Angiogenesis

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The Alphabet of

Summary

Upon reaching a size of several millimeters a developing embryo cannot meet its metabolic needs by diffusion alone. Solid tumors face the same problem, when expanding to a similar magnitude. Both embryo and tumor can only continue growing by establishing a circulatory system to supply them with oxygen and nutrients. The obvious similarity between these 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 in both embryos and tumors.

Recently we described two proteins homologous to VEGF: *VEGF-C* and *VEGF-D*. The structural similarity between VEGF-C and VEGF-D define a VEGF subfamily. Similar to Platelet-derived growth factor (PDGF) mature VEGF-C and D are generated by the cleavage of a precursor. VEGF-C plays an important role in the development of the lymphatic vascular system. The role of VEGF-D is less well defined.

Topics of our research include the further characterization of VEGF-C (biosynthesis, structure, determinants of receptor specificity) and the identification of the function of VEGF-D.

Figure 1. *VEGF receptor binding domain.* Disulfide bonds are rendered as yellow lines. Amino acids matching a superimposed VEGF-C molecule are shown in red.

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).

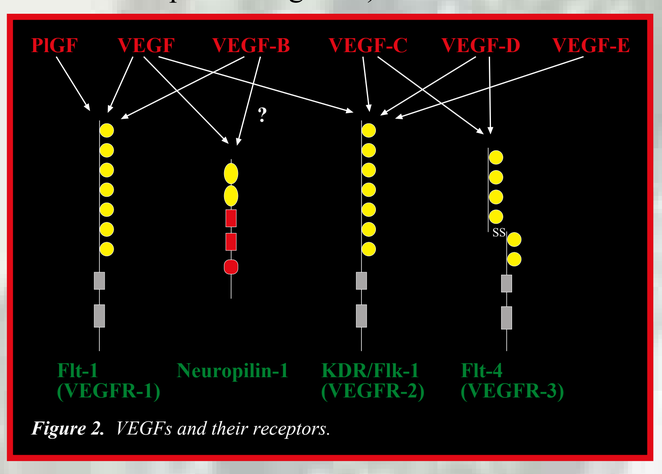

Figure 2. *VEGFs and their receptors.*

We lately cloned two factors homologous to VEGF (JOUKOV, 1997; ACHEN, 1998), designated as VEGF-C (or *VRP*) and VEGF-D (or *FIGF*). Both VEGF-C and 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 developdevelopment (Fig. 2).

Completed Studies

Hyperplasia of lymphatic vessels in VEGF-C transgenic mice

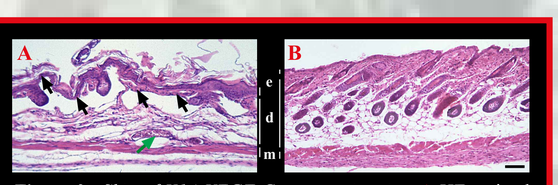

Figure 3. *Skin of K14-VEGF-C transgenic mice.* **A** HE-stained section of the skin of a 8-week-old transgenic mouse. Hyperkeratotic epidermis (e) showed underlying vessel spaces lined with endothelium (black arrows) but devoid of red cells (compare with the dermal vein shown with a green arrow). The dermis (d) is atrophic compared with the control littermate skin (**B**) and the muscle layer (m) is also reduced. Scale bar: 250 µm

VEGF-C overexpression in the basal epidermis of mice promotes the growth of lymphatic structures in the dermis, including large vessel lacunae histologically similar to lymphangioma (Figs. 3, 4, 5; JELTSCH, 1997).

Angiogenesis and lymphangiogenesis in the chorioallantoic membrane (CAM)

Due to the accessibility and regularity of its vascular system the CAM has been used for many years to evaluate potential angiogenic factors (Fig. 6). Less well documented is, that the CAM is drained by a dense network of lymphatic vessels.

The effects of VEGF, PlGF, VEGFxPlGF heterodimers and VEGF-C on the differentiated avian CAM were studied (Figs. 7, 8). Only VEGF-C is chemoattractive for lymphatic endothelial cells, induces their proliferation and the development of new lymphatic sinuses (OH, 1997).

VEGF-D is a ligand of KDR/Flk-1 and Flt-4

VEGF-D was identified by computer-based homology search. It is most closely related to VEGF-C (Fig. 9). VEGF-D activates KDR and Flt-4. A short form of VEGF-D demonstrated receptor-binding resides in the VEGF homology domain which corresponds to the mature form of VEGF-C (Fig. 11; ACHEN, 1998).

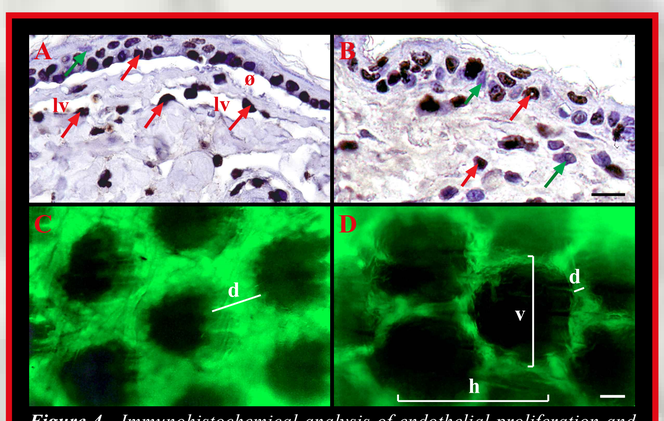

Figure 4. *Immunohistochemical analysis of endothelial proliferation and intravital fluorescence microlymphography of K14-VEGF-C transgenic mice.* **A B**: Staining of cells in the S phase of the cell cycle (BrdU). In 14-day-old transgenic mice, the nuclear staining was observed in many endothelial cells of the lymphatic vessels (lv) as well as in keratinocytes (red arrows in A). In nontransgenic littermates, mainly nuclei of epidermis keratinocytes and some dermal cells are stained (red arrows in B); unstained nuclei were observed in both cases (green arrows). **C D**: Fluorescence microscopy of lymphatic vessels of transgenic and control skin. The measured parameters are diameter (d) (in C, D) and horizontal (h) and vertical (v) mesh sizes (D only). Scale bars: A, B 5 µm; C, D 250 µm.

VEGF-D Function

Transgenic mice with tissue-specific overexpression of VEGF-D

To identify the function of VEGF-D we are establishing animal models with tissue-specific overexpression of VEGF-D analogous to the existing VEGF-C models using the keratin 14 and the rat insulin promoter.

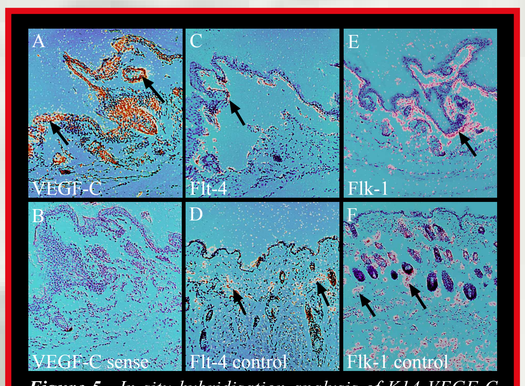

Figure 5. *In-situ hybridisation analysis of K14-VEGF-C transgenic mice skin.* **A B**: VEGF-C antisense and sense probes. **C D**: Flt-4. **E F**: Flk-1. Arrows in A indicate basal keratinocytes; in C through E, they point out the lymphatic endothelium; and in F, arrows show endothelial cells.

Recombinant Growth Factors

Baculoviral and E.coli proteins

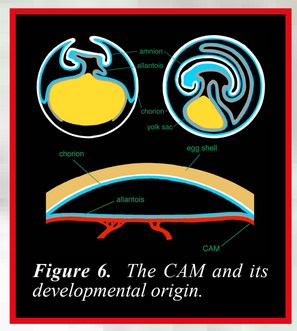

Figure 6. *The CAM and its developmental origin.*

Recombinant VEGF-C produced in the baculovirus system has been used in several studies (PEPPER, 1998; OH, 1997; JELTSCH, 1997; KUKK, 1996). Presently VEGF-C and VEGF-D are produced in E.coli for the production of monoclonal antibodies, in-vivo assays and crystallization purposes.

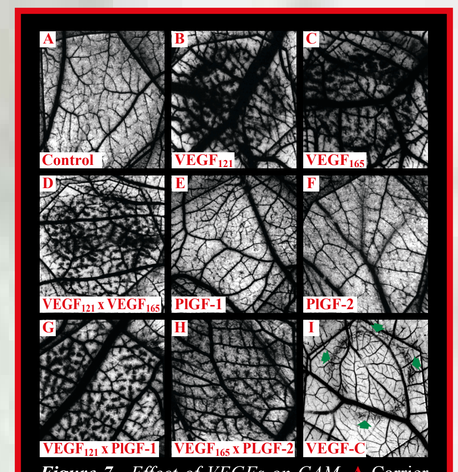

Figure 7. *Effect of VEGFs on CAM.* **A** Carrier alone doesn't induce vascular alterations. **B C D** $VEGF_{121}$, $VEGF_{165}$ and $VEGF_{121}$x$VEGF_{165}$ heterodimers are angiogenic. **E F** PlGF-1 and 2 are not angiogenic. **G H** VEGFxPlGF heterodimers induce angiogenesis. **I** VEGF-C doesn't induce gross changes of the vascular system, its effect shows only in the area of highest growth factor concentration (arrows). Magnification: x12.

Structure & Function

Random mosaic molecules and X-ray crystallography

The crystal structure of VEGF has been resolved as well as the structure of the VEGF-Flt-1 complex. The interaction between VEGF and KDR has not been resolved at structural level and almost nothing is known about the KDR-VEGF-C interaction. To pinpoint the important elements of receptor specificity we started the determination of VEGF-C structure by X-ray crystallography and the creation of a mammalian expression library of 512 different VEGF-VEGF-C mosaic molecules, whose receptor specificities are correlated to the individual composition of the clone. Key features of this approach are the exploitation of the high homology of VEGF to VEGF-C in the core region to create the library and the use of an efficient screening system, that evaluates the receptor affinities of the individual clones.

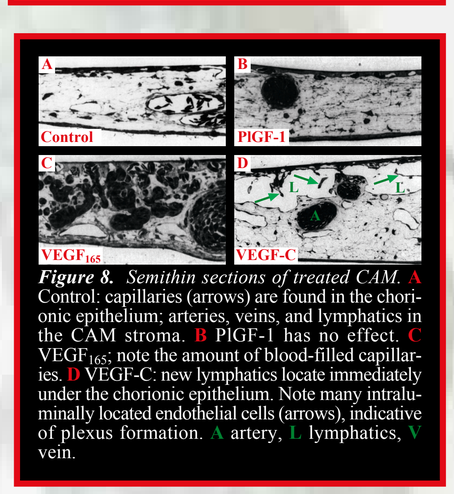

Figure 8. *Semithin sections of treated CAM.* **A** Control: capillaries (arrows) are found in the chorionic epithelium; arteries, veins, and lymphatics in the CAM stroma. **B** PlGF-1 has no effect. **C** $VEGF_{165}$; note the amount of blood-filled capillaries. **D** VEGF-C: new lymphatics locate immediately under the chorionic epithelium. Note many intraluminally located endothelial cells (arrows), indicative of plexus formation. **A** artery, **L** lymphatics, **V** vein.

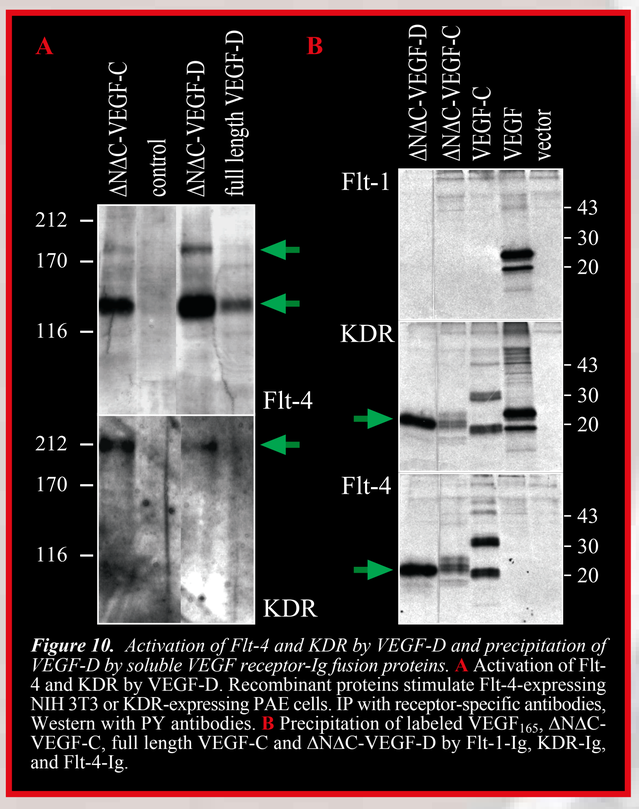

Figure 10. *Activation of Flt-4 and KDR by VEGF-D and precipitation of VEGF-D by soluble VEGF receptor-Ig fusion proteins.* **A** Activation of Flt-4 and KDR by VEGF-D. Recombinant proteins stimulate Flt-4-expressing NIH 3T3 or KDR-expressing PAE cells. IP with receptor-specific antibodies, Western with PY antibodies. **B** 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.

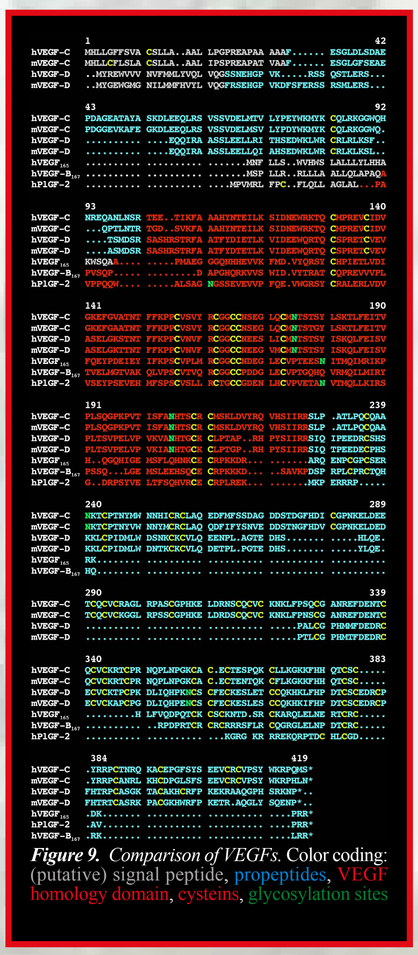

Figure 9. *Comparison of VEGFs.* Color coding: (putative) signal peptide, propeptides, VEGF homology domain, cysteins, glycosylation sites

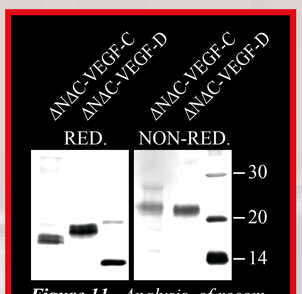

Figure 11. *Analysis of recombinant VEGF-D by Ag staining.* Proteins were produced in HF cells and purified from conditioned medium by Ni^{2+}NTA affinity chromatography

More about VEGF-C & D

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