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Nuclear localization of long-VEGF is associated with hypoxia and tumor angiogenesis

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Abstract

Vascular endothelial growth factor (VEGF) is a potent angiogenic factor that has a pivotal role in normal and pathological angiogenesis. VEGF has a long 5′ untranslated region harboring an open reading frame (ORF) initiated by a CUG codon that is in-frame with the VEGF coding region. The ORF translation leads to the expression of a long isoform termed L-VEGF that is extended by an additional 180 amino acids. In this communication, we provide evidence that L-VEGF is subjected to proteolytic cleavage leading to the detachment of the 180 aa extension from the VEGF moiety. Using immunofluorescence staining, we show that upon hypoxia this 180 aa extension translocates to the nuclei of expressing cells. Accordingly, immunohistochemical staining of both normal and tumor tissue samples demonstrated restricted nuclear localization of the ORF, which was correlated with cytoplasmic localization of VEGF. This suggests that the 180 aa ORF is involved in VEGF-mediated angiogenic processes.

Keywords: VEGF; Angiogenesis; L-VEGF; Hypoxia; Nuclear localization

Angiogenesis is the process of new blood vessel formation from pre-existing networks of capillaries. Vascular endothelial growth factor (VEGF) has a central role in physiological as well as pathological angiogenesis. It is encoded by a single copy gene, however, many isoforms were identified due to alternative splicing. The 26 amino terminal residue of VEGF acts as a leader sequence that is cleaved upon VEGF secretion [1,2]. Being a major angiogenic factor, VEGF is subjected to multilevel regulation to ensure proper expression during embryogenesis and adulthood. The short-lived VEGF mRNA is both induced and stabilized upon stress signals such as hypoxia [3]. It has a very long (1038 bp) 5′ untranslated region (5′UTR), which contains two internal ribosome entry site (IRES) elements (A and B) [4–6]. These two regulatory elements ensure efficient translation under stress conditions such as hypoxia, where normal translation via ribosomal scanning mechanism is severely impaired. In addition, the 5′UTR of the VEGF harbors a highly conserved open reading frame (ORF), which can add 180 amino acids (aa) in front of VEGF. This ORF is initiated by a CUG codon and followed by additional three conserved CUGs. This lengthened isoform was termed Long-VEGF (L-VEGF) and shown to be expressed in VEGF producing cells and in various tissues [7–9].

Cytolocalization studies were performed to gain better insight into the biological activity of L-VEGF. The data presented here clearly show that effective cleavage of L-VEGF results in the detachment of mature VEGF from the 180 aa ORF extension initiated from the upstream in-frame CUG codon. Expression of just the

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180 aa extension of VEGF leads to cytoplasmic staining. However, upon hypoxia this N-terminal segment is translocated to the nucleus via the nuclear localization signal (NLS) confined to the amino-terminus. Further, strong association between the level of VEGF expression and nuclear localization of the 180 aa ORF was observed in various normal angiogenic tissues that was even more profound in the corresponding tumors. Taken together, our results suggest that nuclear localization of 180 aa extension has an important role in the VEGF-mediated angiogenesis; physiological as well as pathological.

Materials and methods

Cell culture, DNA transfections, whole cell lysate, and Western blot analysis. The cell line HEK 293 (originally from ATCC) was grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and antibiotics. These cells (1.5×10^6 cells/10 cm dish) were transfected by the calcium phosphate-DNA coprecipitation method with 15 μg of expression plasmid DNAs and 0.5 μg pMDISRLuc (SV40 promoter driving the expression of *Renilla* luciferase to monitor transfection efficiencies). The cells were subjected to hypoxia as previously described [7]. Under these conditions oxygen level was 5% as determined by gas chromatography. Each culture plate was harvested 48 h after transfection with 100 μl lysis buffer of the Luciferase assay kit (Promega) according to the manufacturer's instructions. Each transfection sample was normalized for protein concentration and corrected for transfection efficiency. Each set of transfection experiments was repeated at least three times generating similar results.

Proteins were separated on 12.5–15% SDS–PAGE and transferred to PVDF membrane. The membranes were subjected to Western blot analyses with affinity purified 1:100 dilution of rabbit anti-ORF antibody. This antibody was generated against bacterially expressed 180 aa extension of L-VEGF fused to glutathione-*S*-transferase (GST). This antibody was affinity purified over bacterially expressed ORF [7] that was covalently bound to cyanogen bromide activated Sepharose beads (Pharmacia). In addition, the membranes were also reacted with 1:100 dilution of monoclonal antibody directed against VEGF (Santa Cruz Biotechnology) or affinity-purified rabbit polyclonal antibody directed against VEGF [7], and 1:500 dilution of anti-GFP antibody (Santa Cruz Biotechnology). The specific bands were identified by ECL analysis using SuperSignal (Pierce).

Plasmids. The plasmids p5′UTR-VEGF in which the 5′UTR of VEGF was fused either to the coding region of $VEGF_{165}$ or $VEGF_{121}$ in the expression vector pcDNA3.1/Hygro (Invitrogen), and the same expression plasmid harboring just the 180 aa ORF extension decorated with His-tag at the carboxyl terminus (pORF), were previously described [7].

To generate a mammalian expression vector allowing the expression of the 180 aa extension of L-VEGF either upstream or downstream to GFP (p180 up and p180 down, respectively), the 180 aa ORF was amplified from the plasmid p5′UTR-$VEGF_{165}$ using a 5′ primer (GGACTAGTAGATCTATGACGGACAGACAGA) harboring *Spe*I and *Bgl*II restriction sites, and a 3′ primer (CCAAGCTTACCGGT GTTTCGGAGGCCCGACC) harboring *Age*I and *Hin*dIII restriction sites. The amplified product was sub-cloned into pGEM-T vector (Promega) and the inserted fragment was subsequently cloned to pEGFP-C1 (Clontech). For upstream fusion the *Spe*I and *Age*I restriction sites were used to clone the ORF fragment to the *Nhe*I and *Age*I sites in the plasmid pEGFP-C1. To generate the downstream fusion, the restriction sites *Bgl*II and *Hin*dIII on both donor and acceptor plasmids were utilized.

To generate a vector that expresses the 100 N-terminal aa of the ORF (p1-100ORF), the corresponding fragment was amplified from the plasmid p5′UTR-$VEGF_{165}$ using the same 5′primer described above and a 3′ primer that harbors the *Hin*dIII restriction site (CCAAGCTTA CCGGTTTCTCTTCTTCCTCCTCCC). The amplified product was cloned into pGEM-T vector (Promega) and the inserted fragment was digested by *Spe*I and *Hin*dIII, and ligated to *Nhe*I and *Hin*dIII sites in the mammalian expression vector pCDNA3.1/Hygro.

In order to generate vectors expressing N-terminal truncated ORF polypeptides, p100-180ORF and p50-180ORF, the coding fragments were amplified using the 5′ primers (GCTCTAGAATGGAGAAGG AAGAGG) and (GCTCTAGAATGGTCGCACTGAAAC) harboring an ATG translation start site and corresponding to p100-180ORF and p50-180ORF, respectively, and the 3′ primer (GCTCGGATCCTTA GTGATGATG). The amplified products were cloned into pGEM-T vector (Promega) and the segments were released by digesting with *Xba*I and *Bam*HI, and subsequently cloned into pCDNA3.1/Hygro.

The plasmid pHygro$VEGF_{165}$, which allows expression of the coding region of $VEGF_{165}$ without the 5′UTR, was generated by digesting the plasmid pUC18$VEGF_{165}$ [10] with *Bam*HI and cloning the $VEGF_{165}$ coding region into the mammalian expression vector pCDNA3.1/Hygro (Invitrogen).

Immunofluorescence. Cells were grown in duplicate on sterile microscope slides (SuperFrost Plus, Menzel, Germany) in a 10 cm dish and transfected with plasmid DNA. 48 h later the cells were fixed for 15 min at room temperature in 4% paraformaldehyde buffered with PBS. After washing with PBS, cells were permeabilized with 0.1% saponin in PBS for 15 min followed by 1 h blocking with blocking solution (1% BSA, 0.1% saponin, and 1 mM EDTA in PBS). Primary antibody (affinity purified αORF) diluted 1:5 in blocking solution was incubated overnight at 4 °C. Following the incubation, the slides were washed with PBS and incubated for 1 h with Alexa Fluor 488-conjugated secondary antibody (Molecular Probes) diluted 1:300 in blocking solution. Finally, slides were washed with PBS containing 1 μg/ml 4′,6-diamidino-2-phenylindole (DAPI, Sigma) for 10 min to stain the nuclei, washed, and mounted with fluoromount G (EMS). All washes were performed three times for 5 min with PBS with gentle agitation.

Immunohistochemistry. Paraffin embedded tissue sections were obtained from the archives of the Pathology Department at Carmel Medical Center, Haifa, Israel. Immunohistochemical staining was performed according to the following protocol. Consecutive sections from formalin-fixed paraffin embedded tissue blocks were cut at 4 μm, deparaffinized, and rehydrated with xylene and graded alcohol. Microwave epitope retrieval was performed in 0.01 M citrate buffer (pH 6.0), followed by cooling for 15 min at room temperature. Endogenous peroxidase activity was quenched by incubation with 3% H_2O_2 in methanol. Sections were then blocked with Avidin–Biotin blocking kit (Zymed Laboratories) according to manufacturer's instructions followed by Cas-block solution (Zyme Laboratories) for 30 min at room temperature. The following primary antibodies were used: affinity purified rabbit-anti-ORF polyclonal antibody (1:5 dilution, [7]), goat anti-VEGF antibody (Santa Cruz Biotechnology), and rabbit-anti-VEGF polyclonal antibody (1:100 dilution, [7]). The slides were incubated with primary antibodies for 16–18 h at 4 °C followed by incubation with the EnVision Plus anti-rabbit or anti-goat kit (DAKO, Glostrup, Denmark) using AEC+ chromogen. Following detection the sections were counterstained with Mayer's hematoxylin and mounted.

Results

L-VEGF is effectively cleaved separating the 180 aa extension from the VEGF moiety

In order to follow the cellular localization of L-VEGF, affinity purified antibody directed against the

180 aa extension of L-VEGF fused to GST was prepared (for details see Materials and methods). This purified antibody reacted only with 180 aa ORF and not with GST (data not shown). To follow the fate of the 180 aa extension of L-VEGF in expressing cells, it was fused in-frame either upstream or downstream to GFP. In these two constructs (Up 180 or Down 180, Fig. 1), the initiation CUG codon was replaced by an AUG codon. These constructs as well as the control construct, encoding just for GFP, were transfected to HEK 293 cells. Forty-eight hours later extracts were prepared, separated over 12% SDS–PAGE, and subjected to Western blot analysis with antibody directed against either the 180 aa extension (αORF) or against GFP. When antibody directed against GFP was reacted with extracts from cells transfected with the 180 aa ORF fused upstream to the GFP, it was evident that the most upper band was fairly faint (Fig. 1A, lane 1). This band corresponds to a protein with an estimated molecular weight of ~53 kDa, which fits the expected size of ORF–GFP fusion product. In addition, three stronger bands were detected. Two adjacent bands with an estimated molecular weight of 34 kDa corresponding to GFP fused to a ~5 kDa segment from the C-terminal end of the 5′UTR-ORF of VEGF. This suggests the existence of an efficient cleavage site ~5 kDa 5′ to the initiation AUG of the GFP. The third intense band has a molecular weight of 29 kDa corresponding to GFP (Fig. 1A, lane 1). This expression of GFP without the fused ORF is not due to proteolytic cleavage but rather due to an effective translation mediated by IRES-A, which spans over 200 bp at the 3′ end of the 5′UTR-ORF extension of VEGF (see illustration in Fig. 1B). The same cell extract was also reacted with antibody directed against the 180 aa extension (αORF). In principle, a similar pattern was seen except for the band corresponding to GFP. These reciprocal results with anti-ORF antibody confirmed that the most upper band is a fusion product of 180 aa ORF and GFP, and that the two adjacent bands with a molecular weight of 34 kDa represent cleavage products ~5 kDa 5′ upstream to the initiation AUG of the GFP. Further conformation for the existence of this effective cleavage site came from similar analyses with extracts from cells transfected with a plasmid construct in which the 180 aa were fused downstream to the GFP. As seen in Fig. 1A complementary data were retrieved with both antibodies. A single slow migrating band with an estimated molecular weight of 48 kDa was observed (Fig. 1A, lanes 2 and 5). This fusion product is shorter by about 5 kDa from the expected size. This also points to the existence of an effective cleavage site ~5 kDa upstream to the C-terminus of the 180 aa extension of L-VEGF (see illustration in Fig. 1B). The nature of the fast migrating band detected with the anti-GFP antibody (Fig. 1A, lane 2) will be discussed henceforward.

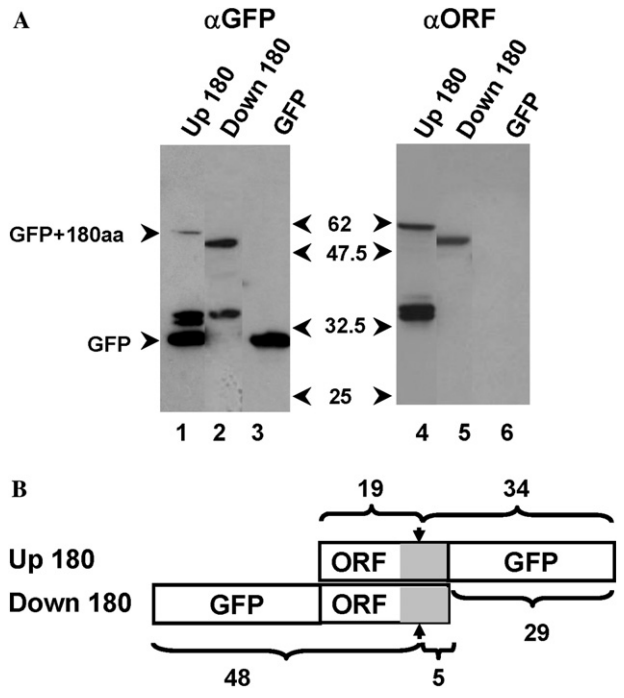

Fig. 1. Expression pattern of the 180 aa ORF of L-VEGF fused either upstream or downstream to GFP in HEK 293 cells. (A) HEK 293 cells were transiently transfected with the mammalian GFP vector pEGFP-C1 alone (GFP) or encoding for a fusion peptide of the 180 aa ORF extension of L-VEGF either upstream (Up 180) or downstream (Down 180) to GFP. Forty-eight hours later cells were harvested and subjected to Western blot analysis with commercial anti-GFP antibody or affinity purified anti-ORF antibody. Arrowheads indicate GFP, GFP fusion product, and molecular weight standards in kDa. (B) Schematic illustration of the 180 aa ORF extension of L-VEGF fused either upstream or downstream to GFP. The putative cleavage site identified by Western blot is indicated by arrowhead and the position of IRES A within the ORF is indicated by shaded area. The sizes of either the proteolytic products or IRES-mediated translation of GFP are shown in kDa.

To show that L-VEGF is also cleaved in a similar manner, the 5′UTR of VEGF fused either to cDNA of $VEGF_{165}$ or $VEGF_{121}$ was cloned in mammalian expression vector. In these two constructs, L-VEGF is intact and expected to initiate from the authentic CUG. In addition, the 180 aa ORF extension alone was cloned in the same expression vector. These plasmid constructs were transfected to HEK 293 and the expression patterns of cellular L-VEGFs and secreted VEGFs were determined by Western blot analyses. In extracts of cells transfected with 5′UTR-$VEGF_{165}$ or 5′UTR-$VEGF_{121}$, two major groups of bands were detected; high molecular weight bands representing L-VEGF and derivatives, and lower molecular bands with much stronger intensity representing the 180 aa ORF extension and derivatives (Fig. 2A, lanes 4 and 3, respectively). As expected L-VEGF originated from $VEGF_{121}$ cDNA is shorter than that of $VEGF_{165}$ (Fig. 2A, compare lanes 3 and 4). More than one discrete band corresponding to

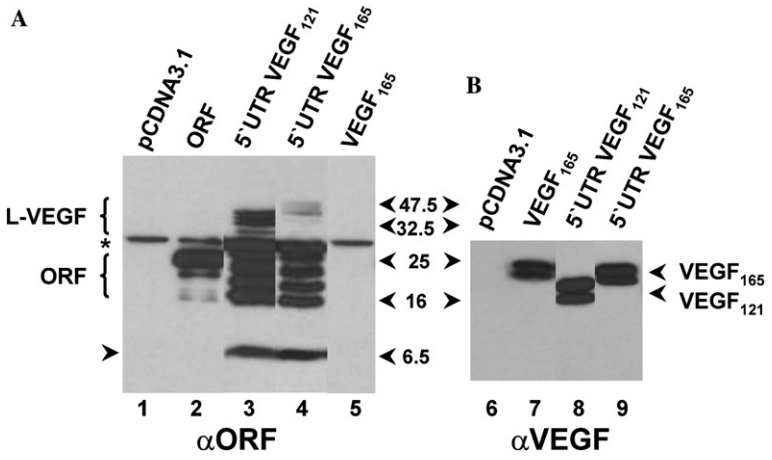

Fig. 2. The 180 aa ORF extension of L-VEGF is cleaved from the VEGF moiety. HEK 293 cells were transfected with either empty vector (pCDNA3.1), or pCDNA3.1 driving the expression of the ORF, the 5′UTR-$VEGF_{165}$, the 5′UTR-$VEGF_{121}$, and just the coding region of $VEGF_{165}$ originating from initiation methionine. Forty-eight hours later, the cells were lysed and ~50 μg of proteins was separated on 15% SDS–PAGE and subjected to Western blot analysis with anti-ORF antibody (A). In addition, the growth medium was collected and 40 μl was taken for Western blot analysis with anti-VEGF antibody (B). Arrowheads indicate the position of the ~5 kDa cleavage products, $VEGF_{165}$ and $VEGF_{121}$. Brackets indicate the L-VEGF and the ORF bands. Asterisk indicates the position of a non-specific band. The positions of the molecular weight size markers are indicated in kDa.

L-VEGF was observed, which results from L-VEGF post-translation modification and/or initiation from alternative CUGs as described before [7]. However, the series of the shorter bands corresponding to the ORF extension were more intense and demonstrated identical gel migration pattern for the two expressed cDNAs. This shows that an efficient cleavage of L-VEGF is taking place resulting in ORF derivatives (Fig. 2A, compare lanes 3 and 4). The fact that this effective cleavage is accompanied by massive secretion of mature VEGF, $VEGF_{121}$, and $VEGF_{165}$ (Fig. 2A, lanes 8 and 9, respectively) implies that the leader sequence was detached. In these series of "shorter molecular weight" bands, the most upper band represents the expected size of the 180 aa extension of VEGF connected to the 26 aa leader sequence (~27 kDa). This band is fainter than just the 180 aa ORF band (Fig. 2, compare lanes 3 and 4 to lane 2, respectively). This is probably due to the fact that the 180 aa ORF is initiated from AUG instead of a CUG codon. The following three bands with estimated molecular weights of 24, 20, and 17 kDa probably reflect cleavage products of the 27 kDa band at one of the two potential proteolytic sites; within the leader sequence of VEGF or 5 kDa upstream to VEGF's initiation methionine. The shortest fragment of ~5–6 kDa is the product of dual proteolytic cleavage at both sites (Fig. 2A, lanes 3 and 4, indicated by left arrowhead). In contrast, when the 180 aa ORF is expressed alone no major degradation products are observed as will be discussed later (Fig. 2A lane 2). As also seen in Fig. 2B, the conditioned media of the cells transfected with L-$VEGF_{165}$ and L-$VEGF_{121}$ produced relatively high levels of secreted VEGF (Fig. 2B, lanes 8 and 9, respectively). The VEGFs are of expected size as compared to $VEGF_{165}$ that was overexpressed in the same cells from a construct that lacked the 5′UTR (Fig. 2B, lane 7). No detectable levels of L-VEGF were observed in the conditioned media of cells expressing just the 180 aa ORF extension (data not shown).

These results point to the fact that both L-$VEGF_{165}$ and L-$VEGF_{121}$ are effectively cleaved not only at the leader sequence but also within the carboxy terminus of the 180 aa extension. The final result of this proteolytic process is a "mature" (secreted) VEGF and a truncated C-terminal 180 aa ORF peptide.

The 180 aa extension of L-VEGF translocates to the nucleus upon hypoxia via a nuclear localization signal confined to the 50 N-terminal aa

The data presented above suggest that L-VEGF is effectively cleaved resulting in separation between the 180 aa ORF moiety and VEGF. To follow the cellular fate of the 180 aa extension of L-VEGF, a series of truncation in the 180 aa extension of L-VEGF were performed (see schematic illustration in Fig. 3A). These constructs were transiently transfected to HEK 293 cell line and the expression level was determined by Western blot analysis with our anti-ORF antibody (Fig. 3B). In addition, the cells transfected with the various constructs were plated in duplicate on microscope slides and 18 h later were either not treated or subjected to hypoxia for additional 18 h. The cells were fixed, double stained with DAPI, to observe nuclei, and with affinity purified anti-ORF antibody, to follow the cellular localization of the 180 aa ORF extension of VEGF (for details see Materials and methods). As seen in Fig. 3C, cytoplasmic staining was observed under normoxia with

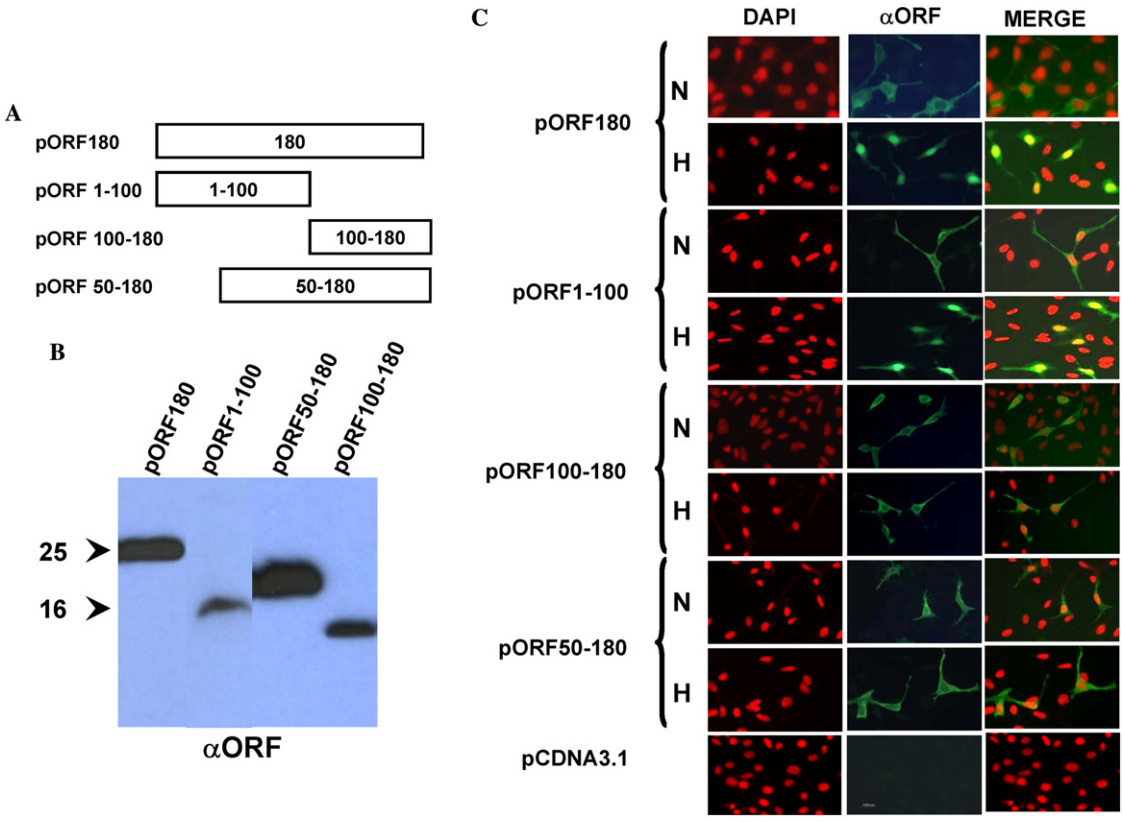

Fig. 3. The first 50 aa of the ORF extension of L-VEGF are essential for nuclear translocation upon hypoxia. HEK 293 cells were transfected in duplicate with either empty mammalian expression vector, pCDNA3.1 or with vectors driving the expression of the full 180 aa ORF of L-VEGF, or various truncations as indicated (A). (B) The expression level of the transfected plasmids was determined by transient experiments and subsequent Western blot analysis with anti-ORF antibody as described in Fig. 1. (C) The cells were transfected with the various constructs (as indicated) on a microscope slide. After 18 h, the cells were either not treated (N) or subjected to hypoxia (H) for additional 18 h. The cells were then fixed, permeabilized, and subjected to double staining with DAPI, to stain the nuclei, and with antibody directed against the ORF. The slides were subsequently reacted with fluorescent secondary antibodies, mounted, and visualized under fluorescent microscope using appropriate filters for detection of the two fluorescent dyes (for details see Materials and methods). Merging DAPI and anti-ORF staining pictures were performed using confocal assistant program.

the antibody directed against the 180 aa ORF with all transfected constructs. However, nuclear staining was observed upon hypoxia in cells transfected only with constructs harboring the full-length ORF or the first 100 aa (Fig. 3C, constructs pORF180 and pORF1-100). The products of the other two constructs, pORF50–180 and pORF100–180 missing the first 50 N-terminal aa, were confined to the cytoplasm even under hypoxia. The fact that the polypeptide expressed from the construct pORF50–180 was restricted to the cytoplasm clearly demonstrates that a NLS is localized within the 50 aa N-terminal fragment of ORF.

The 180 aa ORF extension of L-VEGF is identified in the nuclei of angiogenic tissues: normal and tumorigenic

We next searched for cellular localization of the 180 aa extension of L-VEGF in normal and angiogenic tumor samples. The specificity of our antibody was tested with preimmune serum (data not shown) or by including a competing peptide during the incubation with the first antibody; purified bacterially expressed 180 aa peptide [7], or baculovirus expressed $VEGF_{165}$ [10], and bacterially expressed and purified GST. Only incubation of the specific peptide with the corresponding antibody led to the elimination of the staining (data not shown).

Sections from normal human kidney and breast tissues were compared to sections from renal cell carcinoma (RCC), and invasive duct carcinoma, which are highly angiogenic tumors [11–13]. In sections from normal kidney, cellular staining was evident with anti-ORF antibody mainly in tubular epithelium cells but not in the glomeruli, it was very clear that this staining is restricted to the nuclei of these cells (Fig. 4A, panel N). This staining was not observed with preimmune serum that was retrieved from the same rabbit and subjected to a similar affinity purification procedure (data not shown). When RCC tumor sections were investigated, a more intense nuclear staining was observed. However, unlike normal kidney sections, this staining was evident in the majority of the tumor cells in the section (Fig. 4A, panel T). Similarly, when normal breast tissue

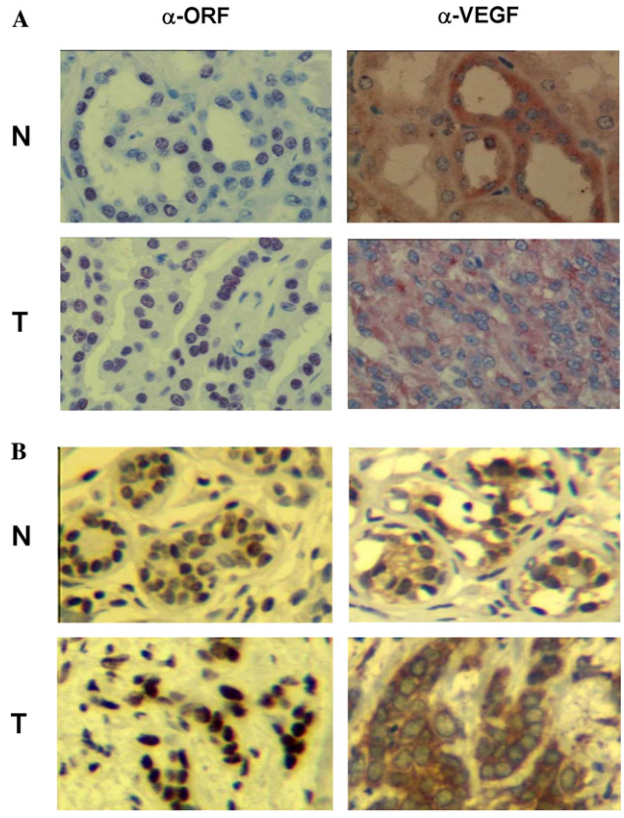

Fig. 4. Immunohistochemical staining of tissue sections from normal kidney, RCC, normal breast, and breast cancer sections with anti-ORF and anti-VEGF antibodies. Paraffin embedded serial sections of normal kidney and RCC (A: panels N and T, respectively) as well as normal breast and invasive duct carcinoma (B: panels N and T, respectively) were fixed and following blocking, the sections were incubated with affinity purified anti-ORF antibody and anti-VEGF antibody as described under Material and methods. Sections were viewed under microscope. Purple-antibodies stain, blue-Mayer's Hematoxylin counterstain.

sections were stained a clear nuclear staining was observed with anti-ORF antibody only in epithelial cells coating the epithelium of the mammary ducts (Fig. 4B, panel N). In sections from an invasive duct carcinoma, it was clearly evident that strong nuclear expression of ORF was observed in many cells in the section (Fig. 4B, panel T).

In principle, a similar staining pattern was observed with anti-VEGF antibody except that it was restricted to the cytoplasm of the cells. The cytoplasm of tubular epithelium in normal kidney sections and the cytoplasm of majority of the tumor cells in RCC section were intensely stained (Fig. 4A). Similarly, in sections from normal breasts and invasive duct carcinoma it is clearly evident that strong cytoplasmic expression of VEGF was observed in the same cells in which their nucleus was stained with anti-ORF (Fig. 4B).

These results were repeated with sections from at least 162 different individuals with similar results. The discrete partitioning of VEGF and ORF within producing cells (cytoplasmic vs nucleus, respectively) clearly demonstrates that L-VEGF is subjected to an effective cleavage process in vivo. Furthermore, the studied tumors exhibited a significantly stronger staining of both proteins in the same neoplastic cells.

Discussion

Cellular localization of VEGF and the 180 aa ORF extension

VEGF is a potent angiogenic factor with numerous isoforms. Recently, we and others demonstrated the existence of an elongated isoform of VEGF termed L-VEGF [7–9]. This is characterized by an extension of a highly conserved 180 aa peptide initiated from upstream alternative translation initiation CUG codon. The biological activity of L-VEGF is not defined, yet a recent study demonstrated that a single nucleotide polymorphism in the *VEGF* gene (-634C-G) led to 17% reduction in L-VEGF expression that was correlated with increased risk in motor neuron degeneration in amyotrophic lateral sclerosis (ALS) [14]. To gain a better insight into the biological roles of L-VEGF, we studied the expression pattern of L-VEGF in cell lines and angiogenic tissues as well as their corresponding tumors. In this study, we provide evidence demonstrating that L-VEGF is efficiently cleaved into its N-terminus fragment and VEGF itself. The 180 N-terminal segment of L-VEGF is confined to the nuclei of VEGF expressing cells. Cleavage of L-VEGF was recently suggested and two potential cleavage sites were described [8,9]. One was identified within the 180 aa extension approximately 10 kDa upstream to VEGF initiation methionine [8]. The second cleavage occurs in the vicinity of the hydrophobic leader peptide of VEGF, which is located in the central part of the L-VEGF. This leader sequence functions as a signal peptide essential for the efficient secretion of VEGF initiated from the canonical AUG [9]. Our results point to the existence of both cleavage sites. With the GFP fusion constructs it was evident that an effective cleavage site is present ~5 kDa upstream to the GFP initiation AUG. This was confirmed by Western blot analyses with antibody directed against the 180 aa extension that was reacted with extracts of cells transfected with the 5′UTR fused to the cDNAs of either $VEGF_{165}$ or $VEGF_{121}$. In these studies, several bands of "ORF derivatives" were observed due to cleavage at both potential sites and due to post-translation modifications. The fact the shortest cleavage product of ~5–6 kDa was observed only when the ORF was attached to VEGF, but not when the ORF was expressed alone, suggests that it is a product of dual cleavages occurring at the leader sequence site as well as at the C-terminus of the ORF (Fig. 2, lanes 3 and 4). In these

transfection studies, the overexpressing cells secreted just "one form" of VEGF: either $VEGF_{165}$ or $VEGF_{121}$ with expected molecular weight. Taken together, L-VEGF is effectively cleaved at two sites. Using various truncation of the 180 aa ORF fused either upstream or downstream to GFP we have identified another putative cleavage site, which fits with the additional band seen in Fig. 1A lane 2 and the lack of a 19 kDa band in lane 1. However, in this communication we concentrate only on the major site just 5 kDa upstream to the canonical AUG. Our results define more accurately the position of the cleavage site described previously [8].

The finding that the 180aa ORF extension of L-VEGF is confined to the nucleus under hypoxia implies a biological function. Huez et al. [9] proposed that L-VEGF is localized in the Golgi apparatus, which could not be confirmed by our studies. Bioinformatic analysis of the 180 aa extension of L-VEGF revealed a 70% chance for the peptide to be localized in the nucleus, pointing to a putative NLS at the N-terminus (http://psort.nibb.ac.jp/form2.html) [7]. It should be noted that other angiogenic growth factors exhibit a similar behavior, being translated from alternative initiation sites. In particular, human bFGF is produced as five isoforms and four of them are directed to the nucleus, where they appear to modulate processes during cell division [15–17]. While the role of the elongated factors initiated from upstream CUG still remains unclear, it appears to have considerable significance because its sequence is highly conserved among mammals. The unique cleavage of L-VEGF and its restriction to the nucleus suggests a regulatory function most probably related to the biological activity of VEGF.

Expression pattern in normal kidney and renal cell carcinoma

While normal development and function of the kidney are critically dependent on adequate vascularization, increased angiogenesis is a prerequisite for tumor growth in the kidney. VEGF expression was shown to be strongly increased in renal tumors in comparison to healthy tissue [18,19]. In these studies, weak cytoplasmic staining of VEGF was observed in tubular epithelial cells of normal tissue, which was also seen here (Fig. 4A). Tubular epithelial cells are involved in absorption and therefore VEGF might be needed in these cells as a permeability factor. In addition, the 180 aa ORF extension of L-VEGF was present in the nucleus of the same cells (Fig. 4A). In the renal cell carcinoma tissue VEGF staining was much stronger in the majority of the tumor cells. Accordingly, an intense nuclear staining was noted with antibody directed against the 180 aa extension demonstrating the correlated expression of the two peptides.

Related to its crucial role in tumor progression and metastasis, increased VEGF expression has been shown to have prognostic value [18]. In this study, 16 sections of various RCC types were examined and an interesting pattern was observed in the expression of the 180 aa L-VEGF peptide. Whereas clear and papillary RCC tissue samples (six samples of each) showed strong ORF expression, weak ORF staining was seen in sections of chromophobe RCC, the RCC type associated with the best prognosis. This suggests that the expression evaluation of the 180 aa extension of L-VEGF may be used as a prognostic factor for survival. Interestingly, patients with lower $VEGF_{121}$ mRNA levels have a significantly longer survival time compared with those with higher levels [20]. The fact that $VEGF_{121}$ is shown to be translated only from upstream CUG [21] together with the correlation between RCC tumorigenicity and ORF nuclear staining is intriguing. It should be noted that our study is based on a limited number of cases and therefore this issue should be examined more attentively, taking into account a greater number of samples.

Expression pattern in normal breast and breast cancer

VEGF expression in breast tumors has been shown to be a significant prognostic factor. Studies have shown that in breast tumor tissue the production of VEGF is as much as 7 times higher than in normal tissue [13]. The detection of excessive expression means poor prognosis, as it leads to invasion and consequent metastasis. In accordance with recent studies, we have found strong VEGF expression in the ductal epithelial cells in normal breast tissue (Fig. 4B). In addition, weak staining of anti-ORF antibody was observed in the nuclei of corresponding cells (Fig. 4B). In contrast, increased VEGF staining in carcinoma tissue was accompanied by a striking enhanced ORF staining in the nuclei. This asymmetrical increase in production of the two proteins suggests that L-VEGF is specifically required in the process of tumor development and progression. As recently reported, the mRNA of $VEGF_{121}$ promotes translation from CUG codon, which leads to L-VEGF expression followed by detachment of leader sequence and maturation of VEGF [21]. Indeed, a correlation between $VEGF_{121}$ expression and increase in the tumorigenicity of a breast cancer cell line was reported [22].

In summary, this study demonstrates for the first time that the 180 aa ORF of L-VEGF can shuttle from the cytoplasm to the nucleus upon hypoxia. In addition, a strong correlation was observed between high levels of VEGF production and nuclear localization of the ORF. This was observed in normal tissues as well as in their corresponding tumors. The fact that $VEGF_{121}$ expression from an alternative translation start site might be the main source for ORF staining and its correlation with tumorigenicity should be further investigated.

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