VEGF IN SKIN MELANOMA – ROLE AND GENETIC POLYMORPHYSMS

Asen Anastasov[1], Tatyana Vlaykova[1]
[1]*Dept. of Chemistry and Biochemistry, Medical Faculty, Trakia University, Stara Zagora*
asen_anastasov@hotmail.com

ABSTRACT

Vascular endothelial growth factor VEGF and its isoforms are potent mitogens, regulating the angio- and lymphangiogenesis, as well as the functions and integrity of blood and lymph vessels. VEGFs are implicated in number of physiological conditions, but impairment of their levels is associated with many diseases, including cancers. Vascularisation is a crucial process for tumor growth and especially for tumor spread. Recent data showed that high vascular density in tumors may be positive condition for successful radio and chemotherapy. Other scientific groups are working on invention of different VEGF inhibitors. This requires good understanding and deeper investigation of VEGF biology and principles of action.

The number of new reported melanoma cases yearly is increasing with lowering of patients age limit, so better knowledge of its development and progression is required. A lot of data suggest that melanoma vascularisation might be a suitable target of the therapy and marker for patient survival.

Introduction

Melanoma cells derive from transformed normal melanocytes exposed under various extrinsic and intrinsic factors. During the transformation they acquire growth autonomy due to inhibition of tumor suppressing genes and activation of proto-oncogenes. The immune system of the patient is strongly influencing the development and progression of melanoma.

This type of cancer is considered as comparatively chemotherapy resistant and it is associated with an extremely poor prognosis once systemic metastases are found, representing about 13,5% mortality. After distant metastases are found the survival median is between 9 and 12 months (Jemal, Siegel et al. 2008; Mehnert, McCarthy et al. 2009).

It is now clarified that the tumor development and growth comprises two phases: the first is prevascular also called avasculare associated with 1-2mm^3 lesion mass and is rarely associated with metastases, whereas the second phase called vascular is connected with rapid tumor growth and aggressive metastasis (Leung, Cachianes et al. 1989; Howell, Bateman et al. 2002; Vlaykova 2004). The transition from prevascular to vascular phase is triggered by number of biochemical and genetic factors, also the induction of angiogenes is regulated through the expression of different angiogenic and anti-angiogenic regulators which local equilibrium may be impaired (Tischer, Mitchell et al. 1991; Howell, Bateman et al. 2002; Vlaykova 2004).

Angiogenesis is complicated process involving a variety of different factors and their receptors. Angiogenesis has been recognized as an essential process in several physiological conditions, such as placental formation, development of embryonic tissue, etc. and it is also well known that the angiogenesis is a key process in the growth and invasion of malignant tumors, including melanoma (Leung, Cachianes et al. 1989; Folkman and Shing 1992; Vlaykova, Laurila et al. 1999; Howell, Bateman et al. 2002; Mehnert, McCarthy et al. 2009). It is well known that the increased vascular density associated with a much higher incidence of metastases leading to worse prognosis for patients with different malignant neoplasms, such as lung cancer, prostate cancer, head and neck squamous cell carcinoma, gastric carcinoma, breast carcinoma and melanoma (Vlaykova, Laurila et al. 1999).

As a pro-angiogenic factors are recognized variety of proteins such as VEGF, FGFs, PDGF, angiotensins, CSFs, TGF-α, ILs (IL-2, -8 and -10), some proteinases and their inhibitors (MMPs, PAs, kathepsins), adhesion molecules (VCAM, ICAM, integrins), etc., as long as anti-angiogenic factors are considered to be angiostatin, arrestin, endostatin, platelet factor 4, thrombospondines

variety of cytokines (TGF-β, TNF-α, IL-1, -4, -10, -12 -18, IFNs) (Vlaykova 2002; Vlaykova 2004; Anastasov 2011).

VEGF family

The vascular endothelial growth factor (VEGF) was firstly described in 1983 by Senger and coworkers (Senger, Galli et al. 1983). The VEGF family plays a critical role in vascular development, permeability, angiogenesis and lymphangiogenesis , hypoxia-induced tissue angiogenesis, as well as in physiological and pathological conditions (Ferrara and Davis-Smyth 1997; Ikeda, Yonemitsu et al. 2006; Sathasivam 2008; Zygalaki, Kaklamanis et al. 2008; Mehnert, McCarthy et al. 2009).

The VEGF family known in humans consists of five different homologous factors (isoforms) VEGF-A (also known as vascular permeability factor or VEGF, VEGF/PF), -B, -C, -D and placental growth factor (PlGF) (Ikeda, Yonemitsu et al. 2006; Sathasivam 2008; Mehnert, McCarthy et al. 2009). Scientifically studied are other two VEGFs - VEGF-E (encoded from *Parapoxvirus*) and VEGF-F (isolated from snake venom) (Grunewald, Prota et al. 2010). VEGF isoforms are result of proteolytic processing and alternative splicing and possess distinct biological activities (Grunewald, Prota et al. 2010).

VEGFs are dimeric glycoproteins which are mostly homodimers, but they can be also heterodimers. These dimeres are covalently linked by two intermolecular disulfide bonds in receptor binding place. They have two domains: receptor binding and heparin binding, and cysteine knot motif (Grunewald, Prota et al. 2010).

VEGF family members are polypeptide growth factors encoded by several genes with strong endothelium-specific mitogen effect. After secretion they regulate mitogenesis, vascular tone, permeability, vasodilatation, blood and lymph vessel formation during embryonic development, wound healing, and maintain vessel homeostasis in adult organisms (Schmidt-Lucke, Belgore et al. 2005; Grunewald, Prota et al. 2010). VEGF interacts specifically with ancestors of hematopoietic and endothelial cells (angioblasts), as well as in process of differentiation and mature endothelial cells (Grunewald, Prota et al. 2010). Binding of VEGF and other ligands to the VEGF-receptor (VEGFR) of endothelial cells activates the angiogenic pathway (Morales-Gutierrez, Abad-Barahona et al. 2011) and increases endothelial release of nitric oxide (NO) by the activation of NO synthases (NOSs), leading to vasodilatation (Schmidt-Lucke, Belgore et al. 2005).

Receptors and Isoforms

The multiple forms of VEGF-A e.g. $VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$ $VEGF_{206}$, $VEGF_{189}$, $VEGF_{183}$, $VEGF_{148}$, (subscripts denote the number of amino acids after signal sequence cleavage) (Vlaykova 2002; Sathasivam 2008; Grunewald, Prota et al. 2010) are result of alternative splicing of a single VEGF gene, which is located on chromosomes 6p21.5 (Paavonen, Horelli-Kuitunen et al. 1996; Howell, Bateman et al. 2002) or of proteolytic processing of the product from this gene (Zygalaki, Kaklamanis et al. 2008). Most VEGF-producing cells express $VEGF_{121}$, $VEGF_{165}$ and $VEGF_{189}$ (Ferrara and Davis-Smyth 1997). $VEGF_{183}$ also has a wide tissue distribution (Zygalaki, Kaklamanis et al. 2008).

The VEGF family members differ in their molecular mass, solubility, binding affinity for heparan sulfate proteoglycans in the extracellular matrix, receptor subtypes, biological functions and distribution in tissues (Sathasivam 2008; Zygalaki, Kaklamanis et al. 2008). It is thought that VEGF-A, VEGF-B , VEGF-D, VEGF-E and PIGF are important for the growth of blood vessels and VEGF-A, VEGF-B and VEGF-C directly affect neural cells, VEGF-C also affects the development of lymphatic vessels (Raab and Plate 2007).

The proper vessel organization is strictly defined by the correct spatial distribution of specific VEGF members by differential ECM adherence and the coordinated signal output initiated by distinct VEGFs (Grunewald, Prota et al. 2010). The interaction between VEGFs, their receptors (VEGFRs) and co-receptors is strictly controlled as it is needed for shaping and maintaining the

functionality of blood vessels. Two main receptor classes: tyrosine and non-tyrosine kinases are known to interact with VEGFs (Schmidt-Lucke, Belgore et al. 2005; Ikeda, Yonemitsu et al. 2006; Sathasivam 2008).

The tyrosine kinase signaling receptor complexes are VEGFR-1, VEGFR-2 and VEGFR-3.

VEGFR-1 also known as *fms-l*ike *t*yrosine kinase (Flt-1) has high affinity to the ligand, resulting in weak response (autophosphorillation) (Mehnert, McCarthy et al. 2009). VEGFR-2 is called *k*inase-*d*omain insert containing *r*eceptor (KDR or Flk-1) shows low affinity and intense response resulting in angiogenic, mitogenic and permeability-enhancing effects (Shalaby, Rossant et al. 1995; Risau 1997; Neufeld, Cohen et al. 1999; Vlaykova 2002). The third receptor subtype is VEGFR-3 or Flt-4. It is known that there is also a soluble Flt-1receptor (sFlt-1, sVEGFR-1) which is result of alternative splicing of the Flt-1 gene (Schmidt-Lucke, Belgore et al. 2005; Ikeda, Yonemitsu et al. 2006; Sathasivam 2008; Zygalaki, Kaklamanis et al. 2008). Recent studies indicate that sFlt-1 can form heterodimers with membrane-bound Flt-1 and KDR, and thus it can inhibit the signal transduction (Kendall and Thomas 1993).

The nontyrosine kinase receptors are neuropilin-1 (NP-1) and neuropilin-2 (NP-2) (Takahashi and Shibuya 2005). The specific receptor for the isoform VEGF165 is NP-1, while NP-2 is specific for VEGF145 and VEGF165 as well (Ferrara, Gerber et al. 2003; Ruhrberg 2003).

It is known that VEGF-C and VEGF-D bind primarily to VEGFR-3 and less to VEGFR-2. VEGFR-3-mediated pathway is thought to be essential for the development of lymphatic vessels in normal and tumor lymphangiogenesis (Ferrara, Gerber et al. 2003). VEGFR-3 has always been found to co-localize with VEGFR-2 in vascular but not in lymphatic endothelium and it is recognized as one of the useful markers of a proliferative vascular endothelial phenotype (Partanen, Alitalo et al. 1999; Witmer, Dai et al. 2002; Ikeda, Yonemitsu et al. 2006).

VEGF-A is a homodimer and is a key regulator of endothelial cell functions with a potent endothelial cell mitogen effect – this factor stimulates the proliferation and migration of endothelial cells. VEGF signals primarily through binding and activating VEGFR-1 and VEGFR-2 (de Vries, Escobedo et al. 1992; Grunewald, Prota et al. 2010) Conditions of hypoxia highly induce VEGF-A expression by hypoxia-inducible factor-1 (HIF-1) (Shweiki, Itin et al. 1992; Hata, Nakagawa et al. 1995; Liu, Cox et al. 1995). All other VEGFs have homologous structure to VEGF-A (Ikeda, Yonemitsu et al. 2006; Grunewald, Prota et al. 2010).

VEGF-B has two isoforms, VEGF-B_{167} and VEGF-B_{186}, which are result of alternative splicing. They interact only with VEGFR-1 from the tyrosine kinase family and with NP-1 from the non-tyrosine kinase family (Olofsson, Pajusola et al. 1996; Olofsson, Korpelainen et al. 1998).

VEGF-C and VEGF-D are synthesized as precursor forms with long peptides in amino- and carboxyl ends, flanking the VEGFR-binding domain. Mature VEGF-C and VEGF-D are result of proteolytic cleavage which releases the receptor-binding domain. Both VEGFs show increased affinity to VEGFR-2 and VEGFR-3 (Joukov, Pajusola et al. 1996; Joukov, Sorsa et al. 1997; Achen, Jeltsch et al. 1998). VEGF-D expression is also induced by hypoxia (Teng, Li et al. 2002) whereas VEGF-C expression is not affected (Enholm, Paavonen et al. 1997). Serum and growth factors induce expression of VEGF-C mRNA (Enholm, Paavonen et al. 1997). Moreover, calcium-dependent cadherin-11-mediated cell-cell adhesion is found to up-regulate VEGF-D mRNA expression, whereas beta-catenin down-regulates its expression (Orlandini and Oliviero 2001; Orlandini, Semboloni et al. 2003).

VEGF-C is supposed to stimulate only lymphangiogenesis whereas VEGF-D stimulates both angiogenesis and lymphangiogenesis but some data indicated the opposite that VEGF-C and -D regulate the formation of lymphatic vessels, as well as VEGF-A, -B, -C and placenta growth factor (PlGF) are required for blood vessel formation (Jussila and Alitalo 2002).

VEGF-E has no heparan-binding domain and varies in its abilities to bind neuropilins (Grunewald, Prota et al. 2010). VEGF-F, isolated from snake venoms shows biological activity

similar to VEGF-A_{165} and it has been reported to stimulate prominently proliferation of vascular endothelial cells in vitro and decreases the blood pressure in rats (Grunewald, Prota et al. 2010).

Expression in norma and pathology

Normal expression is seen in ontogenetic development of blood vessels (vasculogenesis) and in cases with increased tissues oxygen, nutrients and growth factors uptake, such as re-vascularisation of ischemic tissues, hypoxia induced angiogenesis, wound healing and regeneration, at the time of pregnancy and menstrual cycle.

Abnormal VEGF levels through excess or reduced production results in imbalanced formation of blood or lymphatic vessels and causes many human diseases (Takahashi and Shibuya 2005).

Elevated expression is seen in patients with atherosclerosis, hypertonia, ischemic heart disease or ischemic periphery vessels, diabetes, proliferative retinopathy and ocular vascular pathogenesis, rheumatoid arthritis and other inflammatory diseases (Schmidt-Lucke, Belgore et al. 2005; Zygalaki, Kaklamanis et al. 2008). Overexpression is present in different cancer diseases such as intestinal and colorectal carcinoma, carcinomas of the female reproductive system in ovarian and breast cancer tissue, oesophageal carcinomas (Howell, Bateman et al. 2002; Takahashi and Shibuya 2005; Zygalaki, Kaklamanis et al. 2008)], during the progression of early stage melanocytic lesions, transformation of melanoma from radial growth to vertical growth (Erhard, Rietveld et al. 1997; Vlaykova 2002; Vlaykova 2004).

The enhanced expression of this growth factor is correlated with metastatic tumors and respectively with disease progression and lower overall survival (Steffensen, Waldstrom et al.; Salven, Heikkila et al. 1997; Morales-Gutierrez, Abad-Barahona et al. 2011). Indeed in hepatocellular carcinoma and melanoma, it is considered marker of poor prognosis. Low grade of VEGF expression is seen in benign naevi, but dysplastic naevi and melanoma have drastic increase of expression (Vlaykova, Laurila et al. 1999; Pisacane and Risio 2005).

VEGFR-1 and VEGFR-2 are expressed in melanoma and it is hypothesized that VEGFR-2 expression is associated with thicker and more invasive tumors. High levels of VEGFR-3 are present in both tumor cells and serum of patients, whereas there is no elevation in benign melanocytic lesions potentially implicating this receptor in initiation of metastazing. High levels of this receptor are associated with chemotherapy resistance, but low levels are related positively to disease free survival (Vlaykova, Laurila et al. 1999; Straume and Akslen 2001; Pisacane and Risio 2005). Low levels of circulating sFlt-1 are revealed in healthy smokers in comparison to non-smokers (Belgore, Lip et al. 2000).

It is well known that VEGF effects via paracrine fashion, but studies have revealed that in melanoma VEGF has also autocrine effect (Gitay-Goren, Halaban et al. 1993; Graeven, Fiedler et al. 1999). It is thought that VEGFR-1 binds VEGF as this leads to weakening the ligand binding with VEGFR-2, and thus the majority of effects of VEGF in malignancy are mediated through VEGFR-2 (Mehnert, McCarthy et al. 2009).

Expression and genetic polymorphysms of VEGF in melanoma

Increased VEGF expression has been noticed in malignant tumors, which is not noticed in benign tumors. VEGF is expressed with low levels in benign naevi in early cutaneous melanocytic lesions and increases significantly in dysplastic naevi and malignant melanoma (Pisacane and Risio 2005; Einspahr, Thomas et al. 2007). This higher expression is associated with more invasive phenotypes and transition of melanomas from the radial to the aggressive vertical growth phases (Erhard, Rietveld et al. 1997). Prevalence of tumors expressing VEGF cannot be verified as related to prognosis as it varies widely (Howell, Bateman et al. 2002).

A large number of single nucleotide polymorphisms (SNPs) have been recently described in the VEGF gene (chromosome 6p12), reported to be associated with differential VEGF expression *in vitro*. Two of these SNPs (positions −2578C>A and −1154G>A) are located in the VEGF promoter region, one SNP (+405G>C) in exon 1 of the VEGF gene and a further SNP (+936C>T) located in exon 8, corresponding to the 3′untranslated (UTR) region of the gene (Brogan, Khan et al. 1999;

Renner, Kotschan et al. 2000; Watson, Webb et al. 2000; Shahbazi, Fryer et al. 2002). Other SNPs have also been described, although a relationship with VEGF expression has not been demonstrated. VEGF −1154 AA genotype has been associated with significantly lower VEGF expression and low Breslow thickness than GA or GG genotypes. VEGF – 1154 GG genotype has been shown to be associated with high VEGF expression. VEGF −1154 AA is called 'low expression' genotype has significantly increased frequency among cutaneous malignant melanoma (CMM) patients with Stage I and decreased in frequency among CMM patients with Stage II–IV disease. VEGF −2578, −1154, +405 CAC haplotype is associated with less advanced stage of disease and slower disease progression. The effect of the VEGF −1154 GG 'high expression' genotype to promote tumor angiogenesis is associated with significantly greater mean Breslow tumor thickness and may influence tumor invasion among CMM patients compared with patients of VEGF −1154 AA genotype . The stage of disease is associated with the rare VEGF −2578, −1154, +405 CAC haplotype. This revealed a non-significant trend towards better survival amongst individuals wih VEGF −1154 AA genotype compared with VEGF −1154 GA and GG genotypes. The results obtained showed evidence for strong link disequilibrium between the VEGF −2578, −1154 and +405 SNPs, but not between these SNPs and SNP +936 which is only slightly associated with the other SNPs (Yapijakis, Vairaktaris et al. 2007). VEGF +936 TT genotype is very rare (Howell, Bateman et al. 2002). In patients and controls no significant differences in haplotype frequencies were detected. This gives no indications that any of the four VEGF SNPs or associated haplotypes investigated may have a crucial role in susceptibility to CMM (Yapijakis, Vairaktaris et al. 2007)..

A certain number of studies demonstrate that well and rich vascularized tumors respond better to radiotherapy and combined chemotherapy compared to tumors with a poor vascularization (Revesz 1991; Volm, Koomagi et al. 1996; Giatromanolaki, Koukourakis et al. 1999). On the other hand a strong correlation between the tumor vessel density and VEGF expression is also seen. Statistic significant association between the therapeutic response and the level of VEGF expression has been reported. Patients with high VEGF expression respond more frequently and prolonged to the therapy and vice versa – patients with low level of VEGF respond less frequently and short, which can be a good predictor of objective therapeutic response. A tendency in increasing the number of blood vessels and expression of VEGF in previously treated metastasis of patients with objective response (CR or PR) in comparison with the ones not responding to chemoimmunetherapy was reported (Vlaykova 2004).

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