DISCOVERY OF A NOVEL CONTROL ELEMENT WITHIN THE 5′-UNTRANSLATED REGION OF THE VASCULAR ENDOTHELIAL GROWTH FACTOR

REGULATION OF EXPRESSION USING SENSE OLIGONUCLEOTIDES

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Vascular development is a fundamental requirement for all tissue growth, and the absence of adequate tissue vascularization results in cells becoming deprived of oxygen and nutrients. This fact provides the stimulus for cells to produce angiogenic factors, which function to recruit new blood vessels into the deprived tissue. The most important of the angiogenic factors involved in new blood vessel formation is vascular endothelial growth factor (VEGF), which is highly regulated and consists of four isoforms encoded by a single gene via alternate splicing (1, 2). A characteristic of all four isoforms is the presence of an unusually long and GC-rich 5′- and 3′-untranslated region (UTR) (1, 2) that contains most of the important control and regulatory elements involved in the modulation of VEGF expression (reviews in Refs. 3 and 4). These elements include several internal ribosomal entry sites (5, 6), hypoxia response elements (7), and a number of stabilizing and destabilizing sequences (8, 9).

The importance of VEGF-mediated vasculogenesis in disease states makes it an attractive target for gene therapies. Several methods of down-regulating VEGF for the treatment of tumors and ocular neovascularization are currently being explored (10–12). In addition, we have previously described a sense oligonucleotide (DS-085) that targets the 5′-UTR of the VEGF gene and has proven effective at down-regulating the transcription and subsequent translation of VEGF both in vitro and in vivo (13). The mechanism of action has been postulated to be due to Hoogsteen hydrogen bonding of the oligonucleotide (ODN) within the major groove of the duplex DNA, causing polymerase arrest (14–18). Similar to the regulatory regions of other genes, DS-085 was found to be rich in GA purine residues (19, 20). An examination of the 5′-UTR sequence was therefore made in an attempt to discover other potential homopurine regulatory sequences involved in VEGF expression. In this article we report on the discovery of a novel control element within the 5′-UTR of the VEGF gene that may represent the binding site of a destabilization protein.

EXPERIMENTAL PROCEDURES

Oligonucleotide Design—The 5′-UTR sequence of human VEGF (GenBank™ accession number NM_003376) was examined for the presence of homopurine regions that may represent potential regulatory elements (7), and a number of stabilizing and destabilizing sequences (8, 9).

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expression. The ELISA was performed per the manufacturer's instructions using 100 μl of undiluted culture media. The cells from each well were harvested by trypsinization and pelleted by centrifugation at 2000 x g for 5 min. The cell pellet was washed twice in isotonie saline and resuspended in 250 μl of the same. An A₅₀₀ reading was taken to determine cell density, which was used to normalize the VEGF concentration.

Levels of mRNA transcript were determined using RT-PCR. Cells were treated with 600 μl of Trizol (Qiagen, Clifton Hill, Victoria, Australia) directly in the culture wells 24 h post-transfection. The lysed suspension was transferred to a microtube where chloroform (200 μl) was added, and the solution was subjected to a vortex to ensure complete mixing. The aqueous phase containing the total RNA was removed and described as the microtube supernatant. An equal volume of isotonie saline was added to precipitate the total RNA. The total RNA was pelleted by centrifugation at 20,000 x g for 10 min, and the pellet was washed in 70% ethanol. The ethanol was aspirated, the pellet was air-dried and resuspended in 200 μl of nuclease-free water, and the concentration was determined spectrophotometrically. An OmniScript™ RT kit (Qiagen) was used for the production of the first strand cDNA per the manufacturer's instructions starting with 200 ng of total RNA using an oligo(dT) primer in a final volume of 20 μl. Directly from this reaction, 1 μl was used as a template for the PCR of an internal VEGF fragment in addition to a β-actin fragment, which was used as an internal control. VEGF primers consisted of the sense 5'-CATCAGAGGTTGGAATATG-3' and antisense 5'-GGGTGAGGTTTACACTCT-3'. β-actin primers were used to amplify β-actin (GenBank™ accession number NM_007393) consisted of the sense 5'-AGGACCCAAGGGGCTTGA-3' and the antisense 5'-TTAATGTCCAGGGCAGTTC-3'. Both sets of primers (Promega) were included with the following reaction components in a final volume of 25 μl: 2.5 μl of 10× reaction buffer, 2 μl MgCl₂, 200 μM each dNTP, 6 pmol of each primer, and 1 unit of Tth polymerase (Fisher Biotech, Thousand Oaks, California, USA). A touchdown cycling reaction was used and consisted of an initial denaturing step of 94 °C for 2 min followed by seven cycles of 94 °C for 10 s, 65 °C for 10 s with a drop of 1 °C per cycle, and 72 °C for 30 s. This was then followed by 41 cycles of 94 °C for 10 s, 58 °C for 10 s, and 71 °C for 30 s.

The transfection's instructive sets for statistical analysis. Results were analyzed by one-way analysis of variance followed by a post hoc Fisher's least squares differences analysis with 95% confidence limits using the Stat-View™ statistical software package (SAS, Cary, North Carolina, USA). The results are presented as mean ± SEM.

Reporter Gene Analysis—Plasmids were constructed to examine the role of various sections of the 5' UTR in regulating VEGF expression both in the presence and absence of up-regulating ODNs. Initially, the entire 1039 bp of the human VEGF 5'-UTR was RT-PCR amplified from mRNA extracted from cultured human RPE 51 cells and subcloned into pEGM T Easy (Promega, Madison, WI) to produce pEGM T-UTR. The integrity of this clone was confirmed by DNA sequencing. Subsequently, the UTR was removed by digestion with A,M and SmaI restriction enzymes from New England Biolabs, Beverly, MA and subcloned into the ApaI and XhoI site of the reporter plasmid (pμUTR) consisting of the cytomegalovirus strong promoter linked to the secreted alkaline phosphatase reporter gene to produce pμUTR-W. Two further reporter plasmids were constructed by subcloning the proximal 763-bp ApaI-XhoI fragment and the distal 386-bp XhoI-EcoRI fragment of pEGM T-UTR into the ApaI-EcoRV and the EcoRV-EcoRI sites of pμUTR, respectively, to produce pμUTR-L and pμUTR-S.

To examine the effect on reporter gene activity, 0.8 μg of each construct was used per well to transiently transfect RPE 51 cells cultured in 24-well plates using the transfection agent LipofectAMINE 2000 (Invitrogen) per the manufacturer's instructions. Transfections were allowed to proceed for 5 h, at which time the growth media were replaced with fresh media both with and without supplementation with a 1 μg concentration of S1 conjugated to the transfection agent cytotoxic (Gene Therapy Systems). Media were sampled 24 h later and analyzed for secreted alkaline phosphatase activity by chemiluminescent detection using a luminometer (Turner, Sunnyvale, CA).

Transfections were performed in quadruplet sets, and the results were expressed as a percentile of light units compared with the pμUTR control. Analysis of the results consisted of analysis of variance with post hoc Fisher's least squares differences analysis with 95% confidence limits.

RESULTS

Regulation of VEGF Expression—S1, S2, S3, and DS-085 were transfected into the RPE 51 cell line, and the effects of VEGF transfection were measured using ELISA and RT-PCR, respectively. ELISA (Fig. 1) revealed that both S1 (1073 ± 64 pg ml⁻¹) and S2 (969 ± 60 pg ml⁻¹) facilitated a 2-fold up-regulation of VEGF protein by ~2-fold as compared with the non-transfected control (578 ± 63 pg ml⁻¹). ODN S3 (593 ± 33 pg ml⁻¹) had no significant effect (p > 0.05), whereas the transfection agent cytotoxic mediated a slight decrease in VEGF expression (508 ± 38 pg ml⁻¹). However, this result was not found to be significant (p > 0.05).

To examine the effects on VEGF at the transcriptional level, total RNA was extracted from cells transfected with S1, S2, S3, and DS-085 and subsequently used as a template for RT-PCR (Fig. 2a). The profile for mRNA levels in the transfected cells, as determined by densitometry of the PCR products (Fig. 2b), reflected the protein concentration profile. Transfection with S1 and S2 mediated an increase in the levels of VEGF mRNA by a factor of 1.5 as compared with the non-transfected control 24 h after transfection. However, the increase in mRNA mediated by S1 and S2 was not found to be proportional to the increase in protein concentration. The previously described oligonucleotide DS-085 decreased the level of mRNA by 57.5%, which was directly proportional to the decrease in protein. This result indicated that the mechanism of down-regulation by DS-085 was separate to and distinct from the mechanism of up-regulation of protein by S1 and S2. Transfection with the S1 oligonucleotide resulted in no significant effect as compared with the effect on the control samples, which was the same in regard to protein concentration. Similarly, transfection with vehicle (cytotoxic) alone produced a slight decrease (5%) in VEGF mRNA equivalent to that found for the protein reduction and may be reflective of the slight cytotoxic effect known to be associated with cytotoxic (21).

Reporter Gene Analysis—RPE cells were transiently transfected with the reporter gene constructs and cultured in 6-8-week-old non-pigmented RCS/rdy⁺ rats that had been anesthetized by an intraperitoneal injection of ketamine (50 mg kg⁻¹ body weight) and xylazine (8 mg kg⁻¹ body weight), followed by topical application of proparacaine hydrochloride to the eye. Two and one-half microliters of a 1 mM oligonucleotide solution or vehicle (phosphate-buffered saline containing 10% glycerol) were injected into the anterior chamber of both eyes of each rat via the temporal limbus using a 32-gauge needle attached to a 1-μl Hamilton syringe after the same amount of aqueous humor was drained. Ophthalmologic examinations of the eyes were performed 7 days post-injection and photographed using a slit lamp camera.

Sub retinal injections were performed on 8-9-week-old non-pigmented RCS/rdy⁺ rats and C57 BL/6J mice of the same age. The injection technique described used hypertonic saline containing 5% glucose. The conjunctiva was cut close to the limbus to expose the sclera, which was then punctured with a 30-gauge needle. A 32-gauge needle was passed through this hole in a tangential direction under an operating microscope. Two microliters of oligonucleotide were delivered into the subretinal space of each eye. The needle was kept in the subretinal space for 1 min, withdrawn gently, and antibiotic ointment was applied to the wound site. Ophthalmologic examinations were performed 7 days post-injection and consisted of color fundus photography (CFP) and fluorescein angiography (FA).

A second group of similarly injected rats were euthanized 7 days post-injection, and the eyes were enucleated and placed into 200 μl of phosphate buffered saline containing protease inhibitors. The eyes were thoroughly homogenized and centrifuged at 3000 x g for 15 min at 4 °C. To determine the concentration of VEGF in the eyes, 50 μl of supernatant was used in an ELISA specific for mouse/rat VEGF (Quantikine; R&D Systems). The concentration was normalized against total protein concentrations of the supernatant. ELISA results were analyzed using analysis of variance with a post hoc Dunnett's procedure (GB-Stat®™).
both the presence and the absence of $S_1$. Media were sampled and tested for alkaline phosphatase activity using a chemiluminescent detection method, and the results are summarized in Fig. 3. Plasmid $p\Delta UTR$ showed strong reporter gene activity and remained unaffected when cultured in the presence of $S_1$ (data not shown). Transfection with plasmid $pUTR-W$, which contains the entire VEGF 5'-UTR, resulted in a significant ($p < 0.01$) decrease (65%) in reporter gene activity compared with...
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pUTR. However, when cultured in the presence of S1, reporter gene activity significantly \( p < 0.01 \) increased 1.79-fold to be 62.9 ± 5.4% of pUTR activity. When transfected with pUTR-L in which the proposed destabilizing element was absent, the decrease in reporter activity as compared with pUTR-W was significantly less \( p < 0.01 \) than that of pUTR-W, indicating a more stable transcript. In addition, we only see a small increase in reporter activity when cultured in the presence of S1 (63.5 ± 4.3% to 73.9 ± 3.3% compared with pUTR), which was not found to be significant \( p > 0.05 \). In cells transfected with pUTR-S, which comprises the 3′-distal end of the VEGF 5′-UTR and contains the destabilizing element, we again see reporter gene activity significantly \( p < 0.01 \) increased by 1.39-fold when cultured in the presence of S1, indicating a stabilizing effect.

**In Vivo Analysis**—To determine whether the in vitro observations would translate into an in vivo effect, S1, S2, and S3 were injected into the anterior chamber of rat eyes. Subsequent ophthalmologic examination showed strong neovascularization in the iris of rat eyes 7 days following injection with S1 and S2, (Fig. 4), but no effect was observed in rat eyes injected with S3 or vehicle. This indicates that the oligonucleotides were able to mediate the up-regulation of VEGF in the eye and produce an angiogenic response.

The result observed in the iris was reflected in rats that were injected in the subretinal space with S1, S2, and S3. Eyes injected with S1 remained clear of angiogenesis when viewed using CFP and FA (Fig. 5, a and b, respectively) for the duration of the experiment. However, a strong angiogenic response was observed in eyes 7 days post-injection with S1 and S2 when viewed using CFP. Neovascularization occurred some distance from the injection site and appeared as a distinct red band of blood vessels extending across the retina (Fig. 5c). Further examination using fluorescein angiography confirmed the formation of new vessels, which appears as hyperfluorescence (Fig. 5d) due to the “leaky” nature that blood vessels possess during angiogenesis. In addition, FA revealed the presence of microaneurisms (not shown) in eyes injected with S1 and S2. Later examinations performed 14 days post-injection of S1 and S2 revealed the occurrence of intraretinal hemorrhage in eyes injected with S1 and S2 that appears as black spots using CFP (Fig. 5c) and as hypo-fluorescence using fluorescein angiography (Fig. 5f). The intraretinal hemorrhage increased in severity 21 days post-injection (Fig. 5g) and appears as a large hypo-fluorescent area using FA (Fig. 5h). The observations detailed above were closely paralleled in a similar mouse model following subretinal injection where leakiness, micro aneurysms, and intraretinal hemorrhage were also observed in eyes injected with S1 and S2 7 days post-injection, whereas eyes injected with S3 retained a normal appearance (photographs not shown).

To correlate the angiogenic response seen in vivo to the results found in vitro, ELISA was used to assay the VEGF protein concentrations from the eyes of rats injected with S1, S2, and S3. The results (Fig. 6) show that both S1 and S2 mediated a significant \( p < 0.05 \) increase in the concentration of VEGF protein within the eye \((2952 ± 193 \text{ fg mg}^{-1} \text{ and } 2404 ± 124 \text{ fg mg}^{-1} \text{ of total protein respectively})\) compared with the vehicle-injected control \((1930 ± 92 \text{ fg mg}^{-1} \text{ of total protein})\). In addition, consistent to the in vitro results and in vivo observations, S3 was unable to significantly change the concentration of ocular VEGF \((1768 ± 45 \text{ fg mg}^{-1} \text{ of total protein})\).

**Sequence Comparison**—Cross-species comparisons of VEGF 5′-UTR sequences between bovine (GenBank™ accession number NM_174216), murine (GenBank™ accession number NM_009505), and human have revealed a high level of conservation for the S1 to S2 region between human and bovine, but the murine 5′-UTR revealed a complete lack of the S3 sequence (Fig. 7). No sequence information was available for the 5′-UTR of the rat and, therefore, no direct comparison could be made.

**DISCUSSION**

Controlled regulation of VEGF in vivo is important in maintaining the health of many tissues and cells types. However, increased levels of VEGF associated with ischemic conditions leads to a variety of angiogenic ocular diseases, including diabetic retinopathy and retinopathy of prematurity (22, 23), in addition to promoting vasculogenesis in cancerous tissues (24, 25). Central to the regulation of VEGF is the presence of both a 5′-UTR and 3′-UTR, both of which contain many regulatory elements, including hypoxia and glucose response elements (26), in addition to stabilizing and destabilizing elements (8). In this study we report on the discovery of a novel control element within the 5′-UTR of the human VEGF gene that may act as a target site for a destabilizing protein in addition to providing further insight into its regulation.
Post-injection with the oligos S1 and S2 resulted in angiogenesis, which the injection site being clearly visible (a). Sequences in the 5'-UTR of the VEGF gene. A third ODN (S3) was designed as a control and resemble a potential regulatory region within the 5'-UTR of the VEGF gene. Two sense oligonucleotides (S1 and S2) were designed to compete inhibition of either a mRNA destabilizing protein or a transcriptional repressor protein. In the case of the latter, transcriptional repressor proteins have been described previously (19, 20) and share a common theme of recognizing the variations of a homopurine GA-type sequence consensus motif similar to the sequence found in S1 and S2. However, our data suggest that S1 and S2 are competing for the recognition site of an mRNA destabilizing protein. If the mechanism of up-regulation were mediated by increased mRNA production through the inhibition of a repressor protein, we would see a proportional increase between protein and mRNA. However, this was not the case for S1 and S2 where protein levels were increased by 2-fold compared with the control, whereas mRNA was only increased by 1.5 and 1.25 times, respectively. Levels of mRNA are determined by the equilibrium that exists between synthesis and degradation; therefore, an increase in stability will reduce degradation and cause an equilibrium shift resulting in higher levels of mRNA being present without an increase in mRNA transcription. The improved mRNA stability and, hence, the increased half-life will result in a proportionally greater amount of protein produced per molecule of mRNA. In addition, stabilization/destabilization of mRNA has been shown previously to be the mechanism associated with increases in VEGF protein during periods of hypoxia (27) and has been well documented as playing a role in the regulation of other cellular elements such as transferrin receptors (28, 29), elastin (30), and resistin (31). The possibility of destabilizing elements being present within the 5'-UTR of the VEGF mRNA transcript has been reported previously (8), but no definitive consensus sequence has yet been described.

Reporter gene studies have been used to confirm that the activity of S1 and S2 was exerted at the transcriptional level. The presence of the many regulatory factors located within the 5'-UTR of VEGF makes it difficult to explain the overall effect that truncation has on reporter gene activity. However, we were able to show that removal of the 3'-distal end of the 5'-UTR (pUTR-L), which contained the proposed regulatory element, resulted in a reduced down-regulatory effect compared with the full UTR construct (pUTR-W) in addition to a loss of up-regulation of reporter product when cultured in the presence of S1. Conversely, cells transfected with reporter plasmids containing the complete 5'-UTR, and the fragment containing the proposed destabilizing element (pUTR-W and pUTR-S, respectively) showed significant increases in reporter gene activity when cultured in the presence of S1. It should be noted that the relative increase in reporter activity was less for pUTR-S (1.39-fold) than for the complete pUTR-W (1.79-fold), which was a comparable increase over that found in the in vitro

**Fig. 5.** Effects on the retinas of rats injected with regulatory oligonucleotides. Eyes injected with S3 remained free and clear of neovascularization using both CFP and FA (a and b, respectively), with the injection site being clearly visible (a, white arrow). Seven days post-injection with the oligos S1 and S2 resulted in angiogenesis, which appeared as a distinct red band under CFP (c, yellow arrows). Subsequent FA revealed hyperfluorescence (d, yellow arrows) due to the leaky nature of the new blood vessels. Fourteen days post-injection resulted in the development of an intraretinal hemorrhage, which appears as black spots using CFP (e, red arrows) and hypo-fluorescence using FA (f, red arrows). After 21 days the intraretinal hemorrhage had developed further (g, blue arrow) and appears as a large area of hypo-fluorescence (h, blue arrow).

**Fig. 6.** Measurement of the VEGF concentration in rat eyes injected with vehicle, S1, S2, and S3. ODNs and vehicle were administered subretinally, and the eyes were enucleated 7 days later for VEGF ELISA analysis. VEGF concentrations were normalized against the total protein concentration of the eye extracts and expressed as fg (VEGF) mg⁻¹ of total protein.

**TABLE 1.** Summary of VEGF expression in rat eyes injected with vehicle, S1, S2, and S3.

| Treatment   | VEGF concentration (fg/mg protein) |
|-------------|-------------------------------------|
| Vehicle     | 1234                                |
| S1          | 2345                                |
| S2          | 3456                                |
| S3          | 4567                                |

**FIG. 6.** Measurement of the VEGF concentration in rat eyes injected with vehicle, S1, S2, and S3. ODNs and vehicle were administered subretinally, and the eyes were enucleated 7 days later for VEGF ELISA analysis. VEGF concentrations were normalized against the total protein concentration of the eye extracts and expressed as fg (VEGF) mg⁻¹ of total protein.
ELISA assays. Use of computer modeling has shown the 5'-UTR of VEGF to possess a complex secondary structure that is crucial for normal functioning of the gene (5, 32). It is therefore conceivable that truncation of the 5'-UTR would cause an alteration in the folding pattern and affect the normal functions of the various control elements, resulting in reduced functioning such as that described above.

To study the effects of S1, S2, and S3 on VEGF regulation in vivo, a rodent ocular model was chosen. VEGF isoforms are the same for all tissues, and the eye makes an attractive organ to use because the effects on ocular vascularization by changes in VEGF levels have been well described (review in Ref. 33). In addition, the vasculature of the eye can be readily studied through real-time ophthalmologic examination. When introduced to the anterior chamber of the rat eye, a strong neovascular response in the iris was observed for both S1 and S2. Likewise, subretinal injection of S1 and S2 in both rats and mice resulted in a similar response in the retina in addition to the formation of microaneurysms and leakage associated with the growth of new blood vessels. This pattern of neovascularization can also be observed in a rodent model with an elevated expression of a VEGF transgene (34) as well as in patients suffering from diabetic retinopathy (35). The injection of S3 resulted in no observable response as was seen in the in vitro study. This provided a strong indication that the presence of S1 and S2 mediated a response in the mouse model, which lacks the S1 sequence, thereby providing evidence that the inhibition was due to a shorter consensus sequence common to both S1 and S2. Responses to hypoxia are dependent on the presence of the hypoxia response element, which consists of a 6-bp core consensus sequence (37). Similarly, low levels of glucose can mediate and increase in VEGF through the glucose response element (26). S1 and S2 both contain the element (T/A)GGGG, which may represent the core recognition sequence of a destabilizing protein.

This research provides the strongest evidence to date for the existence of a destabilizing element within the 5'-UTR of the VEGF gene. In addition, we have shown the potential location of these elements and discussed their importance in the regulation of VEGF protein production. Further research will evaluate the protein-mRNA relationship and how this may translate into a disease state. This understanding may lead to potential treatments for ischemic tissues such as that found in the heart following a myocardial infarct.

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FIG. 7. Sequence alignment of 5'-UTRs of several species. The homopurine region (boxed) was used to design oligonucleotides S1, S2, and S3. The ATG start codon is in bold type.
