrate that the mechanism of HIF-1/VEGF–induced RGC-32 expression inhibits angiogenesis via RGC-32–dependent attenuation of the FGF2 pathway for cyclin E expression. Several lines of evidence support our finding. First, RGC-32 expression was induced by hypoxia at both the mRNA and protein levels. Second, inhibitory studies indicated that hypoxia-induced RGC-32 expression is dependent on HIF-1α stabilization, and HIF-1 is able to stimulate RGC-32 promoter activity. Third, RGC-32 was induced by VEGF but did not affect VEGF signaling. Instead, RGC-32 significantly downregulated FGF2 and attenuated FGF2–dependent cyclin E expression. Finally, the ability of RGC-32 to inhibit endothelial cell proliferation and migration could be
blocked by inhibition of RGC-32. Injection of RGC-32 in mouse hind-limb ischemia and tumor xenograph models can reduce the number of blood vessels in association with downregulation of FGF2 and cyclin E.

Our data provide evidence that hypoxia induced RGC-32 expression via the HIF-1/VEGF pathway. The results for RGC-32 as an inducible gene were consistent with previous reports indicating that RGC-32 mRNA expression is induced by C5b-9 complement activation, steroid hormones, and TGF-β. However, we did not observe TGF-β-induced RGC-32 expression in endothelial cells, although it might result from TGF-β activating RGC-32 through specific Smad and RhoA signaling to initiate cell differentiation in smooth muscle cells.

Unlike other VEGF-induced genes such as COX-2, RGC-32 did not follow the canonical VEGF-induced angiogenic pathway. One possible mechanism that might explain the role of RGC32 during proliferation and vascular growth is that RGC-32 could be a VEGF-negative feedback regulator. Similarly, delta-like ligand 4 (Dll4) was dynamically induced by VEGF, but Dll4 blockade enhanced angiogenic sprouting while suppressing ectopic pathological neovascularization in the retinal vasculature. In the present study, RGC-32 did not influence VEGF-mediated signaling pathways indicated by VEGF-stimulated phosphorylation of endothelial nitric oxide synthase, Akt, and mitogen-activated protein kinase (ERK, P38), which showed no significant differences in RGC-32-overexpressed endothelial cells compared with control cells. We demonstrated in this report that RGC-32 significantly attenuated expression of FGF2, but it did not interrupt the expressions of the FGF receptor 1 and angiopointin-1/Tie2 pathways. The antiangiogenic proteins angiostatin and endostatin also were not regulated by RGC-32.

Although FGF2 is involved in angiogenesis, its expression regulation during hypoxia is poorly documented. In HUVECs, we have observed that FGF2 protein was downregulated in the 23- and 17-kd isoforms under hypoxic conditions. This result contradicted another report showing that FGF2 was induced at a protein level concomitant with a decrease in FGF2 mRNA caused by internal ribosome entry site during hypoxia. However, our data are in agreement with previous reports suggesting that hypoxia induced expression of VEGF but not FGF2. Interestingly, despite the fact that expression of FGF2 was reduced by RGC-32, it had no effect on the FGF2-stimulated signaling pathways.

In contrast to these active constitutive forms, we found in RGC-32-overexpressing cells that cyclin E expression was attenuated in response to FGF2. Cyclin E is one of the major cyclins involved in the G1-to-S-phase transition. This result is consistent with a previous report showing that RGC-32 suppressed the growth of glioma cells via p53 regulation. However, RGC-32 did not directly alter activation of cyclin E in endothelial cells. Our results suggest that RGC-32 inhibits endothelial cell proliferation not by directly interrupting the cell cycle but by influencing FGF2 pathways in which cyclin E and cyclin-dependent kinase 2 and 4 were expressed after FGF2 infusion. Furthermore, FGF2 decreased levels of the cyclin-dependent kinase inhibitor p27 (Kip1) to enhance the association of cyclin E-cyclin-dependent kinase 2.

RGC-32 had no impact on vascular structure formation, but it did decrease its stability. This finding suggests that RGC-32
not only inhibits angiogenesis but also stimulates apoptosis. RGC-32 has been shown to be a direct transcriptional target of p53 in human cells of various tissue origins.\(^6\) Cell cycle regulation and apoptosis are the most important features of p53-dependent tumor suppression. The regulator of FGF2 transcription is a transcriptional repressor that induces glioma cell death by its overexpression, suggesting that it regulates the G1-to-S transition and apoptosis via the p53/p21\(^{\text{null}}\) pathway.\(^{25}\) Thus, downregulation of FGF2 expression by RGC-32 may trigger suppression of cell growth, especially mitotic progression through the p53 pathway in endothelial cells. Moreover, the FGF2 treatment in hind-limb ischemia increased angiogenesis and the number of collateral vessels after ligation of the femoral artery;\(^{26}\) therefore, downregulation of FGF2 expression by RGC-32 could affect the return of blood flow to the ischemic limb and inhibit progressive neovascularization.

As a cell cycle regulator, RGC-32 has shown 2 opposite results for tumor growth. In addition to targeting the G2/M phase transition and suppressing tumor growth,\(^6\) RGC-32 has also reportedly activated cdc2 kinases to induces cell cycle activation.\(^{17}\) These contradictory results have been explained by the possibility that RGC-32 may play dual roles by enhancing cell proliferation and acting as a tumor suppressor gene in certain types of cancers.\(^7\) The model in the present study demonstrated that RGC-32 reduced colon cancer tumor size with a reduced number of vessels. We also observed a significant decrease in VEGF-induced blood vessel growth by RGC-32 in Matrigels. Many stimuli, including hypoxia, can increase a major angiogenic factor (e.g., VEGF) expression in tumor cells, which is correlated with increased microvessel counts and poor prognosis\(^{27}\) in many human cancers. However, hypoxia/ischemia-induced gene regulation in tumor growth is not fully understood. We document here
that RGC-32 has the ability to promote both angiogenesis and proapoptosis in endothelial cells, which results in decreased blood vessel growth during colon cancer proliferation and, in turn, reduced tumor size. Overexpression of FGF2 mRNA was associated with significantly increased risk for tumor recurrence. Targeting FGF receptors and/or FGF signaling can affect both the tumor cells directly and tumor angiogenesis. Our study indicated that decreased FGF2 expression in the RGC-32–treated xenograph model may not be limited to vessels as a result of the FGF signaling involved in both the cancer cells and surrounding vasculature to enhance proliferation and resistance to cell death, thereby enhancing tumor progression. Our results add an additional mechanism, antiangiogenesis, for RGC-32 as a tumor suppressor.

RGC-32 inhibited endothelial cell proliferation and migration. However, it is not clear how this finding may correlate to other recently reported functions such as cell differentiation or inflammation. Further studies are required to determine whether RGC-32 as a hypoxia-inducible gene affects hypoxia/ischemia-related cell dysfunction in other pathways.

Conclusions

We demonstrated for the first time that RGC-32 is a hypoxia-inducible gene dependent on HIF-1 and VEGF. Induced RGC-32 performed antiangiogenic activity through downregulating endothelial cyclin E via the FGF2 pathway. This study reveals the important role that RGC-32 plays in homeostasis of hypoxic endothelial cells. This may contribute to understanding the crosstalk between different angiogenic gene pathways and highlights the potential for ischemic disorder and tumor treatment by targeting RGC-32.

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Disclosures

None.

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**CLINICAL PERSPECTIVE**

We and others have suggested that response gene to complement 32 (RGC-32) is involved in cell cycle regulation. Our data prove the novel ability of the expression of RGC-32 to increase in hypoxia/ischemia and to inhibit angiogenesis in endothelial cells. Ischemia is characterized by reduced blood supply to the organs. Although angiogenesis occurs in response to ischemia, angiogenesis induced by natural compensatory processes is often inadequate. Many unsuccessful clinical trials have tested the proangiogenic potential of vascular endothelial growth factor or fibroblast growth factor, and the role of growth factor feedback molecules in attenuating angiogenic response in ischemic disease is not completely understood. RGC-32 as a downstream gene induced by hypoxia/ischemia and vascular endothelial growth factor possesses antiangiogenesis capability. Inhibiting the negative feedback of vascular endothelial growth factor is a significant potential angiogenic therapy. In addition, given that angiogenesis is an important process in tumor growth, angiogenic factors can block the fundamental requirements of a tumor. Thus, RGC-32 has clinical application for tumor retardation through its inhibition of angiogenesis. We have demonstrated that injection of RGC-32 in the xenograft tumor model resulted in reduced growth of blood vessels that is consistent with reduced colon tumor size. Therefore, it is conceivable that RGC-32 provides a new target for ischemic disorder and tumor therapies.

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SUPPLEMENTAL MATERIAL

Title: Response Gene to Complement 32 (RGC-32), a novel hypoxia-regulated angiogenic inhibitor
Supplemental Methods

Western Blot Analysis

HUVECs were lysed in RIPA buffer (BioProducts Inc, MA) containing cocktailed protease inhibitors (Roche, NJ). Samples were subjected to 12% SDS-PAGE and transferred to polyvinylidene fluoride membranes (Millipore, MA). Membranes were incubated with the indicated antibodies including anti-RGC32 (a kind gift from Dr. Rus, University of Maryland and custom synthesized by Genemed, CA); anti-FGF2, anti-cyclin B1, D1, E, E2F, Tie-2, angiopointin, FGFR1, actin (Santa Cruz Biotechnology, Inc, (Santa Cruz, CA); anti-Akt, pAkt, eNOS, p-eNOS, ERK, pERK, p38, p-p38, VEGF receptor 2, and caspase 3 (Cell signaling, MA); anti-HIF-1α (BD Transduction Laboratories, CA) and anti-vinculin (Sigma, MO) followed by incubation with HRP anti-mouse or anti-rabbit IgG (Calbiochem, CA). The blots were developed using the ECL detection system.
Supplemental Figures

Figure 1

A. RGC-32 half-life was detected by Northern blot after HUVECs were exposed to either normoxia or hypoxia for 5 h before administering actinomycin D (ACD; 5 ug/ml). Total RNA was extracted from HUVECs exposed to normoxia or hypoxia at 1 hour intervals for 5 hours after ACD administration and subjected to Northern blot with a RGC-32 RNA probe. Signals were quantified and plotted as a percentage of the 0-h value against time. Hypoxia extended the half-life of RGC-32 mRNA from 1.96 to 6.05 h.

B. 

Supplementary figure 1. RGC-32 half-life was detected by Northern blot after HUVECs were exposed to either normoxia or hypoxia for 5 h before administering actinomycin D (ACD; 5 ug/ml). Total RNA was extracted from HUVECs exposed to normoxia or hypoxia at 1 hour intervals for 5 hours after ACD administration and subjected to Northern blot with a RGC-32 RNA probe. Signals were quantified and plotted as a percentage of the 0-h value against time. Hypoxia extended the half-life of RGC-32 mRNA from 1.96 to 6.05 h.
Supplementary figure 2. (A) Western blotting indicates that there were no significant differences for expression levels of these VEGF downstream signaling pathway genes between RGC-32 o/e and control cells. (B) Western blotting indicates that RGC-32 did not effect FGF2 stimulated MAPK pathway.

Supplementary figure 3. GFP expression showed the successful RGC-32 gene delivery from quadriceps sections.