Vaccination against tumor neovascularization: Promise and reality

Immunization against angiogenic-associated antigens selectively expressed on tumor vasculature provides for a novel strategy to block tumor growth. The feasibility of this approach has recently been borne out in several reports demonstrating that the protein or DNA of angiogenic molecules, such as VEGF-R2, can be used as vaccines to generate an effective cytotoxic T cell and antibody response against tumor vessels, thereby blocking tumor growth and metastasis.

Accumulating evidence suggests that inhibition of tumor angiogenesis is an effective strategy to block the growth of certain tumors. The vascular endothelial growth factor (VEGF) family of angiogenic factors has been shown to play an important role in tumor angiogenesis. VEGF, through interaction with its cognate tyrosine-kinase receptor, VEGF-receptor-2 (VEGF-R2, also known as KDR, Flk-1), conveys signals that support proliferation, survival, and motility of endothelial cells (Figure 1). Anti-VEGF-R2 agents, including small molecule tyrosine kinase inhibitors targeted at VEGF-R2, are highly effective in blocking tumor angiogenesis and growth in murine tumor models. However, translation of these strategies to a clinical setting has proven difficult, in part due to toxicities, particularly when these agents are used in combination with chemotherapeutic agents (Marx et al., 2002). Another obstacle in the clinical application of antiangiogenic factors is the delivery of sufficient amounts of bioavailable anti-VEGF-R2 agents selectively to the tumor vasculature to faithfully block VEGF-R2 activity. In most cases, chronic long-term delivery of high doses is necessary to achieve a clinical response. One strategy to circumvent the hurdles associated with chronic therapy has been to take advantage of inherent immune response to selectively target tumor neovascularization, thereby eliminating the need for long-term expensive delivery of high doses of potentially toxic agents.

Breaking immune tolerance against angiogenic-associated molecules, including VEGF-R2, which is upregulated in proliferating tumor endothelial cells, provides a novel strategy to block tumor angiogenesis (Figure 1A). Tumor endothelial cells are genetically stable and readily accessible to the bloodstream; therefore, breaking immune tolerance can deliver cytotoxic T lymphocytes and antibodies directly to the neoangiogenic tumor VEGF-R2+ endothelial cells. Several groups have succeeded in inducing the immune response against VEGF-R2 by breaking immune tolerance. In one pioneer study, the mice were immunized against VEGF-R2 by pulsing in vitro-generated dendritic cells with soluble VEGF-R2 (Li et al., 2002). This immunization strategy generated VEGF-R2-specific neutralizing antibody and CD8+ cytotoxic T cell responses (CTL), thereby breaking tolerance to self-VEGF-R2 antigen. Development of pulmonary metastasis was strongly inhibited in mice challenged with B16 melanoma or Lewis lung (LL) carcinoma cells. Development of cytotoxic CD8+ cells targeted at VEGF-R2 was shown to effectively destroy tumor endothelial cells (Figure 1B). Another mechanism for the antiangiogenic effect was mediated through the generation of specific neutralizing monoclonal that blocked the binding of VEGF to VEGF-R2, thereby blocking tumor angiogenesis (Figure 1C).

In a similar study reported by Niethammer et al., an oral DNA vaccine delivered by attenuated Salmonella typhimurium genetically engineered to express VEGF-R2 was used to break immune tolerance to self-VEGF-R2 antigen (Niethammer et al., 2002). This strategy was highly effective in inhibiting tumor growth in mice challenged with subcutaneous injection of B16 melanoma and LL carcinoma. Oral DNA vaccination with VEGF-R2 was also effective in diminishing tumors in mice inoculated with CT-26 colon carcinoma with fully established pulmonary metastasis. All such treated mice survived and showed only a few small lung metastatic foci, while all of the unvaccinated control mice succumbed to tumor metastasis. The mechanism for inhibition of tumor angiogenesis was cell-mediated immunity with cytotoxic CD8+ cells selectively targeting VEGF-R2+ tumor endothelium (Figure 1B).

Other immunogene therapy strategies to target tumor angiogenesis have used crossimmunization with xenogeneic endothelial cells as a vaccine to induce endothelial-specific immune responses against tumor vasculature (Wei et al., 2000). In this model, the immune response was directed toward integrin α_v and VEGF-R2, again underscoring the significance of VEGF-R2 in regulating tumor angiogenesis. In support of this approach, xenogenic crossimmunization with $\alpha_v\beta_3$ integrin has also been shown to inhibit tumor growth in mice (Lou et al., 2002). Immunization with liposomal basic-fibroblast growth factor (FGF-2) peptide vaccine (Plum et al., 2000) or VEGF DNA vaccine (Wei et al., 2001) has also resulted in the generation of neutralizing antibodies, which effectively blocked tumor angiogenesis. Vaccination against calreticulin in conjunction with a model viral tumor antigen was used to generate both an antitumor and an antiangiogenesis effect (Cheng et al., 2001). Collectively, these studies identify angiogenic factors and their receptors as effective tumor-specific targets for immunogene therapy to block tumor angiogenesis and growth.

But could these immunogene therapy strategies against tumor angiogenesis ever succeed in a clinical setting? Although expression of VEGF-R2 during embryonic development is absolutely essential for angiogenesis and vasculogenesis, the exact functional role of VEGF-R2 expression in adults is not fully known. As upregulation of functional VEGF-R2 also plays a critical role in regulating various postnatal physiological processes, including wound healing and fertility, acute or long-term inhibition of VEGF-R2 might result in the generation of life-threatening complications.

So what are the potential clinical toxicities that may be expected from immunogene therapy against VEGF-R2? Remarkably, only a modest delay in wound healing was detected in mice vaccinated with oral VEGF-R2 DNA, while fertility, neuromuscular function, and hematopoietic reconstitution were preserved (Niethammer et al., 2002). In contrast, mice vaccinated with VEGF-R2-pulsed dendritic cells showed significant impairment in fertility, while wound healing and hematopoiesis remained intact (Li et al., 2002). The differences in the toxicity profile of these two approaches may be dependent on the affinity or specificity of autoantibodies to epitopes

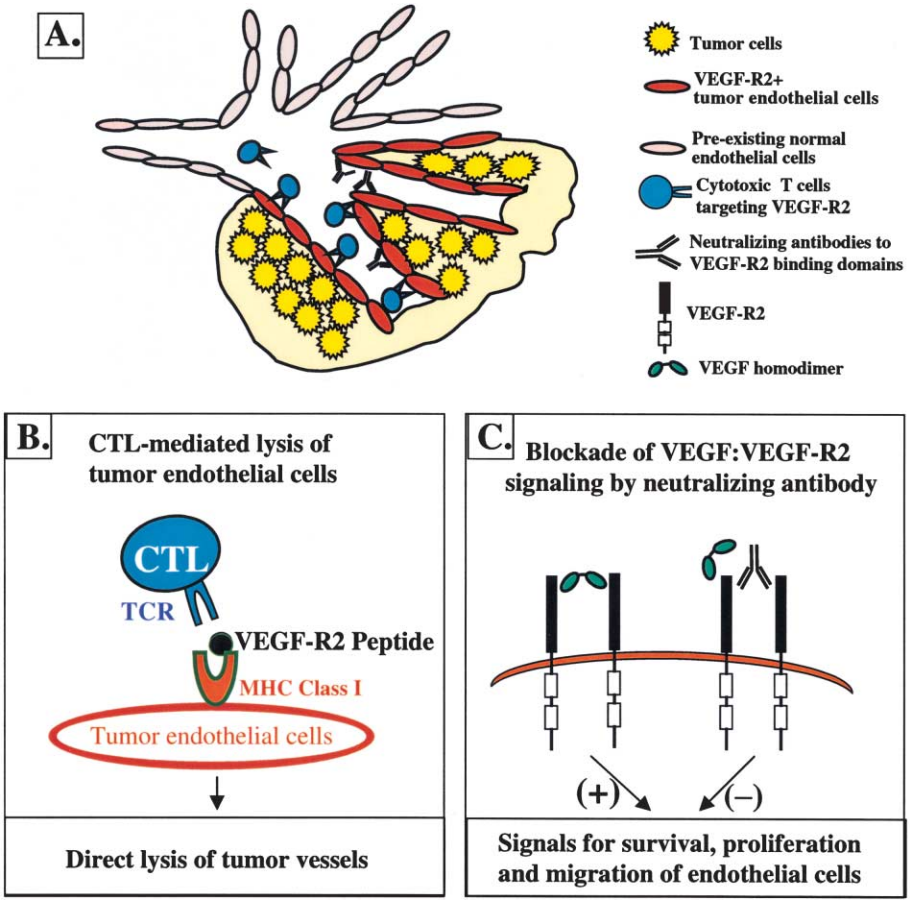

Figure 1. Inhibition of tumor angiogenesis by immunogene therapy against VEGF:VEGF-R2 signaling pathway

Differential expression of certain angiogenic-associated molecules, such as VEGF-R2, provides ideal readily accessible antigenic targets to activate immune response against tumor endothelial cells.

A: Although VEGF-R2 is expressed in certain normal adult vasculature, its expression is sufficiently upregulated in tumor vasculature to allow for generation of cell-mediated immunity against tumor endothelial cells.

B: Immunogene therapy against VEGF-R2 takes advantage of the capacity of endothelial cells to express MHC class I antigen. Tumor endothelial cells present processed VEGF-R2 peptide fragments in conjunction with the MHC class I to the T cell receptor (TCR) of primed cytotoxic CD8+ T cells (CTLs). As tumor endothelial cells express higher amounts of VEGF-R2 than normal vessels, CTLs selectively localize to tumor vasculature and induce endothelial cell lysis.

C: Vaccination against VEGF-R2 also results in the generation of neutralizing antibodies to VEGF-R2 that selectively blocks binding of VEGF homodimers to VEGF-R2. This results in impaired VEGF-R2 tyrosine kinase signaling that is necessary for endothelial cell proliferation, survival, and migration, thereby blocking tumor angiogenesis.

of VEGF-R2. As signaling through VEGF-R2 is modulated through collaboration with other membrane-bound molecules, such as neuropilins or integrins (i.e., $\alpha_v\beta_3$), it is possible, then, that the two different approaches block different epitopes, thereby interfering with separate physiological functions of VEGF-R2. Understanding the exact mechanisms by which immunogene therapy blocks tumor angiogenesis is critical to obviate toxicity to the normal vasculature.

One potential toxicity in humans that is difficult to assess in animal models is the duration of immune response that is associated with immunogene therapy models. In murine models described above, immune response to VEGF-R2 persisted for several months (Li et al., 2002; Niethammer et al., 2002). Therefore, it is very difficult to predict how long such an approach will confer immunity against VEGF-R2 in humans. Prolonged VEGF-R2 inhibition may also contribute to long-term end-organ toxicities, indicating that strategies to turn off immune response to VEGF-R2 should be implemented.

Despite these expected toxicities, the aforementioned immunotherapy studies have several advantages over conventional tumor immunotherapy, where tumor antigens are the main targets of cytotoxic T cells. For example, tumor cells can downregulate HLA class I antigen expression (Hicklin et al., 1999), whereby the tumor cells become resistant to the immune response. However, since endothelial cells are genetically stable, it is unlikely that immunotherapy will result in the development of resistance or evasion of immune response. Moreover, antiangiogenic vaccines can theoretically target different types of tumors, as long as their growth is angiogenesis-dependent. It is also possible to retarget the same tumor with booster shots to reactivate the cytotoxic response without generation of resistance thereby, obviating the need for long-term chronic and economically overbearing therapy. Finally, there is no doubt that many other angiogenesis-associated antigens besides VEGF-R2 are selectively expressed or upregulated on human tumor endothelial cells, each representing a potential target for immunotherapy (St Croix et al., 2000). As formation of tumor vessels is governed by activation of several angiogenic pathways, future immunotherapy protocols targeting one single angiogenic pathway may be inadequate to completely block tumor growth. Immunogene therapy targeted at several tumor-specific angiogenic epitopes may be more effective to completely block tumor angiogenesis and growth while diminishing long-term toxicity to end-organ vasculature.

Rigorous controlled clinical studies are in order to assess the efficacy of immunogene therapy against tumor angiogenesis. In particular, preclinical studies should focus on identifying potential toxicities that may be associated with long-term immune-mediated VEGF-R2 inhibition in human subjects. For example, as angiogenesis plays a critical role in arteriogenesis and collateral vessel formation, chronic long-term or even short-term inhibition of VEGF-R2 may result in major hemostatic complications after cardiac or cerebral vascular ischemia. Moreover, since VEGF isoforms may serve functions to maintain pulmonary and kidney function, chronic VEGF-R2 inhibition may also be associated with pulmonary or renal complica-

tions. However, the potential toxicities that may be associated with immunotherapy targeted at angiogenic agents should not dampen our enthusiasm for moving these strategies through preclinical studies, but rather should ignite the desire to move forward quickly with a watchful eye toward rationally designed clinical trials.

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Is NF-κB2/p100 a direct activator of programmed cell death?

Transcription factor NF-κB has been implicated in cancer development due to its ability to upregulate expression of genes with potentially prooncogenic functions, such as cell cycle regulators and antiapoptotic proteins (Karin et al., 2002). A recent report by Wang et al. (2002) suggests that a structural motif, a death domain (DD), present in one of the mammalian NF-κB proteins, NF-κB2/p100, allows it to directly activate cell death in a transcription-independent manner. Further, it is suggested that loss of the proapoptotic function of NF-κB2/p100 is directly linked to its oncogenic activity in lymphomas.

Nuclear factor of κB (NF-κB) defines a family of related homo- and heterodimeric transcription factors (Ghosh and Karin, 2002). It includes proteins that are synthesized in their mature form and proteins that are synthesized as large precursors that undergo proteolytic processing. The first group encompasses RelA, RelB, and c-Rel, which have an N-terminal Rel homology domain (RHD), required for DNA binding and dimerization, and a C-terminal region with transactivating properties (Figure 1). These proteins are held in the cytoplasm by "Inhibitors of κB" (IκB) proteins, characterized by 6–7 ankyrin repeats that are required for binding to the RHD of Rel proteins and masking their nuclear localization sequences. Upon cell stimulation, the IκBs are phosphorylated and undergo ubiquitin-dependent degradation. The liberated NF-κB dimers enter the nucleus and act as sequence-specific transcription factors. The second group consists of NF-κB1/p105 and NF-κB2/p100 and their products p50 and p52, respectively. The precursors contain an N-terminal RHD as well as a C-terminal region that contains ankyrin repeats similar to those in IκBs (Figure 1). In their C terminus, the precursors contain a death domain (DD). By virtue of their ankyrin repeats and their ability to dimerize with other Rel proteins, p105 and p100 act like IκBs (Mercurio et al., 1993). While p105 processing is largely constitutive, processing of p100 is triggered by its phosphorylation and ubiquitination in a manner akin to IκB degradation (Xiao et al., 2001). Limited proteolysis results in generation of the mature NF-κB2/p52 transcription factor consisting of the N-terminal RHD.

The pathways that lead to activation of NF-κB and processing of p100 depend on components of the IκB kinase (IKK) complex, which contains two catalytic subunits, IKKα and IKKβ, and a regulatory subunit IKKγ/NEMO (Rothwarf et al., 1998). After activation by stimuli like the proinflammatory cytokine TNFα or bacterial products such as LPS, IKKβ phosphorylates the IκBs, leading to their degradation and nuclear entry of NF-κB dimers. This pathway, named the canonical NF-κB activation pathway, applies primarily to NF-κB dimers composed of RelA, c-Rel, and p50. An alternative NF-κB signaling pathway involving IKKα leading to p100 processing has recently been described (Senftleben et al., 2001). This pathway is triggered upon occupancy of BAFF (B cell activating factor) receptor (BAFF-R) and CD40 on B cells and lymphotoxin β receptor (LTβR) on stromal cells. Activation of this pathway results in disappearance of p100 and nuclear translocation of p52:RelB dimers (Dejardin et al., 2002; Senftleben et al., 2001; Xiao et al., 2001). Processing of p100 depends on the catalytic activities of IKKα and another kinase, NIK, which probably acts upstream of IKKα (Senftleben et al., 2001). The function of this pathway is still not well defined, but it is likely to be required for B cell maturation and formation of secondary lymphoid organs (Senftleben et al., 2001). The DD of p100 is also involved in processing, as its removal leads to constitutive processing as well as inhibition of NIK-dependent phosphorylation and processing (Fong et al., 2002). Notably, mutant forms of NF-κB2 were detected in B and T cell lymphomas, where they are caused by chromosomal translocations that result in truncations of the regulatory C-terminal