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News and Commentary

A new role for NF-κB in angiogenesis inhibition

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Angiogenesis as a Therapeutic Target

Angiogenesis is considered a promising target in the treatment of cancer. Over the last 15 years, considerable progress has been made in the development of therapies based on targeting tumor angiogenesis. Currently, several angiogenesis inhibitors are approved for the treatment of cancer and many are in late-stage clinical testing.[1] Most of these compounds act indirectly either by clearing angiogenic growth factors from the circulation or by blocking the signaling pathways activated by these growth factors. Although this category of angiogenesis inhibitors is well developed, the therapy based on these inhibitors may suffer from several drawbacks. Firstly, most tumors express several different angiogenesis growth factors,[2] suggesting that blocking only one (or a few) may not be sufficient. Secondly, the intrinsic selection by growth factor inhibition, may even force the genetically instable tumor cells to drift to the production of alternative pro-angiogenic factors. This may ultimately lead to drug resistance.[3]

Another group of angiogenesis inhibitors are the direct angiostatic compounds. These agents have a direct effect on the endothelium, affecting cellular regulatory pathways, independently of the tumor cells.[4] It is likely that a therapy based on these inhibitors will reduce the risk of developing drug-induced resistance. The reason that this category of agents is lagging behind regarding their translation to the clinic may be the lack of sufficient knowledge on the mechanism of action of these compounds. Interestingly, from several recent reports a commonality in angiostatic signaling is emerging. These studies report that angiogenesis inhibition is associated to NF-κB activation. This is of special interest since in tumor cells, NF-κB activation has been associated to inhibition of apoptosis and currently novel treatment strategies are being developed based on inhibition of NF-κB.[5] While this would cause an anti-tumor effect, it might coincide with pro-angiogenic activity. This review summarizes the recent reports describing the role of NF-κB in the angiogenesis process as well as in the angiostatic therapy. The paradigm that systemic NF-κB inhibition can serve as an anti-cancer strategy, therefore, might need to be re-evaluated. In view of the recent data, it might be speculated that NF-κB activation, when performed specifically in endothelial cells, can be an efficient strategy for the treatment of cancer.

Relationship between NF-κB and the Angiogenic Process

Recently, Kisseleva *et al*.[6] reported on the role of NF-κB signaling in endothelial cell function *in vivo*. Inoculated tumors grew faster in transgenic mice expressing mutated IκBα, under control by the Tie-2 promoter, resulting in endothelial repression of NF-κB. In addition, histological analysis revealed a striking increase in tumor vascularization in these mice. This study highlighted, for the first time, the *in vivo* role of NF-κB in tumor angiogenesis, indicating an inhibitory role for NF-κB in tumor angiogenesis. It is tempting to speculate that activation of NF-κB in endothelial cells appears to be a way to block angiogenesis. However, it is unknown through which mechanisms NF-κB activation leads to inhibition of tumor angiogenesis and how specific activation of NF-κB in endothelial cells can be realized.

Angiogenesis occurs in a coordinated series of steps, which can be roughly divided into a destabilization, a proliferation and a maturation phase. One of earliest events in angiogenesis is the degradation of the vascular basement membrane and the remodeling of the extracellular matrix (ECM). The role of NF-κB in the regulation of these systems is well documented. In line with a pro-oncogenic activity, NF-κB promotes expression of several matrix metalloproteinases (MMPs), including MMP-2, -3 and -9.[7–9] However, NF-κB could also inhibit endothelial cell migration via the upregulation of tissue inhibitors of metalloproteinase-1 as described in astrocytes.[10] Next to the MMPs, plasmin is a broad-spectrum protease that also hydrolyzes many extracellular proteins, the most notable of which is fibrin. Plasmin is produced from an inactive precursor called plasminogen. uPA (urokinase plasminogen activator) and tPA (tissue-type plasminogen activator) are two proteases with high affinity for plasminogen. The activation of plasminogen into plasmin could be negatively regulated by the physiological inhibitors, namely plasminogen activator inhibitor (PAI)-1 and -2.[11] In endothelial cells, it has been described for both reactive oxygen species and tumor necrosis factor-α (TNF-α) induced expression of PAI-1 via NF-κB.[12] In addition, activation of NF-κB by TNF-α can also lead to the inhibition of the tPA expression.[13] These data suggest that NF-κB activation could lead to a decrease in ECM degradation capacity, thus leading to impaired angiogenesis.

Degradation of the vascular basement membrane and remodeling of the ECM allows endothelial cells to migrate and

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form new blood vessels. Integrins are the principal adhesion receptors used by endothelial cells to interact with the extracellular environment and are necessary for cell migration, proliferation and survival.[14] It has recently been demonstrated that the interaction of $\alpha V\beta 3$-integrin with the ECM activates NF-κB by activation of the IKK complex and degradation of IκB-α. In rat aortic endothelial cells, this activation triggers a prosurvival signal.[15]

Many soluble molecules control the balance between cell proliferation and cell death. While angiogenic factors such as VEGF and bFGF are mitogenic and act as survival factors, angiostatic agents induce cell cycle arrest and promote endothelial cell death.[4] Activation of NF-κB in endothelial cells induces expression of both angiogenic and angiostatic factors. VEGF expression is upregulated by hypoxia-induced mitogenic factor through activation of the NF-κB pathway.[16] On the contrary, vascular endothelial growth inhibitor (VEGI, reported to inhibit endothelial cell proliferation) has also been found to be induced by NF-κB.[17] In addition, the promoter of thrombospondin-1 and -2, the first naturally occurring angiostatic agents discovered, contain NF-κB binding sites.[18]

The role of NF-κB in the progression of cell cycle has also been investigated. NF-κB is able to induce expression of both activators and inhibitors of cell cycle progression.[19] For example, NF-κB induces expression of cyclin D or E[20] as well as expression of p21/cip1[21] demonstrating that the overall effect of NF-κB on cell proliferation is difficult to predict. To our knowledge, there are no reports on a direct relationship between NF-κB activation and proliferation in endothelial cells.

Programmed cell death or apoptosis is regulated in an orderly way by a series of signaling cascades and occurs by two connected pathways. The extrinsic pathway involves activation of caspase-8 by cell surface death receptors while the intrinsic pathway involves cytochrome *c* release from mitochondria and subsequent caspase-9 activation. In tumor cells, NF-κB is generally seen as an anti-apoptotic factor since it induces anti-apoptotic genes such as c-IAP-1, c-IAP-2, XIAP that block the caspase cascade or anti-apoptotic members of the Bcl family described to inhibit cytochrome *c* release. Therefore, activation of NF-κB in cancer cells by chemotherapy or radiation therapy is often associated with the acquisition of resistance to apoptosis. This has emerged as a significant impediment to effective cancer treatment. In combination with chemotherapy, inhibitors of the NF-κB pathway were recently used with success as treatment against cancer. NF-κB activation is a multi-step process that could be blocked efficiently using inhibitors of IKK or inhibitors of the IκB degradation that targets the ubiquitin and proteasome.[5] The most significant clinical data have so far been obtained with bortezomib, a proteasome inhibitor that exerts NF-κB-dependent and -independent biological effects. Bortezomib is presently approved by the Food and Drug Administration for use in multiple myeloma patients who have received at least one prior therapy.[22] In contrast to the negative effects of NF-κB activation, recent reports suggest that in certain situations NF-κB can promote apoptosis and may be viewed as a tumor suppressor gene.[23] NF-κB has been described to induce expression of several pro-apoptotic molecules such as Bcl-Xs, Bax, Fas or FasL.[24] Furthermore, blockade of NF-κB predisposes murine skin to squamous cell carcinoma[25] illustrating the dual activity of NF-κB and the complexity of the systemic use of broad-spectrum NF-κB inhibitors for the treatment of cancer.[23] In endothelial cells, a lot of evidence exists that NF-κB activation plays a pro-apoptotic role. For example, a high concentration of glucose activates production of reactive oxygen species and induces caspase-3 activation in endothelial cells.[26] Recently, it has been demonstrated that this is mediated via NF-κB and subsequent c-Jun N-terminal protein kinase activation.[27] In human cardiac microvascular endothelial cells, IL-18 induces expression of Fas, FasL and Bcl-Xs via NF-κB. These inductions lead to the activation of both the intrinsic and extrinsic apoptotic pathways.[28] Nevertheless, as described previously for tumor cells, diverse activities have also been observed in endothelial cells. Indeed, Silibinin, a cancer chemopreventive agent, was found to induce apoptosis in endothelial cell line by inhibiting NF-κB[29] and a report describes that pro-survival effect of VEGF is mediated by NF-κB activation.[30] In conclusion, NF-κB is involved in a complex network of pathways that control the decision between life and death of cells. As described for apoptosis, recent data indicate that necrotic cell death is controlled and programmed as well.[31] This is an emerging area of research that underlines the role of NF-κB on the crossroads of the cell to die by apoptosis or necrosis.[32]

Overall, activation of NF-κB in endothelial cells appears to have a negative effect on angiogenesis. Indeed, increased activation of NF-κB results in reduced tube formation in matrigel. In HUVEC, Chng *et al*.[33] have investigated the effect of A20, a negative endogenous regulator of NF-κB, in angiogenesis by using RNA interference. In a matrigel assay, it was observed that A20 inhibition, and subsequent activation of NF-κB, effectively leads to reduced tube formation.

NF-κB: A Mediator in Angiostatic Therapy?

The observations described above indicate that NF-κB is involved in the regulation of migration and/or proliferation/survival of endothelial cells and that NF-κB activation could be beneficial in tumor therapy. The following will highlight the role of NF-κB in the angiostatic agent signaling. Indeed, several angiostatic compounds already described to block tumor growth have been reported to act on endothelial cells via NF-κB activation.

The 16 kDa N-terminal fragment of prolactin (16K PRL) is a potent angiostatic agent in various *in vivo* models and has been shown to inhibit endothelial cell migration and proliferation.[34] We have demonstrated that NF-κB activation is required for 16K hPRL-induced caspase-8 and -9 activation and subsequent apoptosis.[35] In addition, it is interesting to note that NF-κB activation appears to be a very proximal event. 16K hPRL is not the only angiostatic agent that has been described to activate NF-κB. Platelet factor-4 (PF4) is an α-chemokine naturally secreted by platelets and is known to inhibit angiogenesis.[36] PF4 promotes the expression of E-selectin in HUVEC. Data provide direct evidence that the NF-κB-binding site is required for PF4 activation of the E-selectin promoter. In addition, EMSA experiments demonstrate that PF4 treatment of HUVECs results in binding of

NF-κB to its DNA-binding site already after 1 h of stimulation.[37] The angiostatic properties of angiostatin, a cleavage product of plasminogen, have also been linked with NF-κB. Chen *et al.*[38] have analyzed the global action of angiostatin in endothelial cells. By microarray screening, they have found an altered expression of 189 genes after treatment with angiostatin. These genes are mainly involved in growth, apoptosis, migration, but also in inflammation. Even if no direct evidence demonstrates activation of NF-κB, angiostatin promotes mRNA expression of RelB as well as many NF-κB target genes, namely E-selectin, intracellular adhesion molecule-1 (ICAM-1), Cyclin D1, p21/cip1 and FasL (for the complete list of altered gene expression, please see Chen *et al*[38]). Based on these data, there is strong suggestion that NF-κB is also activated by angiostatin. Administration of statins has been shown to decrease tumor growth and angiogenesis.[39] It has been recently published that statins upregulate the expression of endothelial and inducible nitric oxide synthase through NF-κB activation.[40] Finally, the angiostatic agent Neovastat, which is currently in phase III clinical studies, inhibits angiogenesis through an increase in tPA activity and it has been shown that this induction is NF-κB dependent.[41]

Based on all these reports, it is suggested that the activity of angiostatic compounds is dependent on activation of NF-κB. Therefore, activation of NF-κB specifically in endothelial cells might be an attractive therapy. TNF-α is one of the most potent NF-κB activators. The clinical use of TNF-α as an anticancer drug is limited to local treatment (e.g. isolated limb perfusion) because of its systemic toxicity.[42] To circumvent this problem, targeted delivery of TNF-α to tumor vessels was achieved by coupling this cytokine with cyclic CNGRC peptide, an aminopeptidase N (CD13) ligand that targets the tumor neovasculature. Administration of this compound leads to a reduced toxicity, a marked endothelial cell apoptosis, destruction of blood vessels and improvement of the anti-tumor activity of doxorubicin.[43] These studies demonstrate that NF-κB activation, specifically in endothelial cells, can be an efficient strategy for the treatment of cancer.

In conclusion, while in tumor cells NF-κB is mostly oncogenic, upregulation of NF-κB in endothelial cells is associated with angiostatic activity. It might therefore be warranted to revisit anti-cancer therapies based on inhibition of NF-κB activity for effects on angiogenesis.

Role of NF-κB in Endothelial Cell Anergy

Next to a direct anti-tumor activity of NF-κB through inhibition of tumor angiogenesis, the activation of NF-κB could also be connected with an indirect anti-tumor activity through reversal of endothelial unresponsiveness to inflammatory signals, a process called endothelial cell anergy. The latter is defined as the inability of tumor endothelial cells to express adhesion molecules such as ICAM-1/-2, vascular endothelial cell adhesion molecule-1 (VCAM-1) or E-selectin, in response to inflammatory cytokines such as TNF-α, interferon-γ and interleukin-1. These adhesion molecules mediate leukocyte rolling along, adhering to, and diapedesis through the vessel

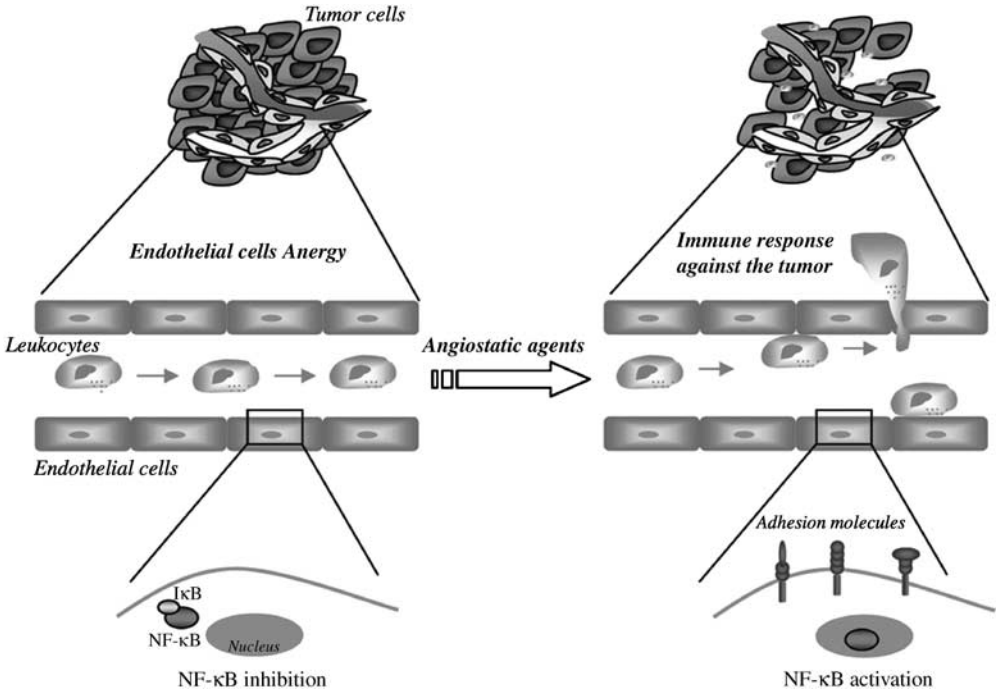

Figure 1 Role of NF-κB in endothelial cell anergy. Tumors escape from immunity by inhibiting NF-κB in endothelial cells. This inhibition leads to the downregulation of adhesion molecules and leukocyte–vessel wall interactions, as well as the inability to induce these upon inflammatory stimulation, a situation called endothelial cell anergy. These phenomena are the result of endothelial cell exposure to angiogenic growth factors. Inhibition of angiogenesis overcomes this escape from immunity called endothelial cell anergy. Angiostatic compounds activate NF-κB and upregulate expression of adhesion molecules such as ICAM-1/2, VCAM and E-selectin. The upregulation of endothelial adhesion molecules in tumor vessels contributes to leukocyte recruitment and infiltration within the tumor. Based on these observations, NF-κB activation in endothelial cells contributes to the anti-tumor activity of the angiostatic compounds

wall, and thus have an important role in the selection of an inflammatory infiltrate.[44]

It has been demonstrated that tumor endothelial cells display a reduced expression of adhesion molecules (ICAM-1 and ICAM-2) as compared with normal endothelial cells.[45] This explains the observation of a reduced number of infiltrated leukocytes in the tumor.[46] *In vitro* and *in vivo* studies on endothelial cell anergy have demonstrated that this reduced expression is caused by exposure to angiogenic growth factors such as VEGF and bFGF.[47] In addition, it has been recently described that bFGF downregulates ICAM-1 expression via NF-κB inhibition.[48] Furthermore, we have demonstrated that suppressed leukocyte–vessel wall interaction in tumor vessels can be normalized by angiostatic compounds, such as endostatin, angiostatin and anginex, as well as by treatment with chemotherapeutic agents.[49] This normalization has been correlated with upregulation in endothelial cells of ICAM-1, VCAM-1 and E-selectin.[37,50] Therefore, activation of NF-κB by angiostatic therapy has apparently not only a direct effect on endothelial cells but also an indirect effect via expression of adhesion molecules and subsequent reversal of endothelial cell anergy (Figure 1). In addition, we have recently described that galectin-1 is a receptor for anginex, and that this protein is crucial for tumor angiogenesis.[51] Since galectin-1 induces chemokines production via the activation of NF-κB,[52] we can speculate that anginex could overcome endothelial cell anergy by activating the NF-κB pathway.

Undoubtedly, NF-κB plays a key role in regulating endothelial cell anergy. While pro-angiogenic factors inhibit NF-κB and the subsequent expression of adhesion molecules, angiostatic agents overcome endothelial cell anergy via the activation of the NF-κB pathway. Such activation of NF-κB resulting in stimulation of anti-tumor immunity but also in inhibition of angiogenesis clearly results in an anti-tumor outcome.

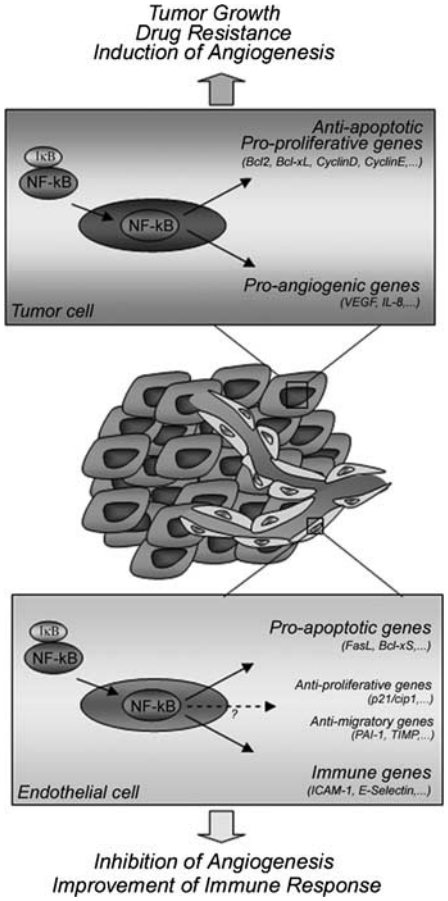

Figure 2 Model of the dual role of NF-κB in tumorigenesis. In tumor cells, activation of NF-κB leads to tumor growth by both direct and indirect mechanisms. The direct mechanism involved expression of anti-apoptotic and pro-proliferative molecules while the indirect mechanism involves promotion of angiogenesis. In endothelial cells, activation of NF-κB could block tumor progression by both, angiostatic activity, via the production of pro-apoptotic molecules and by improvement of immune response via expression of adhesion molecules

Future Directions

Inhibition of angiogenesis is a promising therapeutic approach to fight cancer. Important advances have been made towards the comprehension of the molecular mechanisms induced by angiostatic agents in endothelial cells. Nevertheless, up to now, a clear and precise view of how angiostatic agents act on endothelial cells still remains to be defined. From several recent findings, it is likely that activation of NF-κB is a common mechanism of angiostatic agents, resulting in both inhibition of angiogenesis and stimulation of anti-tumor immunity (Figure 2). While these data raised a cautionary note about the pharmaceutical agents that block NF-κB, they also suggest that a targeted activation of NF-κB, specifically in endothelial cells, could represent a new and promising strategy in cancer treatment.

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