Interleukin 6 Induces the Expression of Vascular Endothelial Growth Factor*

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Angiogenesis, the formation of new blood vessels, is induced by various growth factors and cytokines that act either directly or indirectly. Vascular endothelial growth factor (VEGF) is a specific mitogen for vascular endothelial cells and therefore has a central role in physiological events of angiogenesis. Interleukin-6 (IL-6) expression on the other hand is elevated in tissues that undergo active angiogenesis but does not induce proliferation of endothelial cells. We demonstrate using Northern analysis that treatment of various cell lines with IL-6 for 6–48 h results in a significant induction of VEGF mRNA. The level of induction is comparable to the documented induction of VEGF mRNA by hypoxia or cobalt chloride, an activator of hypoxia-induced genes. In addition, it is demonstrated by transient transfection assays that the effect of IL-6 is mediated not only by DNA elements at the promoter region but also through specific motif(s) located in the 5′-untranslated region (5′-UTR) of VEGF mRNA. Our results imply that IL-6 may induce angiogenesis indirectly by inducing VEGF expression. It is also shown that the 5′-UTR is important for the expression of VEGF. The 5′-UTR of VEGF is exceptionally long (1038 base pairs) and very rich in G + C. This suggests that secondary structures in the 5′-UTR might be essential for VEGF expression through transcriptional and post-transcriptional control mechanisms.

Angiogenesis, the formation of new blood vessels, is a multistep process in which many growth factors and cytokines have an essential role. Two types of angiogenesis promoting agents were characterized: 1) those that indirectly stimulate angiogenesis like tumor necrosis factor α (TNF-α)1 and transforming growth factor β (TGF-β). 2) Those that act as direct angiogens such as acidic and basic fibroblast growth factor (aFGF and bFGF, respectively) or vascular endothelial growth factor (VEGF) (1–6).

VEGF is a very potent angiogenic agent that acts as specific mitogen for vascular endothelial cells through specific cell surface receptors. VEGF is encoded by a single gene, however, 4 isoforms of 206, 189, 165, and 121 amino acids long are produced as a result of alternative splicing (7, 8). The 165-amino acid long isoform is the most abundant. However, no differences in the biological activities of these isoforms have been reported although differences in their receptor binding abilities and their capability to interact with the ECM were demonstrated (9, 10).

The promoter region of VEGF has been cloned, sequenced, and found to contain numerous putative binding sites for various transcription factors such as AP1, AP2, and SP1 (11). Interestingly, VEGF contains a very long (1038 bp) 5′-UTR that was also noted in a related cytokine, Platelet-derived growth factor (PDGF), and found to contain all the DNA binding motifs described above. This 5′-UTR is characterized by a high G + C content upstream to the translation initiation site which is also characteristic of 5′-UTRs corresponding to PDGF, placenta growth factor and TGF-β (12–14). VEGF expression was reported in normal tissues like lung, kidney, adrenal gland, liver, stomach, heart, and peritoneal macrophages and during normal physiological conditions such as cyclical corpus luteum formation, wound healing, and embryonic development. Conversely, the expression of VEGF was also reported during abnormal physiological conditions such as tumor angiogenesis (2, 15–19).

The expression of VEGF promotes the formation of new blood vessels in a regulated mechanism that is sensitive to hypoxia and is not completely characterized. Recently, it was demonstrated that physiological conditions such as hypoxia (or treatment with cobalt, that mimics hypoxia) can induce VEGF expression (20, 21). In addition, induced expression of VEGF was also noted in cells treated with interleukin-1β (IL-1β), TGF-β, PDGF-B, and 12-O-tetradecanoylphorbol-13-acetate (22–25). Yet, the molecular mechanism governing the expression of VEGF are not characterized and it is likely that other cytokines might promote the expression of VEGF as well.

In this work, we have studied the effect of various cytokines on the expression of VEGF. We show that VEGF mRNA is induced by IL-6. This induction is also demonstrated by transient transfection assays in which the promoter region of VEGF including its exceptional 5′-UTR were connected to the reporter gene chloramphenicol acetyltransferase (CAT). We show that DNA element(s) located upstream to the transcription initiation site mediate in part the response to the IL-6. In addition, we demonstrate that the induction of VEGF is also mediated through specific DNA element(s) located at the 5′-UTR of the gene. The data suggests that the 5′-UTR elements cooperate synergistically with DNA elements located upstream to the transcription initiation site. It is also shown that 5′-UTR of VEGF has an important role in the expression of VEGF. Fi-
nally, we propose that IL-6 should be considered as an indirect inducer of angiogenesis that exerts its activity through the induction of VEGF.

MATERIALS AND METHODS

Cell Culture—The cell line A431 was received from ATCC (Rockville, MD). The cell lines C6 and L8 were kindly obtained from Drs. E. Keshet (The Hebrew University, Israel) and S. Salzberg (Bar-Ilan University, Israel), respectively. The cell lines were all grown in Dulbecco's modified Eagle's medium supplemented with gentamicin, glutamine, 10% fetal calf serum for A431 and C6 cells and 15% fetal calf serum for L8 cells.

Northern Blot Analysis—Cells were grown to 75% confluency and a typical set of experiments included untreated cells and cells treated with cytokines and cobalt at indicated times. R-IL-6 (80 ng/ml) was kindly obtained from Inter-Lab Ltd. (Ness-Ziona, Israel) through Drs. M. Revel and J. Chebath (Weizmann Institute, Israel) and from Dr. M. Rubinstein (Weizmann Institute, Israel). Human interferon-β (IFN-β, Fronet, 2500 IU/ml) was kindly obtained from Interpharm Laboratories Ltd. (Rehovot, Israel), Tumor necrosis factor-α (10 ng/ml) was a generous gift from Dr. D. Wallach (Weizmann Institute, Israel). Cobalt chloride (550 μM) was purchased from Spectrum Chemical Manufacturing Corp. (Gardena, CA). Following the incubation, the cells were harvested and RNA was prepared using Tri Reagent (MRC, Cincinnati). Northern blot analyses were carried out as described previously (26).

RESULTS

Construction of VEGF-CAT Constructs—The bacteriophage λ genomic clone (4), originally generated from human lung fibroblasts that contains the first two exons of VEGF and the 3.4-kb sequence upstream to the translation initiation site, was kindly obtained from Dr. J. Abraham (California Biotechnology Inc.) (11). A 5.5-kb EcoRI fragment from λ was cloned into the EcoRI site of pUC18 (pUC18-5.5). This fragment containing pUC18 plasmid was digested with EcoRI and NarI and a 3.3-kb segment corresponding to DNA sequences 83 bp upstream to the first ATG codon of VEGF was cloned into the same restriction sites in pUC19 thus generating pUC19-3.3. This plasmid was digested with NarI and HindIII1 and the resulting fragment (3.3 kb) was cloned into the promoterless CAT construct pBL CAT 3 (28) that was digested with AccI and HindIII1, thus generating p3.4CAT-NR. To generate internal deletions p3.4CAT-NR was digested with Nhel, thus removing 695 bp. The digested plasmid was self-ligated thus generating p3.4CAT-NHe. Similarly p3.4CAT-NR was digested with Nhel and Xhol, thus deleting 989 bp upstream to the translation initiation site, that was then religated to itself following fill-in reaction with Klenow fragment generating the reporter plasmid p3.4CAT-NX. 3 CAT constructs were digested with PstI, self-ligated, and as a result a 1571-bp fragment was deleted from the 5′ region of each clone and the equivalent plasmid were termed p1.8CAT-NR, p1.8CAT-NHe, and p1.8CAT-NX. Polymerase chain reaction was employed to construct the CAT reporter plasmids that contain the missing 83 bp located 5′ to the first ATG codon. The 3′ primer corresponding to the first 15 bp located upstream to the first ATG (to which a Xhol site was engineered (L-4, 5′-CATATGCTCGAGGTTTCCGAGGCGCGG-3′) was amplified with the 5′ primer corresponding to the native PstI site located upstream to the translation initiation site (L-3, 5′-TACACTTGTGTTGTCGGAGGCGGCGG-3′) using pUC18-5.5 as a template and the resultant 1829-bp fragment was digested with PstI and Xhol and was replaced with the PstI Xhol corresponding fragment in plasmid p1.8CAT-NR generating p1.8CAT reporter plasmid. To generate a reporter plasmid that contains the available reporter region of VEGF and the 5′-UTR (from position 3400 up to +1), p3.4CAT-NR was digested with PstI and the resultant 1751-bp fragment was cloned into the PstI site of p1.8CAT generating p3.4CAT. The various CAT constructs are schematically illustrated in Fig. 4, A and B, and their features are described under “Results.”

DNA Transfections and CAT Assays—L8 cells were transfected by the calcium phosphate-DNA coprecipitation method as described before (29). Cells were transfected with 5 μg of various plasmid DNA and 15 μg of pUC19 as carrier DNA. For IL-6 treatments, the cells were washed twice with phosphate-buffered saline 24 h following transfection, then treated with human IL-6 at indicated concentration and harvested 48 h later. For cobalt chloride treatment, the cells were washed twice with phosphate-buffered saline as above and cobalt chloro-

![Fig. 1. Effect of cytokines and cobalt chloride on VEGF mRNA expression in A431 cells. A431 cells were treated with the various cytokines at indicated times or cobalt chloride for 6 h (for details see “Materials and Methods”) and total RNA was prepared. Northern blot analysis was performed using 32P-labeled DNA fragments corresponding to VEGF (upper panels) and interferon regulatory factor-1 (middle panel). Lower panel shows the amounts of 28S rRNA.](image-url)
were either treated with IL-6 (80 ng/ml) or cobalt or not. IL-6 and Cobalt—

Transcription initiation site (see 1.8 CAT plasmids illustrated in Fig. 4). An EcoRI-PstI fragment (1571 bp) was deleted from the 5'-UTR region. The first construct, p3.4CAT (3.4-kb genomic region) as compared to CAT activity of untreated cells. However, in cells transfected with the construct p3.4CAT/NrX, in which most of the 5'-UTR was deleted (except for 49 bp downstream to transcription start site), no significant change in CAT activity in response to IL-6 or cobalt was detected. Interestingly, this construct (p3.4CAT/NrX) still contains "a classical promoter region" which is missing the 5'-UTR. However, the CAT activity derived by this construct in untreated cells was reduced by 50% when compared to that of cells transfected with the full-length construct. We have also studied the effect of shorter internal deletions in the 5'-UTR on the ability to promote CAT activity in response to IL-6 and cobalt. Deletion of only 83 bp in the 5'-UTR near the translation start site (p3.4CAT/NrX) resulted in a significant reduction in CAT activity of the untreated transfected cells (at least by 30%) and in a weak response to IL-6, although the response to cobalt was still sustained. A reporter construct containing an internal deletion of 695 bp out of the 1038 bp of the 5'-UTR (p3.4CAT/NrX) was also tested. When introduced into the cells, the level of CAT activity as compared to the full-length construct was also reduced by 25% but no significant response to either cobalt or IL-6 was noted. These results imply that the 5'-UTR potentiates the expression of the VEGF promoter and that response elements to either IL-6 or cobalt may be located within that region.

Fig. 4B shows the results of transient transfection experiments using the 1.8-kb group of CAT constructs which are similar to the 3.4-kb constructs except for a deletion of 1751 bp at the 5' end of the available DNA sequence of the promoter region of VEGF (see illustrations in Fig. 4B). In general, the results of the 1.8-kb group resemble that of the 3.4-kb group except for the fact that the basal CAT activity and responses to IL-6 or cobalt were reduced by 25–50%. The 1.8CAT construct promoted response to both IL-6 and cobalt. However, in comparison to the 3.4CAT construct the extent of increased CAT activity was only 1.4–1.5-fold and not 3–5-fold as for the 3.4CAT construct. Similarly, deletion of 83 bp (p1.8CAT/NrX)
resulted in a modest response to cobalt treatment and a weak response to IL-6. The remaining constructs, p1.8CAT\Nhe and p1.8CAT\NX, promoted CAT activities in untreated transfected cells that were 4-fold lower as compared to that of cells transfected with the full-length 3.4-kb construct. No significant response to IL-6 was observed. These results indicate that the 1751-bp deleted segment contains DNA motifs that work cooperatively with DNA or RNA elements located at the 5'-UTR and confirm the fact that this region is essential for expression of VEGF.

### DISCUSSION

Formation of new blood vessels, angiogenesis, is mediated through the activation of parental vessels by specific inducers that either act directly or indirectly. VEGF is a direct angiogenic agent and its role in promoting angiogenesis during normal and abnormal physiological conditions is well documented (1). This suggests that its expression is subjected to complex regulatory mechanisms. Several stimulators of VEGF expression have been reported including hypoxia and cobalt chloride (20, 21, 31, 33), and various cytokines and growth factors such as IL-1\beta, TGF-\beta, and PDGF-B (22–34, 35).

We have studied the effect of IL-6 on VEGF expression. IL-6 is a multi-functional cytokine that is produced by many cells and has pleiotropic effects. IL-6 acts on a wide range of tissues and cell lines and promotes cell growth and differentiation on the one hand and growth arrest on the other hand (36–38). Previously, it was demonstrated that the in vivo expression of IL-6 accompanies vascularization in the reproductive tissues (32). Moreover, IL-6 expression has been noted during wound healing and tumor growth (38, 39). It was suggested that IL-6 may induce angiogenesis and may also induce motility of cells such as endothelial cells (32, 38, 40). We provide evidence indicating that IL-6 may promote angiogenesis through the induction of VEGF expression. This induced expression is mediated by specific DNA motifs located on the putative promoter region of VEGF (1, 2, 3, 4).
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region of VEGF as well as by specific elements located in the 5'-UTR. IL-6 exerts its biological effects through association with specific cell surface receptors resulting in the activation of specific transcription factors that interact with two types of cis-acting DNA control elements mediating IL-6 response. Type I elements have a consensus sequence attGGNNGYAA which serves as binding sites for NF-IL-6, and type II IL-6 response elements have a consensus sequence CTGGGA that binds IL-6-RE binding protein (41–43). Interestingly, two type I response elements are located at DNA sequences flanking the translation initiation site of VEGF. The first is located between positions −796 and −804 (ATGCTGGAA) within the 5'- UTR and the second is located between positions −2313 and −2320 (TGAGGGAA) upstream to the transcription initiation site. Our results provide evidence that both elements might be functional. When DNA sequences between positions −1829 and −3400 were deleted, the response to IL-6 was decreased by 2-fold in comparison to the response of the full-length promoter plus 5'-UTR. This suggests that part of the IL-6-REs are confined to the deleted region (Fig. 4). In addition, internal deletions within the 5'- UTR abrogated the effect of IL-6, indicating that a possible functional IL-6-RE is also contained within that region. These findings indicate that the putative IL-6-REs at the promoter region of VEGF and its 5'-UTR are not only functional but also operate in a cooperative manner. Our results, however, do not enable us to determine whether motifs in the 5'-UTR regulate the expression of VEGF at the transcriptional or post-transcriptional level.

Recently, it was reported that IL-6-like VEGF is induced in response to hypoxia (44). Thus, our findings together with these recent results suggest that induction of IL-6 by hypoxia may promote the expression of VEGF that eventually leads to angiogenesis. The involvement of IL-6 in angiogenesis is apparent not limited to the hypoxia conditions since it is also produced during wound healing (39) and ovulation (32). Therefore, IL-6 should be considered as an indirect angiogenic factor. Interestingly, α2-macroglobulin is an acute-phase protein that is also induced by IL-6 (42, 43). It was reported that VEGF is inactivated by binding to α2-macroglobulin (45). Since IL-6 induces α2-macroglobulin it may eventually lead to the inactivation of circulating VEGF.

We also demonstrate that other cytokines such as IFN-β and TNF-α can also induce the expression of VEGF mRNA. The involvement of IFN-β in angiogenic processes has not been described before. However, IFN-β is expressed during inflammation, rheumatoid arthritis, and wound healing. Thus, it is probable that expression of IFN-β in response to these disorders might be one of the signals that triggers the angiogenic process through the induction of VEGF expression. Direct proof for such regulatory mechanisms awaits future studies. On the other hand, TNF-α has been implicated as an indirect angiogenic factor (46). Our data suggests as published very recently, that it acts through the induction of the expression of the direct angiogenic agent, VEGF (47).

The 5'-UTR of VEGF is exceptionally long (1038 bp) and contains several putative DNA motifs for SP1, AP1, AP2, as originally noted by Tischer and colleagues (11), and also the putative IL-6-RE type I element. This region is rich in G + C content. A similar structure was also reported for PDGF-B. It was demonstrated that the PDGF 5'-UTR acts as translational inhibitor and upon deletion translation efficiency increases (13, 48). Furthermore, during megakaryocytic differentiation, in which PDGF-B is produced, translation repression is removed by cis-acting elements (48). Similarly, the 5'-UTR of placenta growth factor, albeit shorter (less then 300 bp), is composed of 73% G + C base pairs with a short open reading frame that has an inhibitory effect on translation (12). Our results also demonstrate a possible role for the 5'-UTR in VEGF expression. However, unlike the 5'-UTRs of PDGF and placenta growth factor that serve as translation inhibitors, VEGF 5'-UTR enhances promoter activity as determined by transient assays. A deletion of 83 bp located in the vicinity of the translation start site resulted in a significant decrease in CAT activity (30–40%), suggesting that this region has a role in VEGF expression. Since this region is characterized by a highly G + C content (83%) it is most probable that it has a role in maintaining proper secondary structure of the 5'-UTR which is crucial for proper translation. A possible role for secondary structure was also suggested for PDGF 5'-UTR in inhibiting scanning mechanisms of ribosomes. It was proposed that such structured 5'-UTRs might serve as internal ribosome entry sites (48). Since the 5'-UTR of VEGF can theoretically form such structures, it is most probable that it is also subjected to translational regulation. DNA motifs that confer response to hypoxic treatment enable the binding of specific hypoxia induced factor (21). Similar DNA motifs were also observed for VEGF, however, only the motif located at the 3'-UTR was functional while the two homologous motifs at the promoter region of VEGF (−2856 to −2847 and −1958 to −1949) were not essential (49). It was found that a putative new hypoxia element was contained within a 100-bp region between locations −1882 to −1782. Our results support this finding and enables the margins of the hypoxia element to be further narrowed to positions −1782 to −1829 (construct p1.8CAT). The magnitude of increased CAT activity for the 3.4CAT construct following treatment with cobalt and for the 1.8CAT construct was similar (2-fold), implying that the hypoxia (cobalt) response element is indeed located downstream to position −1829. When most of 5'-UTR was deleted, the response to cobalt was not observed (Fig. 4). This is not in agreement with the reported results of Minchenko et al. (49) that also used constructs lacking the 5'-UTR to which luciferase was connected as the reporter gene. They demonstrated enhanced luciferase activity in response to cobalt in HeLa cells, and calculated their results as ratio between the activity of the reporter gene in treated cells and untreated cells and not in absolute light units. Therefore, it is likely that the extremely sensitive luciferase assay enabled the detection of variations even at very weak promoter activities. However, their results do not contradict ours showing that the 5'-UTR is essential for VEGF expression. Differences in cell lines should be ruled out since our data were also reproducible in HeLa cells (data not shown).

In conclusion, we provide evidence showing that the role of IL-6 in angiogenesis is mediated through the induction of VEGF, a potent angiogenic agent. We show that putative DNA motifs located at the promoter region as well as in the 5'-UTR are necessary for the responsiveness to IL-6. In addition, it is demonstrated that 5'-UTR enhances the basal promoter activity of VEGF as determined by transient transfection assays. These results indicate that the secondary structure of 5'-UTR may be important for an efficient expression of VEGF and suggest that transcription and post-transcription control mechanisms are involved.

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