Induction of VEGF Gene Expression by Retinoic Acid through Sp1-Binding Sites in Retinoblastoma Y79 Cells

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PURPOSE. Vascular endothelial growth factor (VEGF) is an angiogenic peptide that has been implicated in many retinopathies. Although all trans-retinoic acid (atRA) has long been known as an essential factor in the visual cycle, the role of atRA in the pathogenesis of retinal disease remains elusive. In this study, we investigated the effects of atRA on expression of the VEGF gene in retinoblastoma Y79 cells.

METHODS. Total RNA prepared from Y79 cells, with or without atRA, was subjected to Northern blot analyses. Reporter constructs consisting of the VEGF promoter-luciferase gene were transfected into Y79 cells. Nuclear factors binding to the VEGF promoter were analyzed by electrophoretic mobility shift assays (EMSAs).

RESULTS. The levels of VEGF transcripts were increased by atRA in a time- and dose-dependent manner. Progressive deletion and site-specific mutation analyses indicated that atRA increased VEGF promoter activity through a G+C-rich sequence that was shown to be an Sp1-binding site by supershift assays. EMSAs showed that Sp1 binding was increased by atRA stimulation. Although no measurable change was observed in Sp1 mRNA levels, Western blot analysis showed an increase in Sp1 protein levels in the atRA-treated cells. These data suggest that atRA increases Sp1 protein levels by posttranscriptional mechanisms, and elevated levels of Sp1 protein induce the expression of VEGF at the transcriptional level.

CONCLUSIONS. atRA induction of transcription of the VEGF gene through Sp1-binding sites in Y79 cells. Pharmacologic intervention that inhibits the signals elicited by atRA may be effective in treating VEGF-mediated retinopathies. (Invest Ophthalmol Vis Sci. 2002;43:1367–1374)

Retinal neovascular diseases, including age-related macular degeneration (AMD), diabetic retinopathy, and retinopathy of prematurity, are major causes of blindness. Angiogenesis is known to be the most important aggravating factor. VEGF is a potent angiogenic factor and has been postulated to play a key role in the process of retinal disease. VEGF is produced in many of the types of ocular cells of which the retina and choroid are composed.¹ Among many factors that induce VEGF, hypoxia is one of the most primary stimuli for induction of the VEGF gene, which causes retinal and choroidal neovascularization (CNV).² There is accumulating evidence that VEGF is induced by various cytokines, such as advanced glycation end products and IL-8, in intraocular tissues of the eye.³,⁴ In addition, mechanisms of expression, such as the VEGF gene have been studied in many different cell types, including glioma cells, fibroblasts, endothelial cells, vascular smooth muscle cells, and cardiac myocytes. These studies have shown that VEGF gene expression is dramatically increased by hypoxia, which is particularly relevant to ocular neovascularization.² Hypoxia induces VEGF gene expression through a mechanism involving the hypoxia-inducible factor-1α (HIF-1α/ARNT) heterodimer and its cognate binding sequence (hypoxia response element; HRE) located upstream of the VEGF promoter.⁵,⁶ Furthermore, several lines of evidence indicate that proinflammatory cytokines, such as IL-1β, TNF-α, TGF-β, and IL-6, increase the expression of VEGF transcripts in several cell lines.⁷,⁸,⁹,¹⁰

These studies showed that induction of expression of the VEGF gene in response to IL-1β and TNF-α is mediated by Sp1-binding sites, whereas TGF-α-induc ed expression is predominantly mediated by AP-2-binding sites. However, the precise molecular mechanisms for the regulation of VEGF gene expression remain to be described in ocular cells. In addition to its role in the regulation of cellular growth and differentiation, retinoid acid (RA) plays an essential role in the induction and maintenance of the dorsoventral retinal axis¹¹ and in the structure and physiologic function of retinal horizontal cells in eye development.¹²,¹³ RA promotes formation of rod and cone photoreceptors.¹⁴,¹⁵ Illumination of the vertebrate eye causes an increase in RA synthesis¹⁶; light isomerizes 11-cis-retinal, activates rhodopsin, and initiates the process of phototransduction by which the visual sensation is produced. All-trans-retinal, which is a product of photoisomerization, enters into a series of reactions that regenerate the 11-cis configuration.¹⁷ RA serves as a gene regulator through ligand-activated nuclear receptors, including retinoic acid receptors (RARs) and retinoid X receptors (RXRs), and plays an important role in regulating the growth and differentiation of a wide variety of cell types.¹⁸ In mice disrupted with theRAR, the eyes are extremely small, with gross morphologic defects in the choroid and sclera and retinal dysplasia.¹⁹ Furthermore, previous reports have indicated that RA produces rod photoreceptor-selective apoptosis in developing mammalian retina.²⁰ However, the transcriptional process regulated by RA not been identified in mature retina.

Major RA signals are transduced by the RAR-RXR heterodimer that acts as a functional unit controlling the transcription of RA-responsive genes.²¹ Molecular mechanisms by which the RAR-RXR heterodimer regulates gene transcription have been a focus of research interest. Originally, the RAR-RXR heterodimer was thought to activate gene transcription through the cis-regulatory element, 5'GGTCA-3', which is termed a retinoic acid response element (RARE).²² However, increasing numbers of studies have reported that RA-

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dependent augmentation of the gene expression is not regulated through an interaction between RARE and the RAR-RXR heterodimer. For instance, all-trans-retinoic acid (atRA)-induced stimulation of thrombomodulin tissue-type or urokinase-type plasminogen activator genes is regulated by an interaction between Sp1 and RAR.22,23

In this study, we attempted to determine the effects of atRA on expression of the VEGF gene in Y79 cells, a cultured human retinoblastoma cell line, and showed that VEGF transcripts were increased by atRA in a time- and dose-dependent manner. Deletion and site-specific mutation analyses have demonstrated that Sp1-binding sites are necessary for both static and atRA-induced expression of the VEGF gene. Nuclear proteins from atRA-treated Y79 cells displayed an increased binding activity to Sp1 sites of the VEGF promoter. Furthermore, Sp1 protein levels, but not its mRNA levels, were increased by atRA stimulation in Y79 cells.

**METHODS**

**Materials**

atRA and actinomycin D were purchased from Sigma Chemical Co (St. Louis, MO). Affinity-purified rabbit polyclonal antibodies for Sp1, Sp3, AP-2, and cyclic AMP response element binding protein (CREB) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). [α-32P]dCTP (3000 Ci/mmol) and [γ-32P] adenosine triphosphate (ATP; 6000 Ci/mmol) were obtained from Amersham (Arlington Heights, IL). Anti-human VEGF antibody was obtained from Immuno-Biological Laboratories, Gunma, Japan).

**Plasmid Constructions**

We obtained from Judith A. Abraham (Scios Inc., Sunnyvale, CA) the VEGF promoter-pGL3 that contains the DNA fragment from −480 to +338 of the human VEGF gene fused to a luciferase reporter plasmid.24 Plasmids −480 and −89Luc were made by subcloning the Bgfi and Smal insert from −1180Luc into the corresponding site of pGL3 (Promega, Madison, WI). Expression of the vector of Sp1/RSV, which expresses Sp1 under the control of the cytomegalovirus (CMV) promoter was generously provided by Yoshiaki Fuji-Kuriyama (Tohoku University, Sendai, Japan). DNA sequences of deletion constructs were determined by the dideoxy chain-termination method on a gene analyzer (Prism model 310; PE-Applied Biosystems, Foster City, CA).

**Cell Culture and Transfection**

Y79 retinoblastoma and retinal pigmented epithelial (RPE) cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) and antibiotics at 57° C in 5% CO2. Transfection into Y79 cells was performed with a modified calcium phosphate coprecipitation technique, as previously described.25 Cells were transfected with 1 μg reporter plasmid. After transfection, cells were washed twice with phosphate-buffered saline and treated with or without 1 μM atRA. After 48 hours of incubation, cells were harvested for luciferase assay. Luciferase activity was measured with a luminometer (model LB9501; Lumat; Berthold, Wildbad, Germany) and was normalized to cellular protein concentration. Each transfection was repeated, and the mean concentration ± SEM was determined.

**Electrophoretic Mobility Shift and Supershift Assays**

Nuclear extracts from Y79 cells were prepared as previously described.25 The sequences of double-stranded oligonucleotides used as probes or competitors in electrophoretic mobility shift assay (EMSAs) were as follows, with the mutations of wild-type sequences in italic: VEGF −89/−67, 5′-CCCGGGGCGGGCCCGGGCGGG-3′; VEGF −89/−67 (Sp1m), 5′-CCCGGGGAGGGCGGGCGGG-3′; Sp1, 5′-ATTCGAT-GGGCGGCGGGCGG-3′; CREB, 5′-AGAGATGCTAGCGTCA-3′; VEGF −89/−67, 5′-GATGACCTGACGCCCGGCGGCGGCGG-3′; and AP2, 5′-CGATGACCTGACGCCCGGCGGCGGCGG-3′. All probes were 5′ end labeled with T4 polynucleotide kinase and [γ-32P]ATP. Binding reactions, EMSAs and supershift assays were performed as previously described.25 For competition experiments, 200 ng cold oligonucleotide was mixed with nuclear extracts before addition of 0.5 ng labeled probe.

**cDNA Probes and Northern Blot Analyses**

A 641-bp fragment of human VEGF cDNA sequence and a 2.0-kb fragment of the rat Sp1 cDNA sequence were used as a probe for Northern blot analyses. The cDNA probes were labeled with [α-32P]dCTP, using the random primer method.25 Preparation of total cellular RNA and Northern blot analysis were performed as previously described.25

**Western Blot Analyses**

Nuclear extracts from vehicle- or atRA-treated Y79 cells were directly subjected to immunoblot analysis for Sp1. SDS-PAGE was performed with an 8% gel, according to the standard procedure, after boiling with sample buffer, and protein in the gel was transferred electrophoretically to a nitrocellulose membrane at 400 mA for 3.5 hours. Sp1 was visualized by using an affinity-purified rabbit polyclonal antibody and a horseradish peroxidase-linked anti-rabbit IgG secondary antibody (Amersham). The complexes were detected by autoradiography with a chemiluminescence detection system (ECL; Amersham).

**ELISA for VEGF**

The concentration of VEGF produced was measured with a commercially available ELISA kit (Immuno-Biological Laboratories Co., Gunma, Japan), as stated in the figure legend. The culture supernatants were collected after stimulation for 48 hours, and the absorbance was measured at 450 nm. VEGF production was normalized to the volume of the medium and cell number.

**Statistical Analysis**

Statistical analyses were performed by a Student’s t-test, with significant differences set at P < 0.05. Correlation was performed with the use of simple regression analysis.

**RESULTS**

**Induction of VEGF mRNA in Y79 Cells**

To determine whether atRA regulates expression of the VEGF gene in human retinoblastoma cells, Y79 cells were stimulated with atRA (1 μM) and were harvested at various time points for Northern blot analyses. VEGF transcripts were barely detectable before atRA stimulation (Fig. 1A). Treatment of Y79 cells with atRA gradually increased the level of VEGF mRNA in a time-dependent manner, and it reached maximum accumulation after 72 hours (data not shown). Furthermore, we investigated the dose-dependent effects of atRA on expression of VEGF mRNA. Stimulation of Y79 cells with atRA modestly but reproducibly increased levels of VEGF mRNA in a dose-dependent manner (Fig. 1B). Thus, atRA was a reliable inducer of expression of the VEGF gene in Y79 cells.

**Stability of VEGF mRNA**

To determine whether the expression of atRA-induced VEGF mRNA is regulated at the transcriptional level, we performed a standard mRNA decay assay using actinomycin D, which prevents the transcription of the genes. Y79 cells were stimulated with atRA at a concentration of 1 μM for 48 hours. After incubation with or without atRA (1 μM), cells were treated with actinomycin D and harvested at the various time points...
was performed as described in (A).

Northern blot analysis for VEGF mRNA. Representative Northern blot at RA at the indicated concentration for 48 hours was analyzed by cells. Levels in the control cells are set at 1.0. (the 28S signal. Data are relative to VEGF mRNA levels in the control mRNA yielded by scanning the autoradiographs were normalized to RNA (20 H9262 Northern blot is shown. 28S ribosomal RNA indicated that a comparable region spanning between –89 and +338 revealed two consensus Sp1 sites at –85 and –74 and a consensus AP-2 site at –80, located upstream of the transcription start site. To test whether Sp1 sites serve as the atRA-regulatory element, –89(Sp1m)Luc, a plasmid containing mutations within the two Sp1-binding sites, was transiently transfected into Y79 cells. At 24 hours after transfection, cells were treated with vehicle or atRA for 48 hours. The disruption of both Sp1 sites markedly impaired responsiveness to stimulation by atRA (Fig. 4A). The absence of response of –89(Sp1m)Luc to atRA was not due to the disruption of the elements essential for the basal transcription, because the luciferase activity of –89(Sp1m) Luc was significantly higher than that of the pGL3 (data not shown). These results indicate that the activation of the VEGF promoter in response to atRA depends on the integrity of at least one of the two Sp1 sites. To establish the importance of an increase in expression of Sp1 in the transcriptional regulation of the VEGF gene, we performed cotransfection experiments using an Sp1-expression vector, Sp1/RSV. The cotransfection of Sp1/RSV resulted in a 5.9-fold increase in luciferase activity of the –1180Luc (Fig. 4B). These findings imply that Sp1 overexpression can lead to the activation of the VEGF promoter and that Sp1 sites play an important role in mediating this response.

Identification of the Nuclear Factors Binding to the atRA-Responsive Elements in the Human VEGF Promoter

To examine the ability of these sites to interact with Sp1 or related factors, EMSAs were performed with nuclear extracts prepared from either untreated or atRA-treated Y79 cells and the 32P-labeled double-stranded oligonucleotide probe containing the sequence between –89 and –67. The 32P-labeled VEGF –89/–67 probe gave rise to two specific DNAs (Fig. 5A): protein complexes C1 and C2. The intensity of the C1 complex was enhanced by atRA. In contrast, the C2 complex was not affected (Fig. 5A). Both C1 and C2 complexes were shown to be sequence specific because formation of these complexes was competed by the wild-type but not by the mutated version of the probe sequence (Fig. 5B). These complexes were in competition with the consensus Sp1 sequence but not with the AP-2 and CREB-binding sequence (Fig. 5B). To verify that the C1 and C2 complexes contain Sp1 or Sp3-related proteins, we performed supershift assays using Sp1 or Sp3 antisera (Fig. 5C). Addition of an Sp1 antibody resulted in a supershift of complex C1, indicating that Sp1 is a principal DNA-binding component of this complex. An Sp3 antibody caused a complete supershift of complex C2. Addition of the AP-2 and CREB antibodies had no effect on complex formation. These results provide the evidence that Sp1 and Sp3, but not AP-2 or CREB, bind to the

for Northern blot analyses. The rate of decrease in levels of VEGF mRNA was comparable between control and atRA-treated cells (Fig. 2), suggesting that atRA treatment did not affect the stability of VEGF transcripts. These results indicate that atRA-induced expression of the VEGF gene was regulated at the transcriptional level in Y79 cells.

VEGF Promoter Activity

Next, we tested whether atRA increases the promoter activity of the VEGF gene, –1180Luc, a plasmid containing human VEGF promoter sequence between –1180 and +338 relative to the transcription start site, was transiently transfected into Y79 cells. The luciferase activity was increased approximately 3.5-fold by atRA (Fig. 3), whereas β-actin promoter was not responsive to atRA (data not shown).

We then attempted to map the regions that confer both basal and atRA-induced activities in the VEGF promoter. To this end, progressive 5’ deletion mutants that contain the identical 3’ ends were constructed and transiently transfected into Y79 cells. atRA significantly increased the promoter activity of both −480Luc and −89Luc (Fig. 3). Luciferase activity of −89Luc was significantly higher than that of the promoterless construct pGL3, suggesting that the sequences downstream of −89 contain elements required for both basal and atRA-induced expression of the VEGF promoter. The activation of the VEGF promoter in response to atRA appears to be promoter specific, because the β-actin promoter was totally unresponsive (data not shown). These results indicate that a sequence between −89 and +338 is necessary for atRA-induced transcription of the VEGF gene, as well as for basal expression.

Critical Role of Sp1 Sites in Response to atRA

Search for the consensus sequences of the transcription factor-binding sites within the atRA-responsive region spanning between –89 and +338 revealed two consensus Sp1 sites at −85 and −74 and a consensus AP-2 site at −80, located upstream of the transcription start site. To test whether Sp1 sites serve as the atRA-regulatory element, −89(Sp1m)Luc, a plasmid containing mutations within the two Sp1-binding sites, was transiently transfected into Y79 cells. At 24 hours after transfection, cells were treated with vehicle or atRA for 48 hours. The disruption of both Sp1 sites markedly impaired responsiveness to stimulation by atRA (Fig. 4A). The absence of response of −89(Sp1m)Luc to atRA was not due to the disruption of the elements essential for the basal transcription, because the luciferase activity of −89(Sp1m) Luc was significantly higher than that of the pGL3 (data not shown). These results indicate that the activation of the VEGF promoter in response to atRA depends on the integrity of at least one of the two Sp1 sites. To establish the importance of an increase in expression of Sp1 in the transcriptional regulation of the VEGF gene, we performed cotransfection experiments using an Sp1-expression vector, Sp1/RSV. The cotransfection of Sp1/RSV resulted in a 5.9-fold increase in luciferase activity of the –1180Luc (Fig. 4B). These findings imply that Sp1 overexpression can lead to the activation of the VEGF promoter and that Sp1 sites play an important role in mediating this response.

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VEGF −89/−67 probe, and atRA increased the binding of Sp1 to this sequence.

Effects of atRA on Sp1 mRNA and Protein Levels
An increase in Sp1 binding as assessed by EMSAs led us to determine whether atRA-treatment increases the levels of Sp1 transcripts. Northern blot analyses indicated that Sp1 mRNA levels were not measurably affected by atRA treatment in Y79 cells at any time points and at any concentrations of atRA tested (Figs. 6A, 6B). In contrast, Western blot analyses using nuclear extracts from vehicle or RA-treated Y79 cells showed that atRA increased Sp1 protein levels, which correspond to the two distinct bands with molecular mass of either 95 or 105 kDa. These two protein species are generated as the result of differential posttranslational modification of the single Sp1 polypeptide. Both species of Sp1 protein in atRA-treated Y79 cells were significantly increased in a time-dependent manner (Fig. 6C). SDS-polyacrylamide gel stained with Coomassie brilliant blue showed that nuclear extracts prepared from either control or atRA-treated Y79 cells contain the same quantity of protein (Fig. 6D). Taken together, our data suggest that atRA has no effect on Sp1 gene expression, but rather increases steady state Sp1 protein levels, which in turn increases the transcription of the VEGF gene.

Induction of VEGF mRNA in RPE Cells
To determine whether atRA regulates VEGF gene expression in nontransformed cells, RPE cells were stimulated with atRA (1 μM) and were harvested at various time points for Northern

**Figure 2.** Effect of atRA on the half-life of VEGF mRNA in Y79 cells. Y79 cells were exposed to vehicle (top left) or atRA (top right) for 1 hour after stimulation by atRA at a concentration of 1 μM for 48 hours. Total cellular RNA was extracted from the cells at the indicated times after treatment with actinomycin D, and Northern blot analyses for VEGF mRNA were performed. The levels of VEGF mRNA normalized to 28S ribosomal RNA, and the decay rates were plotted as a percentage of the 0-hour level against time.

**Figure 3.** Deletion analysis of activity of the VEGF promoter induced by atRA in Y79 cells. The human VEGF promoter (−1180/+338) fused to the luciferase gene was transiently transfected into Y79 cells, and the luciferase activities were measured in response to atRA (1 μM). A series of the 5′-deletion constructs of the VEGF promoter, −480Luc, −89Luc, and promoterless pGL3 were transfected into Y79 cells, and the luciferase activities were measured in response to atRA (1 μM). The data are relative to −1180Luc activity in the control cells and the mean ± SEM of three separate experiments performed in duplicate. *P < 0.05 compared with levels in untreated control cells.

**Figure 4.** Specific mutation analysis of activity of the VEGF promoter after atRA treatment in Y79 cells. (A) Site-specific mutation analysis. The construct −89Luc contains wild-type sequence between −89 and +338, and −89(Sp1m)Luc contains mutated Sp1 sites. Y79 cells were transfected with either −89Luc or −89(Sp1m)Luc, along with either Sp1/RSV or control vector pRc/RSV, and assayed for luciferase activity. The data are relative to activity of −89Luc (A) or −1180Luc (B) in the control cells and are the mean ± SEM of three separate experiments performed in duplicate. *P < 0.05 compared with values of in untreated control cells.
blot analyses. VEGF transcripts were barely detectable before stimulation with atRA (Fig. 7). Treatment of RPE cells with atRA gradually increased the levels of VEGF mRNA in a time-dependent manner, reaching maximum concentration after 72 hours—an expression profile similar to that in Y79 cells.

Regulation of VEGF Protein Production by atRA

To assess whether the observed increase in VEGF transcripts represents upregulation in VEGF production, specific ELISA was performed. Confluent cultures of Y79 and RPE cells were

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**Figure 5.** EMSAs of the potential Sp1 sites. (A) Nuclear extracts prepared from Y79 cells that had been cultured in the presence (+) or absence (−) of atRA (1 μM) for 48 hours were incubated with a 32P-labeled probe containing sequence between −89 and −67. Positions of the sequence-specific DNA (protein complexes C1 and C2) are indicated. The probe alone did not give rise to a shifted complex. (B) Nuclear extracts prepared from Y79 cells that had been cultured in the presence of atRA (1 μM) for 48 hours were incubated with the same probe in the absence or presence of a 400-fold molar excess of unlabeled competitors. (C) Supershift assays. Nuclear extracts prepared from Y79 cells were incubated with antibodies for Sp1, Sp3, AP-2, or CREB, before the addition of the probe and were assayed for DNA binding activity. Positions of the sequence-specific DNA (protein complexes C1 and C2) and supershifted complexes (SS) are indicated.

**Figure 6.** Effects of atRA on levels of Sp1 mRNA and protein. Time- (A) and dose- (B) dependent response of atRA-induced Sp1 mRNA in Y79 cells. Total cellular RNA (20 μg) from Y79 cells cultured in the presence of 1 μM atRA for the indicated times was analyzed by Northern blot for Sp1 mRNA. (C) Nuclear extracts (4 μg) from vehicle- or atRA-treated Y79 cells were analyzed by Western blot. Both species (105 and 95 kDa) of Sp1 protein increased significantly in atRA-treated cells, compared with control cells. (D) Cell extracts prepared from vehicle- or atRA-treated Y79 cells were separated on 8% SDS-polyacrylamide gel. Coomassie brilliant blue staining showed various size of proteins from ~80 to ~200 kDa.
serum starved for 24 hours and then incubated with fresh serum-free medium containing atRA (0, 0.5, and 1.0 μM) for 72 hours. Culture medium was harvested, and VEGF production was measured. VEGF production was significantly increased by atRA (Fig. 8).

DISCUSSION

In this study, we examined the effects of atRA on expression of the VEGF gene in Y79 cells, a cultured human retinoblastoma cell line, and showed that VEGF transcripts were increased by atRA in a time- and dose-dependent manner. To the best of our knowledge, this is the first report indicating the upregulation of VEGF by atRA. Contrary to our results, previous studies have shown that retinoids downregulate VEGF production at the mRNA and protein levels in normal human keratinocytes or vascular endothelial cells.26–29 A study reported by Diaz et al.28 supports the hypothesis that VEGF is downregulated by retinoids through the inhibition of the AP1 pathway. Because atRA induces expression of VEGF mRNA in cultured RPE cells as well as in Y79 cells, the contradiction in regulation of expression of the VEGF gene by atRA is possibly due to the difference in cell types used in the experiments.

There are several reports indicating that RA and RA-binding proteins and receptors are present within subpopulations of neurons and glia in the central nervous system and retina.30,31 Consistent with these studies and our study, there are previous reports showing the regulation of gene expression in nonretinal cells and retinal cells. In nonretinal cells, atRA induces the expression of several genes at transcriptional levels, such as

![Figure 7](image-url)  
**Figure 7.** Time-response of atRA-induced VEGF mRNA in RPE cells. *Top:* total cellular RNA (20 μg) prepared from RPE cells at the indicated times after stimulation with atRA (1 μM) was analyzed by Northern blot for VEGF mRNA. Representative Northern blot analysis is shown. 28S ribosomal RNA indicates that a comparable amount of total RNA was actually blotted onto a membrane. **Bottom:** Densitometric analysis of the Northern blot. VEGF mRNA levels yielded by scanning the autoradiographs were normalized to the 28S signal. The data are relative to levels of VEGF mRNA in the control cells. Levels in the control cells were set at 1.0.

![Figure 8](image-url)  
**Figure 8.** Effects of atRA on production of VEGF in the culture medium of Y79 and RPE cells. Production of VEGF in Y79 cells (○) and RPE cells (■). Confluent cells were serum starved for 24 hours and then incubated with fresh serum-free medium containing atRA (1 μM) for 72 hours. Culture medium was harvested, and the levels of VEGF protein were measured by specific ELISA. Standard curves were constructed from dilutions of purified VEGF. *P < 0.05 compared with values in untreated control cells (n = 4). **P < 0.05 compared with data in 0.5 and 1.0 μM atRA-treated cells (n = 4).
expression of VEGF is increased in retinal tissue of patients with retinopathies such as retinopathy of prematurity and diabetic retinopathy. This raises the possibility that Sp1 can serve as a factor regulating the transcription in response to a wider variety of the stimuli than we have previously thought. However, we want to emphasize that a variety of nuclear factors besides Sp1 are capable of binding to the Sp1 site, and thus Sp1-related factors are responsible for the atRA-mediated increase in VEGF gene expression, but not Sp1 itself. This question deserves further investigation.

Our studies indicate that atRA increases levels of Sp1 protein but not levels of Sp1 mRNA, suggesting that atRA has a potential to enhance the stability of Sp1 protein. Previous studies have demonstrated that DNA methyltransferase inhibitor enhances expression of TGF-β receptor types I and II in cancer cells, and the mechanisms behind this involve an increase in steady state levels of Sp1 as a result of increased protein stability. Further studies to determine precise molecular mechanisms for increased stability of Sp1 are warranted.

The molecular mechanisms underlying intraocular neovascularization have been a focus of intensive research. VEGF is an angiogenic peptide that is increased greatly in response to hypoxia in retinal cells. To date, it has been well established that the hypoxia-mediated increase in expression of VEGF plays a major role in development of many retinal diseases, such as retinopathy of prematurity and diabetic retinopathy. Although several reports have provided evidence that expression of VEGF is increased in retinal tissue of patients with AMD, the molecular mechanisms underlying this observation remain unclear. Thus, the identification of the mediators that are capable of inducing expression of VEGF may provide a new insight into the mechanism of hypoxia-independent ocular disease. In this regard, our study indicating that atRA increases expression of VEGF in retinal cells seems to have important clinical relevance, because it has been reported that RA is preferentially distributed in retinal cells, and light can increase RA’s synthesis. Thus, it is tempting to speculate that RA may be responsible for light-induced retinal disease.

In conclusion, our current study, atRA induced expression of the VEGF gene through Sp1-binding sites of the human VEGF promoter. In addition, atRA increased levels of Sp1 protein and enhanced binding activity to Sp1 sites within the VEGF promoter. Based on the evidence of light-induced synthesis of RA in retinal tissue, RA may be one of the critical mediators of neovascularization through induction of expression of VEGF. Therefore, our findings raise the possibility that pharmacologic intervention that inhibits the signals elicited by RA may be effective in treating the VEGF-mediated retinopathies.

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References


