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The Vascular Endothelial Growth Factor Family of Polypeptides

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Abstract Vascular endothelial growth factor (VEGF) was identified as a heparin-binding polypeptide mitogen with a target cell specificity restricted to vascular endothelial cells. Molecular cloning reveals the existence of four species of VEGF having 121, 165, 189, and 206 amino acids. These have strikingly different secretion patterns, which suggests multiple physiological roles for this family of polypeptides. The two shorter forms are efficiently secreted, while the longer ones are mostly cell-associated. Alternative splicing of mRNA, rather that transcription from different genes, is the mechanism for their generation. In situ hybridization reveals that the VEGF mRNA is widely distributed in most tissues and organs and expressed at particularly high levels in areas of active vascular proliferation, like the ovarian corpus luteum. Ligand autoradiography on rat tissue sections demonstrates that VEGF binding sites are associated with vascular endothelial cells of both fenestrated and non-fenestrated capillaries and with the endothelium of large vessels, while no displaceable binding is evident on non-endothelial cell types. These findings support the hypothesis that VEGF plays a highly specific role in the maintenance and in the induction of growth of vascular endothelial cells.

Key words: growth factors, endothelial cells, angiogenesis, vascular endothelial growth factor, vascular permeability factor

Angiogenesis, the growth of new capillary vessels, results from a cascade of events consisting of (i) enzymatic degradation of the basement membrane of a local venule, (ii) chemotaxis, and (iii) proliferation of endothelial cells [1–3]. Angiogenesis is required for the development and differentiation of the vascular tree, as well as for a wide variety of fundamental physiological processes like somatic growth, wound healing, tissue and organ regeneration, and cyclical growth of the corpus luteum and endometrium [4,5]. In addition, abnormal proliferation of blood vessels is an important pathogenic component of a variety of disorders, including tumor growth and metastasis, rheumatoid arthritis, retinopathies, psoriasis, and retrolental fibroplasia [4,5]. Several factors which have no direct mitogenic effects on endothelial cells are able to promote angiogenesis in vivo. This effect is thought to be mediated by the paracrine release of direct angiogenic inducers from inflammatory cells [4,5]. In contrast, aFGF, bFGF, and PD-ECGF are able to stimulate both vascular endothelial cell growth in vitro and angiogenesis in vivo [6–8]. However, the lack of a signal sequence in FGFs [9,10], as well as in PD-ECGF [8], suggests that these factors may be available to their target cells only following cell death, such as that which occurs during embryonic and fetal development, in a variety of rapidly proliferating neoplasias, or after an injury. Alternatively, FGF may be incorporated into the basement membrane and be released when specific enzymes degrade this structure [11]. Therefore, it appears that factors which can be released by intact cells are more likely to play a role in the regulation of physiological angiogenesis occurring, for example, in the corpus luteum [12], or in the tonic maintenance of the differentiated state of the endothelium in the vascular tree.

In this article we present a review of the molecular and biological properties, as well as an account of the distribution of mRNA and binding sites for a recently identified family of endothelial cell mitogens and angiogenic factors referred to as vascular endothelial growth factor (VEGF) [13–16], vascular permeability factor (VPF) [17,18], or vasculotropin [19]. VEGF was detected in the media conditioned by pituitary

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folliculo-stellate cells [13] and by a variety of transformed cells lines [14,15]. VPF was identified in the media conditioned by tumor cell lines on the basis of its ability to induce blood vessel permeability and protein extravasation [17,18]. Cloning of the cDNA for these factors revealed that they are encoded by the same gene and arise from alternative splicing of messenger RNA. The resulting four polypeptides have strikingly different secretion patterns, which suggests multiple physiological roles for this family of proteins. Two members of this family are secreted by cells, while the third and fourth are mostly cell-associated, despite the fact that all members have an identical signal sequence.

MOLECULAR PROPERTIES OF VEGFS

Clones encoding bovine VEGF were isolated from a cDNA library prepared from folliculostellate cells [14]. The NH_2-terminal sequence determined by microsequencing is preceded by 26 amino acids corresponding to a typical signal sequence [20]. The mature protein is generated directly following signal sequence cleavage, without any intervening prosequence. Therefore, VEGF is a protein which has the potential to be secreted. The mature bovine monomer is expected to have 164 amino acids with a calculated molecular mass of 19,162. The existence of a potential glycosylation site at Asn^{74} suggests that VEGF is a glycoprotein. Clusters of basic amino acid residues are present at the positions around 110, 123, and 163 and they are likely to be responsible for the binding of the molecule to heparin. The amino acid sequence of VEGF displays limited but significant (18–20%) homologies with those of the A and B chains of PDGF and the product of the sis oncogene [21–23]. All eight cysteine residues found in PDGF are conserved in VEGF. However, VEGF contains eight additional cysteine residues within the COOH-terminal region.

Complementary DNA clones encoding human VEGF were isolated from a cDNA library prepared from phorbol-ester activated HL 60 promyelocytic leukemia cells [14]. One of the clones identified encodes a protein which is 95% identical to bovine VEGF. Human VEGF is expected to have 165 amino acids ($VEGF_{165}$) due to the insertion of a glycine in position 6. Two less abundant human clones encoding for a shorter form with a deletion of 44 amino acids between position 116 and 159 and for a longer form with an insertion of 24 highly basic residues in position 116 were also identified. The mature proteins, $VEGF_{121}$ and $VEGF_{189}$, are expected to have 121 and 189 amino acids, respectively. In both cases Asn^{115} is replaced by a Lys. The insertion in $VEGF_{189}$ is highly homologous to the 15 amino acid extension identified in PDGF-A encoded by exon 6, which is subjected to alternative splicing [24].

In order to identify additional members of the VEGF family, we screened a variety of human cDNA libraries by the polymerase chain reaction technique. In a fetal liver library, a unique transcript which did not correspond to any of the previously cloned forms of VEGF was identified and a full length clone was generated [25]. DNA sequence analysis showed that this novel form contained an insertion of 41 amino acids compared with the most abundant form, $VEGF_{165}$, and included the highly basic 24 amino acid insertion found in $VEGF_{189}$. The mature protein therefore is expected to have 206 amino acids ($VEGF_{206}$). Alternative splicing of mRNA, rather than transcription from different genes, is the most likely explanation for the existence of such multiple forms. This is supported by Southern blot analysis of human genomic DNA, which shows that the restriction pattern is identical using either a probe for $VEGF_{165}$ or one which contains the insertion in $VEGF_{206}$. Further evidence of alternative splicing of mRNA is provided by analysis of genomic clones in the area of putative mRNA splicing. This shows an intron/exon structure consistent with alternative splicing [25]. Figure 1 illustrates a model for the generation of all identified molecular species of VEGF.

BIOLOGICAL AND BIOCHEMICAL CHARACTERIZATION OF VEGFS

VEGF purified from bovine pituitary folliculostellate cells is a heat- and acid-stable, dimeric, heparin-binding protein, with molecular weight of ~45 kDa [13,26]. It is completely inactivated by reducing agents. The isoelectric point of VEGF is ~8.5. Recombinant human $VEGF_{165}$ ($rhVEGF_{165}$) behaves similarly to native folliculostellate cells derived VEGF in terms of binding to heparin and retention in reverse phase columns, as well as bioactivity. The molecular weight of $rhVEGF_{165}$ is ~46 kDa. $rhVEGF_{165}$ can exist both in a glycosylated and a deglycosylated species. This results in two types of monomers, having molecular mass, respectively, of 23 kDa and 18 kDa. Since these two species can form

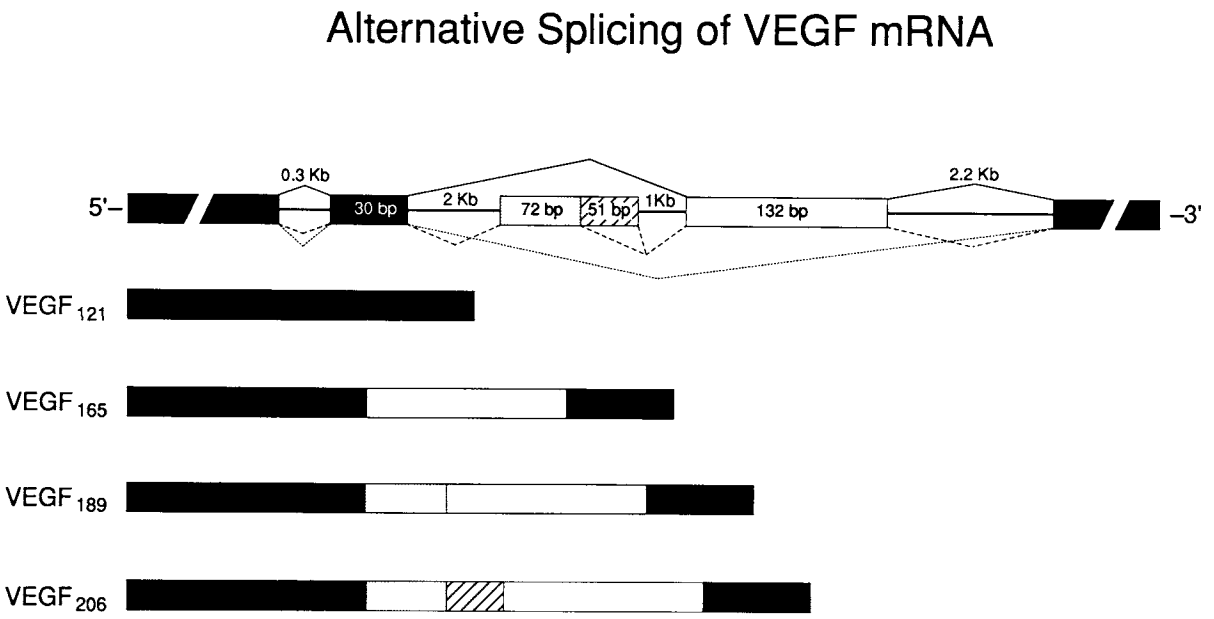

Fig. 1. Schematic model for alternative splicing of mRNA generating four molecular species of VEGF. Exons are represented by boxes and introns by solid lines. The genomic structure of 5'-end and the 3'-end of the VEGF gene has not been determined, as indicated by incomplete boxes.

homodimers and heterodimers, three types of dimers are identified.

Native VEGF or rhVEGF$_{165}$ are able to stimulate the proliferation of endothelial cells isolated from both small and large vessels. These include endothelial cells from bovine adrenal cortex, cerebral cortex, fetal and adult aorta, and human umbilical vein. Half-maximal stimulation of endothelial cells growth was observed at 100–150 pg/ml of VEGF (2–3 pM) and a maximal effect occurred at 1–4 ng/ml (22 pM). VEGF, however, fails to stimulate the proliferation of corneal endothelial cells, vascular smooth muscle cells, BHK-21 fibroblasts, keratynocytes, human sarcoma cells, or lens epithelial cells. This indicates that, unlike aFGF or bFGF, the target cell specificity of VEGF is restricted to vascular endothelial cells. VEGF is also able to induce a marked angiogenic response in the 8-day-old chick chorioallantoic membrane [14]. This in vivo effect demonstrates that VEGF can trigger the entire sequence of events leading to angiogenesis [1–3].

Like VEGF$_{165}$, VEGF$_{121}$ promotes endothelial cell growth and is efficiently secreted by cells, as assessed by metabolic labeling and immunoprecipitation studies. Immunoblot [27] and immunoprecipitation [25] studies indicate that VEGF$_{121}$ exists in two molecular forms having molecular weight of 18 kDa and 14 kDa, respectively. Unlike VEGF$_{165}$, VEGF$_{121}$ binds poorly to heparin and is instead strongly retained by anion exchange columns such as a Mono Q at pH 8.0, indicating that it is an acidic protein. This can be explained on the basis of the absence in VEGF$_{121}$ of the 44 amino acid region, rich in basic residues, which is present in VEGF$_{165}$.

Unlike the two shorter forms, the longer forms appear to be mostly cell-associated. Little or no mitogenic activity can be recovered in the medium conditioned by 293 cells transiently expressing a cDNA encoding VEGF$_{189}$ or VEGF$_{206}$ [25]. Furthermore, immunoprecipitation and SDS/PAGE analysis of both cell lysates and conditioned medium of metabolically labeled, transfected cells reveals the presence of strong bands of the expected size (27 and 29 kDa, respectively) in cell extracts, while only weak bands of lower molecular weight (18 to 14 kDa) are precipitated in the medium [25]. The information for remaining cell-associated is entirely contained within the 24 amino acid insertion, since a mutant of VEGF$_{206}$ lacking the 24 amino acid insertion, but containing the 17 amino acid insertion unique to VEGF$_{206}$, was instead efficiently secreted [25]. Media conditioned by stable cell lines expressing a VEGF$_{189}$ cDNA have low level of mitogenic activity for endothelial cells. Surprisingly, some of the chromatographic properties of such bioactive material are similar

to those of $VEGF_{121}$. It binds weakly to heparin and is retained by a Mono Q column at pH 8.0, indicating that its isoelectric point is in the neutral-acidic range. This is clearly inconsistent with the full length polypeptide, which is expected to be even more basic than $VEGF_{165}$. It is, however, consistent with a cleaved polypeptide, lacking a large portion at the COOH terminus, but rich in basic residues. This hypothesis is supported by the molecular mass of the immunoprecipitated material.

All four molecular species display activity in a Miles-type of assay [28], namely they induce extravasation of Evans Blue when injected intradermally. However, preliminary evidences suggest that significant differences in potency may exist. For example, despite the low amounts of VEGF secreted, media conditioned by cells expressing a cDNA encoding $VEGF_{189}$ or $VEGF_{206}$ are strongly positive in the Miles assay, suggesting that the secreted products of $VEGF_{189}$ or $VEGF_{206}$ may be more potent than the other forms in inducing vascular permeability.

EXPRESSION OF VEGF

Cultured Cells

A variety of human tumor cell lines, including sarcomas and carcinomas, show a 3.7 kb RNA transcript which hybridizes with the VEGF probe in a Northern blot. A minor transcript having the size of 4.2 kb is also observed. Also, several human and rodent tumor cell lines have been shown to secreted VPF [17]. This factor was thought to be a specific product of tumor cells, most likely responsible for the abnormal permeability properties of tumor vessels and for tumor-associated ascites. Interestingly, mouse sarcoma 180 cells express VEGF mRNA and secrete a VEGF-like mitogen [29]. The media conditioned by these cells was used in the first successful long-term culturing of capillary endothelial cells [3].

Little is known regarding the expression of VEGF in normal cells. We recently found that an untransformed cell type other than pituitary folliculo-stellate cells expresses VEGF. Bovine aortic smooth muscle (ASM) cells express the VEGF RNA transcript and secrete a VEGF-like endothelial cell mitogen [27]. The localization of VEGF in ASM cells might have significant implications for the integrity of the endothelial cells in the intima. In a large arterial vessel like the aorta, endothelial cells are exposed to the sheer force of blood flow under high pressure and presumably there is a need for a growth factor capable of maintaining the integrity of endothelial cells and repairing intimal tears. Further studies are required to establish whether or not ASM cells also secrete VEGF in vivo.

Tissue Distribution of VEGF mRNA

To study the cellular localization of VEGF in various tissues, we used the in situ hybridization technique. Probes of ^{35}S-labeled RNA were synthesized from a template of bovine VEGF cDNA and cloned in both orientations into a plasmid vector containing SP6 RNA polymerase promoter [30]. Such a probe, however, would not discriminate among the different molecular species of VEGF. We examined whether or not VEGF mRNA is expressed in pituitary gland, brain, kidney, and ovary [30,31].

Pituitary gland. In adult rat pituitary specific hybridization to the VEGF probe was observed in at least 20% of *pars distalis* cells. Since folliculo-stellate cells account for only 5–10% of the total cell population of the *pars distalis* [32], it appears that at least certain secretory cells have the ability to express VEGF. Modest hybridization was observed in the *pars intermedia*, the least vascularized area of the pituitary. Intense labeling was detected instead in the *pars nervosa*.

The expression of VEGF in the pituitary might contribute to the development and differentiation of the pituitary portal vessels during fetal and early postnatal life and to the maintenance of their differentiated state in the adult animal. Capillaries of the primary portal plexus are observed as early as the 18th fetal day in the rat [33]. The proliferation and maturation of these microvessels is essential for the establishment of a vascular link between median eminence and pituitary, which allows hypothalamic neurohormones to affect secretory cells [34,35].

Brain. Angiogenesis is a prerequisite for the development and differentiation of the nervous system. The neural plate appears as early as the cardiovascular primordia, but the latter reach a functional state, while the neural elements are still relatively undifferentiated [36]. The ingrowth of blood vessels into the early anlagen of mesencephalon and telencephalon correlates well with the onset of neuroectodermal proliferation [37]. Previous studies have documented the angiogenic properties of extracts derived from fetal mesencephalic and telencephalic structures and

provided evidence for the presence of FGF in such extracts [38].

Our evidence indicates that VEGF also is expressed in the brain [31]. In situ hybridization on fetal rat brain sections at day 14 reveals a high level of expression of VEGF mRNA throughout the brain. In sections of fetal bovine cerebral cortex a low level of hybridization was detected throughout, while certain subpopulations of cells in layers II and III gave an especially strong signal.

We also examined a series of coronal sections of adult rat brain at the level of the hypothalamus. A diffuse low level hybridization consistently higher than sections incubated with control sense probe was detected. A particularly high signal was identified in the supraoptic (SO) and paraventricular (PV) nuclei, as well as in the choroid plexus. Significant hybridization was also identified in the median eminence and in the subfornical organ. In both SO and PV nuclei, the hybridization signal was clearly localized around nuclei of cells consistent with the morphology of the magnocellular neurons, which produce ADH and oxytocin [39]. These data demonstrate that the mRNA for the growth factor is present in brain structures in several species and both in fetal and adult life.

Kidney. In adult rat kidney, a diffuse hybridization signal was observed throughout the cortex and medulla. A strikingly higher signal was detected in association with the glomeruli. Silver grains were seen in the external surface of the glomerulus and most of the hybridization signal appeared to be associated with the podocytes, the visceral epithelial lining of Bowman's capsule. This localization is particularly interesting, considering that podocytes are in a unique position to exert trophic effects on capillary endothelial cells, since their feet-like processes closely embrace the entire network of glomerular capillaries [40].

Ovary. The growth of new capillary vessels is a prominent feature of the development and differentiation of the corpus luteum (CL) [12]. In the course of follicular growth, the theca interna becomes richly vascularized. Following ovulation, the thecal vessels grow into the ruptured follicle and form a complex network of vessels which nourish the developing CL. We examined whether or not VEGF is expressed in the rat ovary [30]. Intense hybridization to the VEGF probe was found in the CL, while minimal hybridization was detected in the mural granulosa cells. Most of the hybridization signal was associated with luteal cells. This suggests a temporal relation between VEGF expression and growth of capillary vessels into the CL. This finding also argues for a physiological role played by VEGF in the regulation of development and function of the CL.

VEGF BINDING SITES

Specific binding sites for VEGF/VPF have been recently identified in cultured endothelial and non-endothelial cells [41–43]. Two classes of high affinity sites were identified on the cell surface of bovine endothelial cells, having dissociation constants of 10^{-12} and 10^{-11} M, respectively [41]. Based on cross-linking studies, the molecular weight of the putative receptor has been estimated to be ~180 kDa [41,42] or ~230 kDa [43]. A lower molecular weight (110 kDa) receptor species would be also present in endothelial cells [42]. Surprisingly, high affinity binding was also identified in cell types that do not display a mitogenic response to VEGF, such as lens epithelial cells or corneal endothelial cells [44]. In these cells, however, only the lower molecular weight species of receptor was found.

In order to determine the distribution of cells responsive to VEGF in situ, we performed binding to tissue sections from adult rats with biologically active, iodinated $rhVEGF_{165}$ [45]. Quantitative autoradiography was employed to examine the binding kinetics and simultaneously localize binding sites at both macroscopic and microscopic levels. We identified specific, displaceable, high affinity VEGF binding sites in the majority of tissues and organs. Quantitative analysis of whole body sections revealed that the highest density of binding sites was in brain and spinal cord, adrenal cortex, lung, glandular stomach, spleen, and pancreas. Scatchard analysis of saturation isotherms in sections from brain, kidney, and pancreas revealed a single class of binding sites with high affinity (K_d = 16 to 35 pM) and low capacity (1.9 to 6.8 fmole/mg protein). Throughout the sections, specific binding was heterogeneously distributed in a manner consistent with the known vascular pattern of the various organs. Colocalization of binding with factor VIII-like immunoreactivity demonstrated that the binding was associated with vascular endothelial cells of both fenestrated and non-fenestrated capillaries and with the endothelium of large vessels. In contrast, no displaceable binding was evident on non-endothelial cell

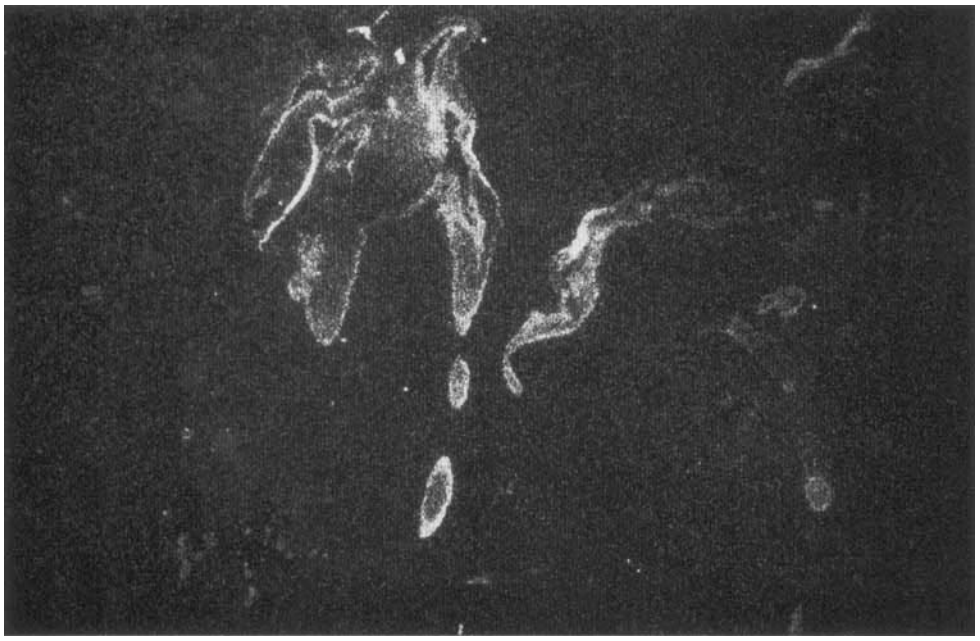

Fig. 2. Specific ^{125}I-VEGF binding to adult rat mitral valves. The binding is found on the lower and upper surface of all valves in the heart, as determined by examination of longitudinal sections through the heart of three rats.

types. VEGF binding sites were associated with quiescent as well as proliferating vessels. Figure 2 illustrates the binding of ^{125}I-rhVEGF to the mitral valve, a structure which displays a particularly high density of binding sites [45].

CONCLUSIONS AND PERSPECTIVES

Although VEGFs have been purified and cloned only very recently, the high degree of structural conservation across species and the widespread tissue distribution of both VEGF mRNA and binding sites already suggest that this family of growth factors plays important biological roles.

The finding that the expression of VEGF mRNA is temporally related to the proliferation of capillary vessels in the ovarian CL is clearly consistent with the hypothesis that one of the physiological roles of VEGF is to promote angiogenesis. Interestingly, VEGF expression was also identified around microvessels in areas where no active angiogenesis is taking place, such as the adult kidney glomerulus, the pituitary, or the brain. Likewise, VEGF binding sites were identified in the vasculature of these areas. This raises the possibility that the tonic presence of the growth factor may be required in order to maintain the differentiated state of those vessels, which otherwise might undergo involution. It is therefore tempting to speculate that the suppressed expression of a positive angiogenic inducer like VEGF and/or its receptors may be at least in part responsible for a variety of regressive phenomena involving blood vessels, both in developing and adult animals. For example, certain tissues like the cartilage or the vitreous body in the eye are vascularized richly during embryonic life, but become avascular at later stages of development [46], suggesting either the action of inhibitors or the suppression of activities capable of exerting trophic effects on the blood vessels.

The finding that VEGF binding sites in adult rat tissues are localized only in endothelial cells [45], provides a direct demonstration of the unique target cell specificity of this growth factor, which had been suggested by in vitro studies.

An especially high density of binding sites for VEGF is present in the endothelium lining areas which are subjected to the sheer force of high flow, such as the atrioventricular valves in the heart. The maintenance of the integrity of the endothelium lining these structures is essential for the prevention of thrombosis or endocarditis [47]. The presence of high VEGF binding in these regions might relate to a need for continual repair and maintenance of the endothelium.

Further research is required in order to address an important biological question like the

elucidation of the factors which regulate VEGF expression. The finding that VEGF mRNA expression in the ovary is related to luteinization suggests that gonadotropins are able to induce ovarian VEGF [48].

A property of this family of growth factors is the ability to induce extravasation of Evans Blue when applied intradermally. All four molecular species display such activity, although significant quantitative differences might exist. One might therefore speculate that one of the physiological roles of these factors is the regulation of vascular permeability. The verification of this hypothesis would probably require the demonstration of a correlation between expression of mRNA and/or receptors and microvascular permeability. However, binding sites for $VEGF_{165}$ are expressed both in fenestrated (e.g., endocrine glands) and tight capillaries (e.g., brain). Likewise, VEGF mRNA is expressed around both types of microvessels. This suggests that the most abundant form of VEGF probably is not directly responsible for inducing vascular leakage and protein extravasation, at least in physiological circumstances.

An intriguing aspect is the existence of four different molecular species of VEGF due to alternative splicing of mRNA. As previously noted, it appears that the 165 amino acid species is the predominant molecular form found in normal cells and tissues, with the exception of the placenta where the short form appears to be the most abundant. Very little is known about the distribution of the longer forms. It is possible that these may be only minor molecular species expressed by all cells that express the VEGF gene or they could be preferentially expressed in specific circumstances. Interestingly, alternative splicing of mRNA takes place also in PDGF-A. The two molecular species of PDGF-A differ by 15 amino acids in the COOH-terminus [24]. This alternative splicing pattern is found in all species examined, including *Xenopus laevis* [49,50]. The 15 amino acid extension in PDGF-A is very rich in basic amino acids and is highly homologous to the 24 amino acid insertion found in human VEGF [51]. These basic motifs have been termed nuclear targeting sites and display homology to highly conserved basic sequences in histones [52,53]. It has been proposed that such sequences impart directional or targeting information on polypeptides. For example, the extended form of PDGF-A would be sequestered into specific domains, while the non-extended form would be mostly secreted [54]. Also, recent studies on acidic FGF, which also contains a nuclear targeting sequence in its amino terminal domain, indicate that such sequences may encode critical information for intracellular signaling and protein trafficking [55]. In the case of VEGF, the nuclear targeting domain confers on the molecule the property of being mostly cell-associated, rather than secreted. Future studies are required to elucidate the significance of such cell-associated molecular species.

The study of VEGF may also provide important insight into the pathogenesis of several disorders characterized by abnormal angiogenesis. As previously indicated, a variety of diseases, most notably tumors, are characterized by uncontrolled proliferation of blood vessels. The hypothesis that the overexpression of VEGF may facilitate tumor growth and metastasis can be tested directly, by analogy with other growth factors, like TGF alpha [56] or bFGF [57], by transfecting cells with plasmids carrying the VEGF cDNA and observing whether or not those cells acquire tumorigenic properties when injected into nude mice. It would be also very interesting to compare the tumorigenic properties of cells transfected with cDNAs encoding the different molecular species of VEGF.

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