Inhibition of Neuroblastoma Progression by Targeting Lymphangiogenesis: Role of an Endogenous Soluble Splice-Variant of VEGFR-2

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Abstract

The endogenous-soluble-vascular-endothelial-growth-factor-receptor-2 (esVEGFR-2) is a novel member of the VEGFR-family. It is generated from the VEGFR-2 gene by alternative splicing and was shown to bind VEGF-C with high affinity, therefore acting as an endogenous inhibitor of lymphangiogenesis. Still little is known about its functions in development and disease. We explored the distribution patterns of esVEGFR-2 in human embryonic tissues and found it expressed in many organs and structures throughout the developing organism. Most interestingly, sympathetic ganglia and the adrenal medulla were positive for the inhibitor, too. Neuroblastoma is an embryonic tumor, developing from sympathetic progenitor cells of the neural crest, which in normal development form the sympathetic nervous system as well as the adrenal medulla. Immunohistology revealed that in neuroblastoma with a differentiating type of histopathology and in differentiated tumors of the ganglioneuroblastoma type, esVEGFR-2 was expressed regularly, whereas in highly malignant neuroblastoma with an undifferentiated phenotype it could be rarely detected. All-trans-retinoic acid (ATRA) is known as crucial compound in embryonic differentiation processes. It has also been shown to induce differentiation of neuroblastoma in vitro, and is a part of clinical regimen for neuroblastoma treatment. Neuroblastoma cells treated with ATRA showed increased expression of esVEGFR-2,

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suggesting that high expression of esVEGFR-2 may indicate differentiated neuroblastoma and vice versa. Real-time RT-PCR analyses revealed that INSS stage 1 and 2 neuroblastomas express higher amounts of esVEGFR-2 transcripts than stage 3 and 4 tumors. Intriguingly, the down-regulation of the transcripts inversely correlates with lymph-node involvement and metastasis formation.

Together, esVEGFR-2 may indicate differentiation in neuroblastoma and provide new insights into cancer progression mechanisms where not only expression of pro-angiogenic molecules may facilitate tumor progression, but also the down regulation of anti-(lymphangiogenic)-molecules could drive malignancy.

Introduction to Tumor Angiogenesis

Angiogenesis, the formation of blood- and lymphatic vessels from preexisting vessels, can be observed under physiological conditions whenever regeneration of blood vessels is needed, namely during the proliferative phase of the endometrial mucosa or during wound healing. In tumor pathology, the supply with nutrients and oxygen via blood vessels is a key prerequisite for tumor growth beyond the borders of normal diffusion. Tumor lymphatics drain interstitial fluid from the tumor. Therefore many clinically relevant tumor entities share the ability to induce and stimulate vessel outgrowth from existing surrounding capillaries or larger vessels and to attract the newly formed vessels to the tumor. This process is termed tumor angiogenesis. Both, angiogenesis and tumor angiogenesis are mediated by secreted molecules and signaling pathways which are often found deregulated in malignancies. Molecules involved are the Hypoxia-Inducible transcription factor (HIFs), Angiopoietins (Ang-1, Ang-2) and their receptors (TIE-1; TIE-2), Integrins, Fibroblast Growth Factors (FGFs) or Transforming Growth Factor beta (TGFbeta), just to mention some of them (Carmeliet 2003). However the strongest regulators in angiogenesis and tumor angiogenesis are the Vascular Endothelial Growth Factor family members, which are VEGF-A, VEGF-B, VEGF-C, VEGF-D and Placenta growth factor (PlGF) in collaboration with their appropriate tyrosin-kinase receptors VEGFR-1 (Flt-1), VEGFR-2 (KDR/Flk1) and VEGFR-3 (Flt-4).

The VEGF-Family and its Receptors

VEGF-family members are predominantly active as homo-dimers, but heterodimers between various VEGF members have also been observed. The complexity of this system is further increased by the fact that several splicing-forms or proteolytic fragments exist for most of the VEGFs. As there are excellent recent reviews only a short overview will be provided here (for review see Adams and Alitalo 2007; Koch et al. 2011). In brief, VEGF-A is the main inducer of blood vessels. It binds to VEGFR-2 homodimers, which are assisted by the co-receptor neuropilin 1 (NRP1). The activation of VEGFR-2 is regulated by VEGFR-1, which has tenfold higher affinity to VEGF-A but only weak signaling capacity. VEGFR-1 seems to have mainly regulatory properties such as the induction of VEGFR-2 expression or attenuation of VEGF-A signaling by trapping the ligand. Of VEGF-B, two isoforms are generated by alternative splicing, both binding exclusively to VEGFR-1. The function of VEGF-B still remains a matter of debate but there is emerging evidence that it is involved in cardiac angiogenesis during development. PlGF is expressed strongly in the placenta, but can also be found in other tissues. It also binds exclusively to VEGFR-1 and its role is not fully understood, however it seems to potentiate VEGF-A induced angiogenesis. VEGF-C and VEGF-D both bind to VEGFR-3 and are the main inducers of lymphangiogenesis.

Since VEGF-C and VEGF-D were identified, it became evident that members of the VEGF-family orchestrate not only hemangiogenesis but also lymphangiogenesis. In tumors, lymphangiogenesis was considered to be non-existing for a long time, until VEGF-C-induced lymphangiogenesis was demonstrated by using VEGF-C expressing tumor cells on the chorioallantoic membrane of the chicken embryo (Papoutsi et al. 2000).

Subsequently a series of experiments could show that this also holds true for mouse models, where VEGF-C induced lymphangiogenesis correlates positively with the formation of lymphnode-metastases (Karpanen et al. 2001; Skobe et al. 2001; Stacker et al. 2001). Even though many human tumor entities also show a positive correlation between the expression of VEGF-C and VEGF-D and lymphnode involvement, this is not the case for all human malignancies (Pepper et al. 2003). Therefore it is reasonable to assume that lymphangiogenesis is not only controlled by activators but could also be counterbalanced by inhibitory molecules.

Despite intensive investigations by many groups the VEGF-system is not fully understood yet. Nevertheless, the discovery of VEGF-A opened the door to access a mighty system of angiogenic regulators, which were consequently identified as targets for anti-angiogenic therapy of cancer and other diseases, although, at least so far, not with the expected success (Leung et al. 1989; Carmeliet 2003; Sitohy et al. 2012).

EsVEGFR-2 is a Novel Endogenous Inhibitor of Lymphangiogenesis

For both, VEGFR-1 and VEGFR-2, soluble fragments, which can be detected in blood and interstitial fluid, are known (sVEGFR-1 and sVEGFR-2). These fragments are often derived by enzymatic shedding of the membrane-bound receptor. Thereby sVEGFR-1 is considered to be a major negative regulator of VEGF-A-induced haemangiogenesis, and it has been shown that especially in the cornea the soluble fragment is not a product of proteinase activity but rather synthesized by alternative splicing from the VEGFR-1 mRNA (Ambati et al. 2006).

Recently, Albuquerque and colleagues also identified a novel soluble splice variant of VEGFR-2, termed sVEGFR-2, in mouse cornea and also in human tissues (Albuquerque et al. 2009). To make strictly clear that this molecule is not a shedded proteolytic fragment, it is subsequently termed endogenous soluble VEGFR-2 (esVEGFR-2) by the author as proposed by Shibata et al. (2010). EsVEGFR-2 is characterized by a unique C-terminal 13 amino-acid sequence in mice or 16 amino-acid sequence in human, and a complete lack of the membrane-spanning and intracellular signalling components of the full-lengthVEGFR-2. The C-terminal amino acid stretches are generated by the use of an alternative stop-codon in intron 13 and proved suitable for the production of specific antibodies as well as specific primers for real-time RT-PCR. Surprisingly, esVEGFR-2 does not seem to bind VEGF-A, but exhibits high binding affinity for VEGF-C. In a series of elegant experiments Albuquerque et al. (2009), demonstrated that tissue specific knock-down of esVEGFR-2 in the mouse cornea, using the PAX6 promotor in a cre-lox system, leads to proliferation and growth of lymphatic vessels into the (normally avascular) cornea (Albuquerque et al. 2009). Additionally, keratin 14 promotor-based cre-lox VEGFR-2 knock-out causes edema in the skin due to dilation and hyperplasia of lymphatics. Cell culture experiments revealed that esVEGFR-2 inhibits VEGF-C induced proliferation of human lymphatic endothelial cells in vitro.

EsVEGFR-2 in Embryonic Development

The above discussed experiments published by Albuquerque et al. (2009), were performed using the offspring of transgenic mice and suggest that esVEGFR-2 exerts its functions during embryonic development. We used sections of human embryos and fetuses for immunohistology in order to determine esVEGFR-2 expression patterns (Becker et al. 2012).

In 8 weeks-old embryos positive staining is detectable in the epithelium of the choroid plexus, the floor plate of the neural tube, the anterior spinal artery and distinct cells of the notochord. In the liver hematopoietic cells (Fig. 6.1a, b) and scattered cells in the adventitia of the portal vein stain positive for esVEGFR-2. Scattered positive cells are also detectable in the developing dermis, and the epidermis of 8 weeks-old embryos stains

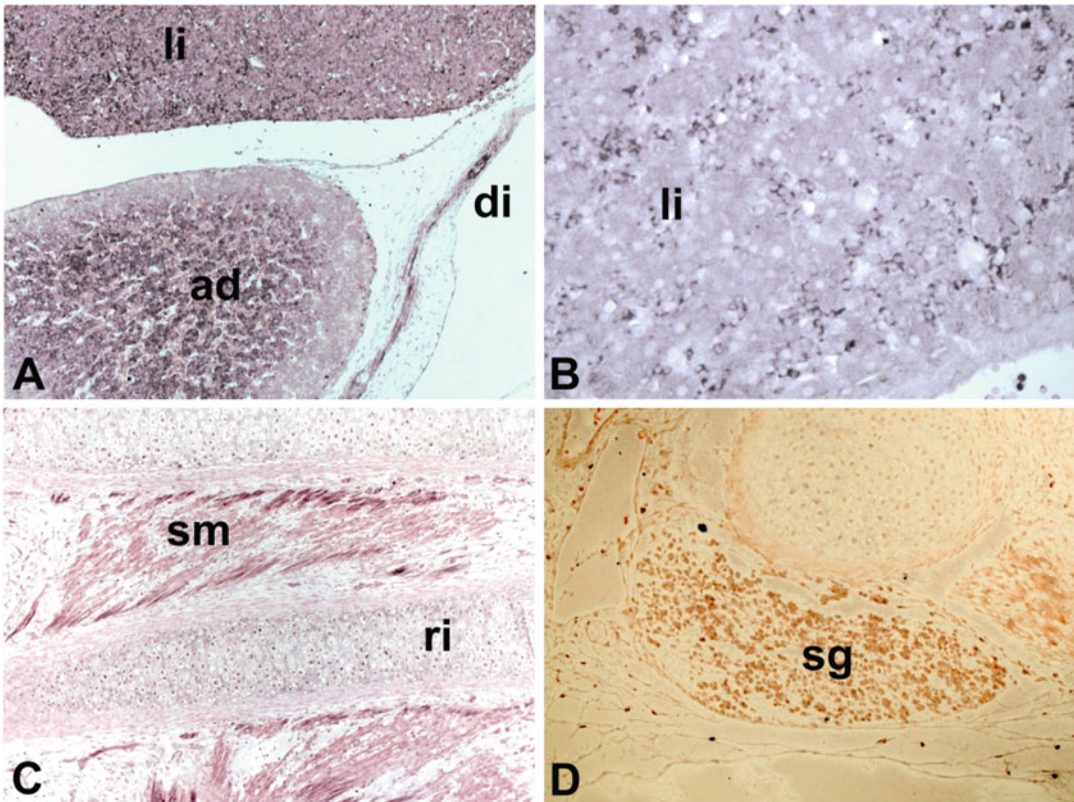

Fig. 6.1 Immunostaining for esVEGFR-2 in tissues of human, 8–10 weeks old, embryos and fetuses. (**a**) adrenal gland (ad), liver (li) and diaphragm (di). (**b**) liver. (**c**) intercostal muscles (im), ribs (ri), note strong immunoreactivity of tendineous parts of the intercostal muscle. (**d**) sympathetic ganglion (sg)

nicely positive, too. However, the identity of the scattered cells found in many tissues is not clear yet. Additional positive organs are the gut mucous-membrane, skeletal muscle cells, the tendons of skeletal muscles (Fig. 6.1c), the adrenal gland (Fig. 6.1a) and sympathetic paravertebral ganglia (Fig. 6.1d). Most strikingly, we could show that arterial endothelial cells are positive for esVEGFR-2 whereas venous endothelial cells are not (Becker et al. 2012). This phenomenon may be explained with regard to co-localization patterns of blood and lymphatic vessels. Larger arteries are accompanied by lymphatics that drain exudates, produced due due to high arterial fluid pressure. Additionally, larger lymphatics and lymphatic trunks, which drain a tributary region, are in most cases co-localized with the arteries that supply this region. Blood endothelial cells are a source of VEGF-C and therefore it can be speculated, that esVEGFR-2 might be expressed and secreted by arterial endothelial cells to block VEGF-C on the abluminal side in order to prevent attraction of lymphatic endothelial cells and thereby preventing formation of anastomoses between lymphatics and blood vessels.

EsVEGFR-2 in Neuroblastoma

Neuroblastoma is an embryonic tumor originating from neural crest cells, which belong to the sympathetic neuroblast lineage. In normal development, these cells migrate from the neural crest and form the sympathetic nervous system. Therefore neuroblastomas usually develop within or nearby sympathetic ganglia, for example along the sympathetic trunk or the adrenal medulla. Both of the latter tissues display esVEGFR-2 positivity in human embryos and fetuses (Fig. 6.1a, d). It remains unclear whether the malignantly

transformed cells follow their normal pathways in the embryo or whether the malignant transformation occurs when the cells have reached their final destination.

VEGF-C-induced lymphangiogenesis and the existence of lymphatic vessels in primary neuroblastoma specimens and experimental tumors has been shown some time ago (Lagodny et al. 2007). The international neuroblastoma staging system (INSS) refers to the lymphnode status as one of the main criteria for the clinical evaluation of tumor progression and prognosis. Albeit this system has currently been revised and transferred into the International Neuroblastoma Risk Group Staging System (INRGSS), which covers more clinical imaging data, the impact of the lymphnode status is still undoubted (Monclair et al. 2009).

Therefore, we decided to investigate the expression of esVEGFR-2 in primary neuroblastoma and the clinical relevance of the expression pattern (Becker et al. 2010). Additionally, neuroblastoma cell-lines were screened for VEGFs and VEGF-receptors, including esVEGFR-2. All tested molecules were expressed in the cell-lines, but as expected for neuroblastoma, a wide heterogeneity of the expression levels could be observed. When we used the same primer sets on untreated (which means without adjuvant chemotherapy before surgery) primary tumor samples, a slight increase of VEGF-A transcripts in stage 4 s tumors and a lower expression of VEGF-D transcripts in tumors of stages 3, 4 and 4 s could be detected. Other VEGFs did not show remarkable differences between clinical stages, except for esVEGFR-2, which was expressed significantly lower in tumor stages 3, 4 and 4 s, whereas membrane-bound VEGFR-2 (mbVEGFR-2) did not show any alterations (Becker et al. 2010).

The most significant marker for neuroblastoma progression is the amplification of the transcription factor MYCN, which strongly correlates with unfavorable outcome (Westermann and Schwab 2002). MYCN amplification can be found in 20–25 % of neuroblastomas and amplification levels may reach up to several hundred copies. This leads to a massive over-expression of MYCN at transcript and protein level. Neuroblastomas with high MYCN expression are aggressive, fast growing, well vascularized and frequently metastasizing to other organs and lymphnodes. In our studies, we could not find differences between MYCN-amplified and non-amplified primary stage 4 neuroblastoma specimens for VEGF-C, VEGFR-2 and VEGFR-1 at mRNA level. However, VEGF-A and VEGF-D transcripts are moderately increased in the MYCN-amplified samples and the inhibitory soluble receptors sVEGFR-1 and esVEGFR-2 are clearly, yet not statistically significantly, down-regulated. Of note, in 26 neuroblastoma cell-lines, mainly derived from primary tumors or metastases, there were no differences between MYCN-amplified and non-amplified specimens detectable, suggesting that in vitro data and cell-line experiments may not adequately reflect tumor behavior in this aspect (Becker et al. 2010).

However, in vitro experiments using WAC2, which are MYCN-transfected and over-expressing cells derived from the neuroblastoma cell line SH-EP, confirmed the correlation between MYCN expression and down-regulation of esVEGFR-2 (Becker et al. 2010). Other Groups reported earlier that MYCN amplified neuroblastomas up-regulate VEGF-C and that VEGF-A is down-regulated after siRNA-mediated knock-down of MYCN (Eggert et al. 2000; Kang et al. 2008). These data suggest that up-regulation of pro-angiogenic factors is one feature that facilitates neuroblastoma progression, but on the other hand, down-regulation of inhibitors of angiogenesis namely esVEGFR-2 and sVEGFR-1 may also severely disrupt the balance of pro- and anti-angiogenic factors and may strongly contribute to neuroblastoma progression.

EsVEGFR-2 Expression Correlates with Differentiation in Neuroblastoma

Typically for embryonic tumors, most neuroblastomas are diagnosed in the first 2 years of life or, with the help of modern imaging techniques even before birth. The median age of neuroblastoma patients is about 24 months. Interestingly many neuroblastomas of younger patients respond better to chemotherapy or even differentiate spontaneously to benign ganglioneuroma or

undergo complete regression. Therefore statistically, younger age strongly correlates with favorable outcome. In the neuroblastoma staging system, attention has been given to this fact with the introduction of the stage 4 s, which encloses children with progressed neuroblastoma including some degree of metastases to the skin and liver, but with an age not exceeding 12 months. Despite metastases formation these patients share an excellent prognosis and can often be cured. In older children with high risk neuroblastoma, retinoic acid derivatives have successfully been used to increase survival when applied together with or after conventional chemo-therapy (Masetti et al. 2012). The differentiating effect of retinoic acid on neuroblasoma cells in vitro has been shown in 1982 by Sidell and was refined in the years since (Sidell 1982; Reynolds et al. 1994).

We sought to identify whether esVEGFR-2 expression is correlated with differentiation in neuroblastoma. By immunohistology using an antibody raised against the specific C-terminal intronic peptide of esVEGFR-2, we found expression predominantly in low grade neuroblastomas (Hughes Grade I), which are stroma-rich with large tumor cells resembling differentiated neuronal cells (Fig. 6.2b). This tumor type is associated with no or only minor, regional lymph node involvement and good prognosis. In contrast, undifferentiated lesions with small round tumor cells (Hughes Grade III; WHO Grade III, IV) are often associated with distant lymph node metastases and dissemination to other organs like bone marrow or liver. This type of neuroblastoma is associated with poor prognosis and in the specimens we probed, no positive staining for esVEGFR-2 was detectable (Fig. 6.2a) (Becker et al. 2012). Especially in neuroblastoma with a histology of the differentiating phenotype, expression was detectable in the cytoplasm of maturing neurons, and in ganglioneuroblastoma and ganglioneuroma (Hughes Grade I) most of the ganglionic cells were strongly positive. This suggests that sympathetic neuroblasts might be able to inhibit tumor lymphangiogenesis by the production of esVEGFR-2.

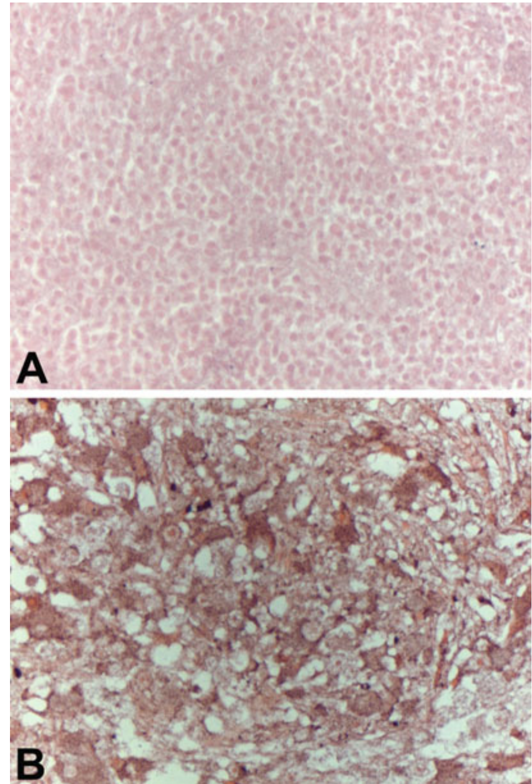

Fig. 6.2 Expression of esVEGFR-2 in human primary neuroblastoma. Paraffin sections of primary neuroblastoma specimens were subjected to immuno-histochemistry using anti-esVEGR-2 antibodies and horseraddish peroxidase-coupled secondary antibodies. (**a**) Neuroblastoma of the undifferentiated small round cells type shows no immunoreactivity for esVEGFR-2. (**b**) Neuroblastoma of the differentiating type: large neuron-like cells show up with strong immunoreactivity for esVEGFR-2 in the cytoplasm

Therapy with ATRA induces differentiation of the neuroblastoma cells and can stop malignant progression of the tumor especially in children younger than 18 months of age (Reynolds et al. 2003). Treatment of neuroblastoma cell-lines with 5–10 μM ATRA induced the maturation of the cells in vitro, which is depicted microscopically by the formation of neurite-like protrusions and by a proliferation delay or even arrest. Screening of ATRA-treated neuroblastoma cells by real-time RT-PCR revealed that esVEGFR-2 is constantly up-regulated over 12 days of treatment in SMS-KAN cells, however not all of the tested cells responded at the same degree (Becker et al. 2012).

EsVEGFR-2 in Other Cancers

So far, the effects of esVEGFR-2 have not been studied in other human cancer entities. Shibata and colleagues used a mouse model with virally inducible mamma carcinoma (Shibata et al. 2010). They treated the experimental mouse tumors with plasmid vectors containing esVEGFR-2 cDNA, introduced into the carcinoma cells by in vivo electroporation. After such treatment, tumor volumes were reduced by 30 % while survival rates increased from 60 % in controls to 90 % in esVEGFR-2-treated animals. Also, metastases to lymph nodes and the number of metastatic foci in organs like the lung were reduced by 50 %. However the number of organs (lung, kidney, adrenal and ovaries) with metastases was not altered, suggesting that the hematogenic spread of tumor cells was not affected. Remarkably, only the number of lymphatics was significantly decreased in treated tumors, while the blood vessel number was not altered. Even more interestingly, the number of lymphatics with tumor cells in the lumen decreased by about 25 % in treated tumors, which is far more than one would assume by the mere reduction of vessel number, indicating that esVEGFR-2 may also affect tumor-vessel interactions. For carcinoid cancer, a tumor derived from enterochromaffin cells of the gut, Silva et al. (2011) reported that migration in vitro is enhanced by VEGF-C binding to VEGFR-3. The authors speculate that the observed expression of esVEGFR-2 in carcinoid tumor cells may bind VEGF-C and thus slow down tumor progression (Silva et al. 2011).

Induction of Alternative Splicing

In 2011 Vorlova and co-workers published their studies on the induction of alternative splicing in tyrosine-kinase receptors (RTK). They show that alternative splicing like in VEGFR-2 can be found regularly in most of the RTK-family members (Vorlova et al. 2011). More interestingly, using VEGFR-2 in human umbilical vein endothelial cells (HUVEC), they demonstrated that alternative splicing can be induced with appropriate morpholino oligonucleotides which block the U1 small nuclear ribonucloparticle (U1 snRNP, here U1) binding site at the 5′ splicing site. As shown both at mRNA and protein levels this led to specific and efficient inhibition of splicing at intron 13 and complete replacement of membrane-bound VEGFR-2 by secreted esVEGFR-2. The authors conclude that their morpholino approach may have new therapeutic prospects by targeting RTK signaling at three levels: (i) the induction of alternative splicing substitutes membrane bound receptor by the soluble inactive form; (ii) secreted soluble receptor may bind ligands and act as a decoy receptor and (iii) the secreted soluble isoforms may dimerize with their membrane-bound counterparts and form binding-active receptors with reduced or even absent signaling capacity (Vorlova et al. 2011).

Lately, Uehara and colleagues (2013) were able to confirm the results published by Vorlova and co-workers (Vorlova et al. 2011; Uehara et al. 2013). They used a similar morpholino specific for the splicing site at the exon 13 – intron 13 boundary and forced polyadenylation at the latent polyA-site of intron 13. The resulting product was correctly glycosylated and exported to the culture fluid. In vivo intravitreal injection of morpholinos induced esVEGFR-2 expression and suppressed neovascularization of the choroidea after laser treatment, therefore the effects of esVEGFR-2 on hemangiogenesis may following the mechanisms proposed by Vorlova et al. (2011). Also in cornea suture experiments, morpholinos injected under the conjunctiva inhibited hemangiogenesis and lymphangiogenesis during cornea healing. Additionally, after cornea transplantation, morpholino-treatment prevented graft rejection effectively (Uehara et al. 2013).

In sum, morpholino based induction of esVEGFR-2 seems to be a promising tool to control lymphangiogenesis and hemangiogenesis in the future.

Conclusions

The novel soluble splice variant of VEGFR-2 is a potent inhibitor of VEGF-C-mediated processes. There is evidence that in neuroblastoma, the down-regulation of esVEGFR-2 correlates with

tumor progression and lymph node involvement. There are several scenarios to explain this assumption. Firstly, with regard to the findings in the human embryonic tissues, esVEGFR-2 expression seems to be a marker of normally differentiating sympatho-adrenal cells and progenitor cells. The down-regulation of the inhibitor may therefore be a sign that the cells have left their differentiation pathway into a malignant direction. This notion is supported by our experiments with the retinoic acid derivative ATRA, which induces differentiation of tumor cells and increases the levels of esVEGFR-2. Secondly, the loss of esVEGFR-2 expression in neuroblastomas of progressed stages may elicit indirect effects in the stroma such as increased lymphatic endothelial proliferation, which facilitates the lymphatic spread of tumor cells. Beside anti-angiogenic effects, the molecule may have further properties, which influence tumor outcome, for example the modulation of immune responses. A connection between the immune system and VEGF-C has earlier been described (Chen et al. 2005). When siRNA directed against VEGF-C was applied to mice bearing experimental mammary-carcinoma the authors found that the knock-down of VEGF-C causes increased infiltration of CD 8^+ T-cells and dendritic cells, indicating an increased immune response, but also decreased lymphangiogenesis. Their anti-VEGF-C treatment resulted in an overall increased survival of the animals. A similar effect may be postulated for esVEGFR-2, which may offer an additional valuable therapeutic option. However further studies are required to fully elucidate the therapeutic value of this promising molecule.

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