The Alphabet of Angiogenesis

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SUMMARY

Upon reaching a size of several millimeters a developing embryo cannot meet its metabolic needs by diffusion alone. Solid tumours face the same problem, when expanding to a similar magnitude. Both embryo and tumour can only continue growing by establishing a circulatory system to supply them with oxygen and nutrients. The obvious similarity between these two processes is reflected on the molecular level: *Vascular endothelial growth factor* (VEGF, Fig. 1) has been identified as a key regulator of blood vessel growth (angiogenesis) in both embryos and tumours.

Recently we described three proteins structurally homologous to VEGF. They were designated as *VEGF-B*, *VEGF-C* and *VEGF-D*. We have shown, that VEGF-C plays an important role in the development of the lymphatic vascular system. Our aim is to identify the function of VEGF-B and VEGF-D. This includes identifying receptors and target cells, determinants of receptor specificity and signal transduction pathways. Finally we aim to therapeutically influence angiogenesis. Main topic of my current research is to pinpoint structural elements that provide VEGFs with receptor specificity.

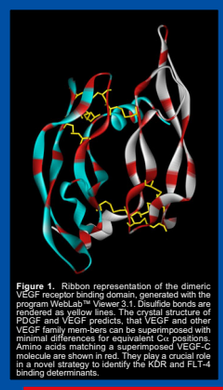

Figure 1. Ribbon representation of the dimeric VEGF receptor binding domain, generated with the program WebLab™ Viewer 3.1. Disulfide bonds are rendered as yellow lines. The crystal structure of PDGF and VEGF predicts, that VEGF and other VEGF family mem-bers can be superimposed with minimal differences for equivalent C_{α} positions. Amino acids matching a superimposed VEGF-C molecule are shown in red. They play a crucial role in a novel strategy to identify the KDR and FLT-4 binding determinants.

THE VEGF FAMILY AND THEIR RECEPTORS

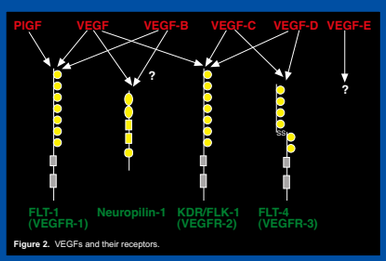

Figure 2. VEGFs and their receptors.

VEGF is an important regulator of endothelial cell proliferation and migration in embryonic vasculogenesis (the *in situ* differentiation of endothelial cell precursors) and angiogenesis (the growth of new blood vessels from preexisting ones) as well as in pathological conditions like tumour angiogenesis.

We lately cloned three factors homologous to VEGF (JOUKOV, 1997; ACHEN, 1998). These were designated as VEGF-B/VRF (VEGF-related factor), VEGF-C/VRP (VEGF-related protein) and VEGF-D/FIGF (*c-fos*-induced growth factor).

VEGF interacts with two receptor tyrosine kinases present on endothelial cells: *FLT-1* and *KDR*, whereas VEGF-B seems to interact only with FLT-1. Both VEGF-C and VEGF-D are ligands for KDR (the main proliferation-inducing receptor on blood vessel endothelial cells) and *FLT-4* (a receptor tyrosine kinase whose expression becomes restricted to lymphatic endothelium during development). The known growth factor-receptor interactions within the VEGF family are summarized in Fig. 2.

COMPLETED STUDIES

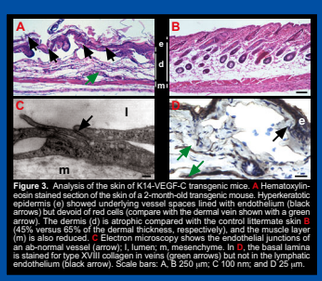

Figure 3. Analysis of the skin of K14-VEGF-C transgenic mice. A Hematoxylin-eosin stained section of the skin of a 2-month-old transgenic mouse. Hyperkeratotic epidermis (e) showed underlying vessel spaces lined with endothelium (black arrows) but devoid of red cells (compare with the dermal vein shown with a green arrow). The dermis (d) is atrophic compared with the control littermate skin B (45% versus 65% of the dermal thickness, respectively), and the muscle layer (m) is also reduced. C Electron microscopy shows the endothelial junctions of an ab-normal vessel (arrow); l, lumen; m, mesenchyme. In D, the basal lamina is stained for type XVIII collagen in veins (green arrows) but not in the lymphatic endothelium (black arrow). Scale bars: A, B 250 μm; C 100 nm; and D 25 μm.

1 Hyperplasia of lymphatic vessels in VEGF-C transgenic mice

VEGF-C over-expression in the basal epidermis of transgenic mice is capable of promoting an abundant growth of extensive lymphatic vessel structures in the dermis, including large vessel lacunae similar in their histopathology the human condition known as lymphangioma (Figs. 3, 4 and 5; JELTSCH, 1997).

In contrast, preliminary data on mice which overexpress VEGF-B, does not yet allow us to draw any conclusions about its possible biological function (JELTSCH, 1997). These mice show a hypercollagenized dermis and cataract and we are trying to identify the role of VEGF-B in this phenotype.

2 Specific induction of angiogenesis and lymphangiogenesis in the differentiated avian chorioallantoic membrane

The chorioallantoic membrane (CAM) of the chick is a transient respiratory organ, which lies just beneath the egg shell. It consists of a mesodermal stroma lined by an outer ectodermal and inner endodermal epithelium (Fig. 6). Due to the accessibility and regularity of its vascular system the CAM has been used for many years to evaluate potential angiogenic factors. Less well documented is, that the CAM is drained by a dense network of lymphatic vessels.

The effects of VEGF, PlGF, VEGF x PlGF heterodimers and VEGF-C on the lymphatics of differentiated (day 13) avian CAM were studied (Figs. 7 & 8). Growth factors containing at least one VEGF chain are angio-genic but do not induce lymph-angiogenesis. PlGFs are neither angiogenic nor lymphangiogenic. Only VEGF-C is chemoattractive for lymphatic endothelial cells, induces their proliferation and the development of new lymphatic sinuses which are directed immediately beneath the chorionic epithelium.

These studies confirm that VEGF and VEGF-C are specific angiogenic and lymph-angiogenic growth factors, respectively (OH, 1997).

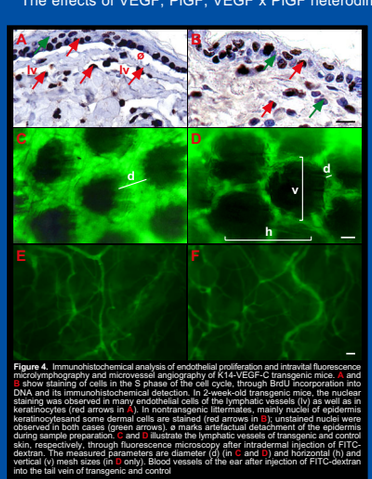

Figure 4. Immunohistochemical analysis of endothelial proliferation and intravital fluorescence microlymphography and microvessel angiography of K14-VEGF-C transgenic mice. A and B show staining of cells in the S phase of the cell cycle, through BrdU incorporation into DNA and its immunohistochemical detection. In 2-week-old transgenic mice, the nuclear staining was observed in many endothelial cells of the lymphatic vessels (lv) as well as in keratinocytes (red arrows in A). In nontransgenic littermates, mainly nuclei of epidermis keratinocytesand some dermal cells are stained (red arrows in B); unstained nuclei were observed in both cases (green arrows). a marks artefactual detachment of the epidermis during sample preparation. C and D illustrate the lymphatic vessels of transgenic and control skin, respectively, through fluorescence microscopy after intradermal injection of FITC-dextran. The measured parameters are diameter (d) (in C and D) and horizontal (h) and vertical (v) mesh sizes (in D only). Blood vessels of the ear after injection of FITC-dextran into the tail vein of transgenic and control

3 VEGF-D is a ligand of KDR/ FLK-1 and FLT-4

VEGF-D was identified by computer-based homology search of the EMBL/ GenBank EST databases. It is most closely related to VEGF-C by virtue of its N- and C-terminal extensions other VEGF family members are devoid of (Fig. 9). In adult human tissues, VEGF-D mRNA is most abundant in heart, lung, skeletal muscle, colon, and small intestine (Fig. 10). VEGF-D is a ligand for VEGF receptors KDR/FLK-1 and FLT-4 and can activate these receptors (Fig. 11). A short form of VEGF-D expressed in insect cells demonstrated that the receptor-binding capacities reside in the *VEGF homology domain* which corresponds to the mature form of VEGF-C (Fig. 12). The structural and functional similarities between VEGF-C and VEGF-D define a VEGF subfamily (ACHEN, 1998).

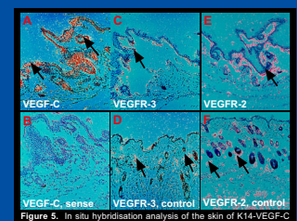

Figure 5. In situ hybridisation analysis of the skin of K14-VEGF-C transgenic mice. A and B show hybridisation of transgenic skin with VEGF-C antisense and sense probes, C and D show in situ hybridisation for FLT-4 and E and F show in situ hybridisation for FLK-2 in the skin of transgenic and litter-mate control animals. Arrows in A indicate basal keratinocytes; in C through E, they point out the lymphatic endothelium; and in F, arrows show endothelial cells. Scale bar: 20 μm.

WHERE TO GO?

1. Identifying the function of VEGF-B and VEGF-D and finding their physiologically relevant target cells.
2. Pinpointing the structural determinants for receptor specificity, especially the KDR and FLT-4 binding determinants.

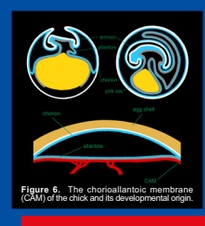

Figure 6. The chorioallantoic membrane (CAM) of the chick and its developmental origin.

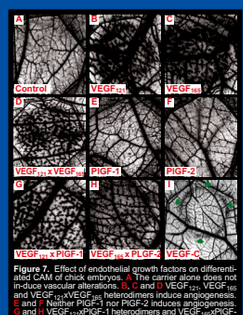

Figure 7. Effect of endothelial growth factors on differentiated CAM of chick embryos. A The carrier alone does not in-duce vascular alterations. B, C and D $VEGF_{121}$, $VEGF_{165}$ and $VEGF_{121}$x$VEGF_{165}$ heterodimers induce angiogenesis. E and F Neither PlGF-1 nor PlGF-2 induces angiogenesis. G and H $VEGF_{121}$/PlGF-1 heterodimers and $VEGF_{165}$/PlGF-2 hetero-dimers induce angiogenesis. I VEGF-C does not induce gross alterations of the vascular system. A circular effect (arrows) is only visible in the area of highest growth factor concen-tration. Magnification: x12.

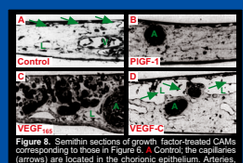

Figure 8. Semithin sections of growth factor-treated CAMs corresponding to those in Figure 6. A Control; the capillaries (arrows) are located in the chorionic epithelium. Arteries, veins, and lymphatics are found in the stroma of the CAM. B PlGF-1; neither an angiogenic nor a lymphangiogenic effect is present. C VEGF-C; note large amount of blood-filled capillaries. Lymphatics are not affected. D VEGF-C; newly developed lymphatics located immediately beneath the chorionic epithelium. Note many intraluminally located endothelial cells (arrows), indicative of plexus formation. No other cell types are affected. A: artery, L: lymphatics, V: vein.

VEGF-B & VEGF-D FUNCTION

Transgenic mice with tissue-specific over-expression of VEGF-B or VEGF-D

To identify the function of VEGF-B and VEGF-D we are establishing animal models with tissue-specific over-expression of VEGF-B and VEGF-D analogous to the existing VEGF-C models.

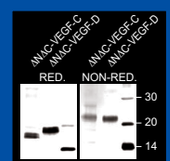

Figure 12. Analysis of recombinant ΔNΔC-VEGF-D by silver staining. Proteins were produced by HF cells and purified from serum-free conditioned medium by Ni^{2+}-NTA affinity chromatography.

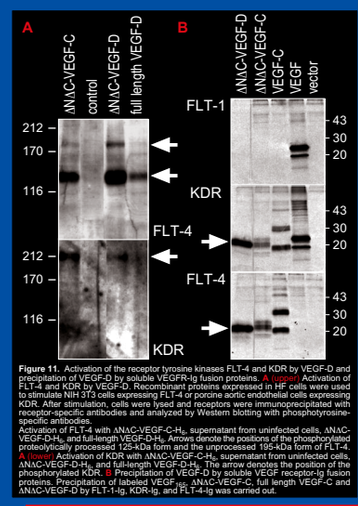

Figure 11. Activation of the receptor tyrosine kinases FLT-4 and KDR by VEGF-D and precipitation of VEGF-D by soluble VEGFR-Ig fusion proteins. A (upper) Activation of FLT-4 and KDR by VEGF-D. Recombinant proteins expressed in HF cells were used to stimulate NIH 3T3 cells expressing FLT-4 or porcine aortic endothelial cells expressing KDR. After stimulation, cells were lysed and receptors were immunoprecipitated with receptor-specific antibodies and analyzed by Western blotting with phosphotyrosine-specific antibodies.
Activation of FLT-4 with ΔNΔC-VEGF-C-H_{is} supernatant from uninfected cells, ΔNΔC-VEGF-D-H_{is} and full-length VEGF-D-H_{is}. Arrows denote the positions of the phosphorylated proteolytically processed 125-kDa form and the unprocessed 195-kDa form of FLT-4.
A (lower) Activation of KDR with ΔNΔC-VEGF-C-H_{is}, supernatant from uninfected cells, ΔNΔC-VEGF-D-H_{is} and full-length VEGF-D-H_{is}. The arrow denotes the position of the phosphorylated KDR. B Precipitation of VEGF-D by soluble VEGF receptor-Ig fusion proteins. Precipitation of labeled $VEGF_{165}$, ΔNΔC-VEGF-C, full length VEGF-C and ΔNΔC-VEGF-D by FLT-1-Ig, KDR-Ig, and FLT-4-Ig was carried out.

Large scale production of recombinant growth factors

The production of recombinant VEGF-B and -C in the baculovirus system has been described (JELTSCH, 1997). Proteins were obtained in the μg-range and used in several *in-vivo* assays (OH, 1997; KUKK, 1996). Our aims are to:

- upscale the production into the mg-range.
- develop a purification procedure for VEGF-B.
- produce VEGF-D for biological *in-vivo* assays.
- produce VEGF-D in E.coli for the production of monoclonal and polyclonal antibodies and crystallization purposes.

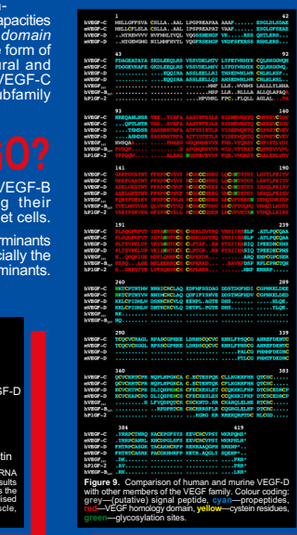

Figure 10. Northern blot analysis for detection of VEGF-D mRNA in polyadenylated RNA from human tissues. A shows the results of hybridisations with a human VEGF-D cDNA probe. B shows the filters after the VEGF-D probe had been stripped and hybridised with with a β-actin cDNA. SK. MUSCLE: skeletal muscle, S.INTESTINE: small intestine (mucosal lining).

Figure 9. Comparison of human and murine VEGF-D with other members of the VEGF family. Colour coding: grey—putative) signal peptide, cyan—propeptides, red—VEGF homology domain, yellow—cystein residues, green—glycosylation sites.

SOLVING THE SPECIFICITY QUESTION

Identification of receptor specificity determinants using a novel mutagenesis approach

Several research groups have been (and are) trying to identify the critical amino acids for receptor binding. However satisfying and unisonous results have not been achieved (KEYT et al., JBC 271 (1996), 5638: *Identification of vascular endothelial growth factor determinants for binding KDR and FLT-1 receptors*; MULLER et al., PNAS 94 (1997), 7192: *Vascular endothelial growth factor: crystal structure and functional mapping of the kinase domain receptor binding site*). The used methods (X-ray crystallography, random mutagenesis and scanning mutagenesis) are all very costly and time-consuming, but give only structural (X-ray crystallography) and loss-of-function (mutagenesis) evidence.

While keeping the mutagenesis approach, our experimental design aims at gain-of-function evidence. It is well acknowledged, that the majority of all possible mutations is neutral or leads to a loss of function. All amino acids which are constant within the VEGF family should therefore be excluded as mutational targets. The target amino acids should not be mutated randomly, but only exchanged against amino acids occurring in other VEGFs at the corresponding position. The idea is to create thus a mammalian expression library of VEGF mosaic molecules. Key features of this approach are the exploitation of the high homology of VEGFs in their core region to create the library and the establishment of an efficient screening system, that evaluates the receptor-affinities of the individual clones.

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