

NF- κ B: a new player in angiostatic therapy

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Abstract Angiogenesis is considered a promising target in the treatment of cancer. Most of the angiogenesis inhibitors in late-stage clinical testing or approved for the treatment of cancer act indirectly on endothelial cells. They either neutralize angiogenic growth factors from the circulation or block the signaling pathways activated by these growth factors. 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. 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. The transcription factor NF- κ B has been recently connected with multiple aspects of angiogenesis. In addition, several recent studies report that angiogenesis inhibition is associated to NF- κ B activation. This is of special interest since in tumor cells NF- κ B activation has been associated to inhibition of apoptosis and currently novel treatment strategies are being developed based on inhibition of NF- κ B. The paradigm that systemic NF- κ B inhibition can serve as an anti-cancer strategy, therefore,

might need to be re-evaluated. Based on recent data, it might be speculated that NF- κ B activation, when performed specifically in endothelial cells, could be an efficient strategy for the treatment of cancer.

Keywords Angiostatic · NF- κ B

Introduction

The NF- κ B/Rel proteins are a family of transcription factors that include five proteins, p50, p52, p65 or RelA, RelB, and c-Rel, that exist as homo- and hetero-dimers. The most common NF- κ B heterodimer is composed of p50 and p65. In resting cells, NF- κ B is mainly sequestered in the cytoplasm by its association with proteins belonging to the I κ B inhibitor family. Stimuli such as the proinflammatory cytokines tumor necrosis factor (TNF)- α , and interleukin-1 (IL-1), or bacterial products such as lipopolysaccharide (LPS) can activate NF- κ B. In the canonical pathway, these stimuli activate I κ B kinases (IKKs), which in turn phosphorylate the main NF- κ B inhibitor, I κ B α . This phosphorylation step leads to the ubiquitination and subsequent degradation by the proteasome of I κ B α . The NF- κ B complex translocates to the nucleus where it binds to κ B enhancers present in the regulatory regions of various genes and where it activates transcription [1]. The NF- κ B target genes are involved in a wide range of biological functions including proliferation, survival, and inflammation (Fig. 1).

NF- κ B activation has been connected with multiple aspects of oncogenesis, including the control of tumor cell proliferation, migration, cell cycle progression, and inhibition of apoptosis [2–4]. Indeed, NF- κ B is constitutively activated in several types of cancer cells and it is

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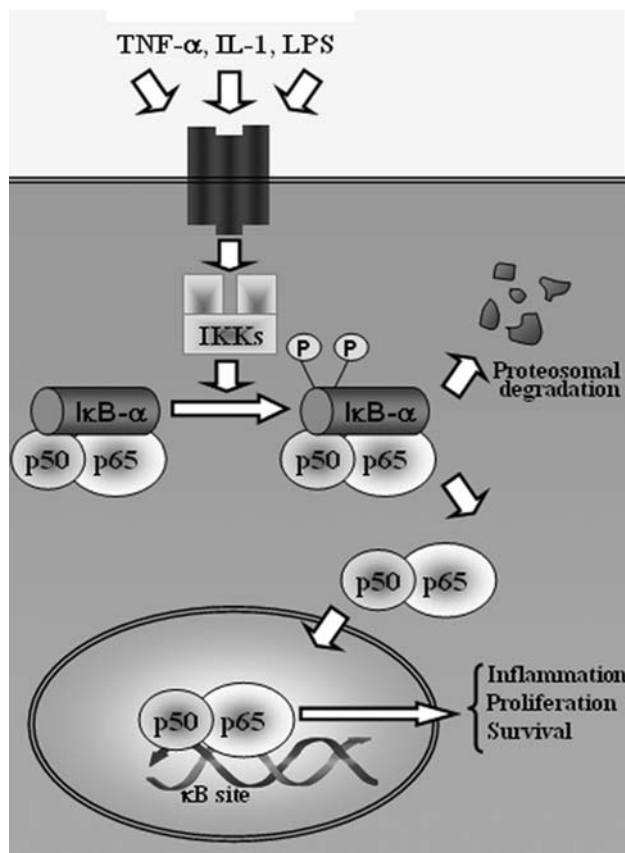


Fig. 1 Schematic NF- κ B pathway. In the canonical activation pathway, NF- κ B (often the dimer composed of p50/p65) is sequestered by its main inhibitor I κ B- α . Upon stimulation, cell surface receptors activate IKK complex, which then phosphorylate I κ B- α . These phosphorylations lead to its degradation by the proteasome and the entry of NF- κ B in the nucleus, which turns on target genes

generally regarded as an anti-apoptotic and pro-oncogenic signal. The most studied and well-established functions of NF- κ B in promoting oncogenesis are its ability to (i) induce growth promoting genes such as cyclin D1 and c-myc and (ii) induce anti-apoptotic genes such as c-IAP-1, c-IAP-2, or XIAP [3]. Therefore, activation of NF- κ B 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- κ B pathway (e.g., proteasome inhibitors) were recently used with success as treatment against cancer [5]. Next to this direct effect, it has also been reported that NF- κ B activity can be tumorigenic by activation of pro-angiogenesis genes, such as VEGF, IL-8, and MMP-9 [6].

In contrast to the negative effects of NF- κ B activation, recent reports suggest that in certain situations NF- κ B can promote apoptosis and may be viewed as a

tumor suppressor gene. For example, blockade of NF- κ B predisposes murine skin to squamous cell carcinoma [7]. This observation could be explained by the fact that in normal human epidermal cells, NF- κ B activation induces cell cycle arrest [8]. In addition, Ryan et al. describe the role of NF- κ B in p53-mediated programmed cell death. The tumor suppressor p53 inhibits cell growth through activation of apoptosis and cell cycle arrest. Using a p53-inducible Saos-2 cell line, it was demonstrated that induction of p53 causes activation of NF- κ B. Furthermore, inhibition of NF- κ B abrogated p53-induced apoptosis demonstrating that inhibition of NF- κ B in tumors that retain wild-type p53 may reduce a therapeutic response [9]. Loss of p65 can also cause resistance to different agents that induce apoptosis through p53 [10]. Independently, it was demonstrated that activation of NF- κ B is essential for the cytotoxic effect of doxorubicin and its analogs [11]. Many hypotheses have been put forward to explain this dual activity. The overall conclusion that is emerging is that the final outcome of NF- κ B activation depends on cell type, the stimulus, and the context of activation [12–14]. The dual activity of NF- κ B complicates the systemic use of broad spectrum NF- κ B inhibitors for the treatment of cancer and it has been suggested to design better therapeutics that specifically unleash the pro-apoptotic activity of NF- κ B [15, 16].

NF- κ B in ongoing angiogenesis

NF- κ B signaling has been found to regulate endothelial cell integrity and vascular homeostasis in vivo. Treatment of zebrafish embryos with NF- κ B inhibitors provokes vascular leakage and alters vessel morphology [17]. The role of NF- κ B signaling in tumor angiogenesis has also been recently investigated. Inoculated tumors grow faster in transgenic mice expressing mutated I κ B α , under control of the Tie-2 promoter, resulting in endothelial repression of NF- κ B [18]. 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- κ B in tumor angiogenesis, indicating an inhibitory role for NF- κ B in tumor angiogenesis. Based on this study, NF- κ B activation in endothelial cells appears to be a way to block angiogenesis. However, it is unknown how specific activation of NF- κ B in endothelial cells can be realized and through which mechanisms NF- κ B activation leads to inhibition of angiogenesis.

Angiogenesis occurs in a coordinated series of steps, which can be divided into a destabilization, a proliferation, and a maturation phase. Whereas inhibition of angiogenesis

can prevent diseases with excessive vessel growth such as cancer, diabetes retinopathy, and arthritis, stimulation of angiogenesis would be beneficial in the treatment of diseases such as coronary artery disease and critical limb ischemia in diabetes [19]. 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- κ B in the regulation of these systems is well documented. In line with a pro-oncogenic activity, NF- κ B promotes expression of several matrix metalloproteinases (MMPs), including MMP-2, -3, and -9 [20–22]. However, NF- κ B could also inhibit endothelial cell migration via the up-regulation of tissue inhibitors of metalloproteinase-1 (TIMP-1) as described in astrocytes [23]. 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 [24]. In endothelial cells, it has been described for both reactive oxygen species as well as for TNF- α induced expression of PAI-1 via NF- κ B [25]. In addition, activation of NF- κ B by TNF- α can also lead to the inhibition of the tPA expression [26]. These data suggest that NF- κ B activation could impair angiogenesis via a decrease in ECM degradation capacity.

Integrins are the principle adhesion receptors used by endothelial cells to interact with the extracellular environment and are necessary for cell migration, proliferation, and survival [27]. It has recently been demonstrated that the interaction of α V β 3-integrin with the ECM activates NF- κ B by activation of the IKK complex and degradation of I κ B- α . This activation triggers a pro-survival signal for example in rat aortic endothelial cells [28].

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 [29]. Activation of NF- κ B in endothelial cell leads to the expression of angiogenic and angiostatic factors. VEGF expression is up-regulated by hypoxia-induced mitogenic factor through activation of the NF- κ B pathway [30]. On the contrary, vascular endothelial growth inhibitor (VEGI, reported to inhibit endothelial cell proliferation) has also been found to be induced by NF- κ B [31]. In addition, the promoters of thrombospondin-1 and -2, which are among the first naturally occurring angiostatic agents discovered, contain NF- κ B binding sites [32].

The role of NF- κ B in the cell cycle progression has been also investigated [33]. NF- κ B induces expression of activators of the cell cycle such as cyclin D or -E [34] as well as expression of inhibitors such as p21/cip1 [35] demonstrating that the overall effect of NF- κ B on cell proliferation is difficult to predict. To our knowledge, there are no reports on a direct relationship between NF- κ B activation and proliferation in endothelial cells.

Programmed cell death or apoptosis occurs mainly 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. As previously described for tumor cells, diverse activities have been observed in endothelial cells. Silibinin, a cancer chemopreventive agent, was found to induce apoptosis in endothelial cell line by inhibiting NF- κ B [36] and a report describes that pro-survival effect of VEGF is mediated by NF- κ B activation [37]. However, a large body of evidence exists that NF- κ B activation plays a pro-apoptotic role in endothelial cells. A high concentration of glucose activates the production of reactive oxygen species and induces caspase-3 activation in endothelial cells [38]. Recently, it has been demonstrated that this is mediated via NF- κ B and subsequent c-Jun N-terminal protein kinase activation [39]. In human cardiac microvascular endothelial cells, IL-18 induces activation of both the intrinsic and extrinsic apoptotic pathways via NF- κ B activation [40]. Angiopoietin-1 inhibits endothelial cell apoptosis induced by growth factor deprivation. This effect is mediated via the activation of an endogenous inhibitor of NF- κ B, namely A20 binding inhibitor of NF- κ B (ABIN-2) [41]. In addition, in endothelial cells, it was observed that A20 inhibition and subsequent activation of NF- κ B, effectively leads to reduced tube formation in a matrigel assay [42].

A role for NF- κ B in angiostatic therapy?

The observations described above indicate that NF- κ B is involved in the regulation of migration and/or proliferation/survival of endothelial cells and suggest a strong link between NF- κ B in angiostatic agent signaling. The following section will highlight the role of NF- κ B 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- κ B activation [29].

Platelet factor-4 (PF4) is an α -chemokine naturally secreted by platelets and is known to inhibit angiogenesis [43]. PF4 promotes the expression of E-Selectin in HUVEC. Data provide direct evidence that the NF- κ B-binding site is required for PF4-mediated activation of the

E-selectin promoter. In addition, EMSA experiments demonstrate that PF4 treatment of HUVECs results in binding of NF- κ B to the E-Selectin promoter already after 1 hour of stimulation [44]. The angiostatic properties of angiostatin, a cleavage product of plasminogen, have also been linked with NF- κ B. Chen et al. 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 not only in growth, apoptosis, migration but also in inflammation [45]. Even though no direct evidence points a role of NF- κ B, angiostatin promotes mRNA expression of RelB as well as many NF- κ B 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, see reference [45]). Based on these data, there is a strong suggestion that NF- κ B is also activated by angiostatin. The 16 kDa N-terminal fragment of prolactin (16 K PRL) is a potent angiostatic agent in various *in vivo* models and has been shown to inhibit endothelial cell migration and proliferation [46–48]. We have demonstrated that NF- κ B activation is required for 16 K hPRL-induced caspase-8 and -9 activation and subsequent apoptosis [49]. In addition, it is interesting to note that NF- κ B activation appears to be a very proximal event. 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- κ B dependent [50]. Finally, administration of statins has been shown to decrease tumor growth and angiogenesis [51]. Statins up-regulate the expression of endothelial and inducible nitric oxide synthase through NF- κ B activation [52].

Based on all these reports, it is suggested that the activity of angiostatic compounds is dependent on activation of NF- κ B. Therefore, activation of NF- κ B specifically in endothelial cells might be an attractive therapy. TNF- α is one of the most potent NF- κ B activators. The clinical use of TNF- α as an anti-cancer drug is limited to local treatment (e.g., isolated limb perfusion) because of its systemic toxicity [53]. 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 [54]. These studies indicate that NF- κ B activation, specifically in endothelial cells, can be an efficient strategy for the treatment of cancer.

In conclusion, while in tumor cells NF- κ B is mostly described as an oncogenic factor, up-regulation of NF- κ B in endothelial cells is associated with angiostatic activity. It

might therefore be warranted to revisit anti-cancer therapies based on inhibition of NF- κ B activity for effects on angiogenesis.

An indirect anti-tumor activity of NF- κ B through circumvention of endothelial cell anergy

Next to a direct anti-tumor activity of NF- κ B through inhibition of tumor angiogenesis, the activation of NF- κ B 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 wall, and thus have an important role in the selection of an inflammatory infiltrate [55].

The observation of a reduced number of infiltrated leukocytes in the tumor [56] has been correlated with the fact that tumor endothelial cells display a reduced expression of adhesion molecules (ICAM-1 and ICAM-2) as compared with normal endothelial cells [57, 58]. *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 [59]. It has been recently described that bFGF down-regulates ICAM-1 expression via NF- κ B inhibition [60]. Furthermore, we have demonstrated that suppressed leukocyte-vessel wall interactions in tumor vessels can be normalized by angiostatic compounds, such as endostatin, angiostatin, anginex, and 16 K hPRL, as well as by treatment with chemotherapeutic agents [61, 62]. This normalization has been correlated with up-regulation of ICAM-1, VCAM-1, and E-selectin in endothelial cells [44, 63]. Therefore, activation of NF- κ B by angiostatic therapy does not directly affect angiogenesis but also has an indirect effect via expression of adhesion molecules and subsequent reversal of endothelial cell anergy. Therefore, such activation of NF- κ B resulting in stimulation of anti-tumor immunity but also in inhibition of angiogenesis clearly results in an anti-tumor outcome.

Where to go from here?

Inhibition of angiogenesis is a promising therapeutic approach to fight cancer. From several recent findings it is likely that activation of NF- κ B is a common mechanism of angiostatic agents, resulting in both inhibition of

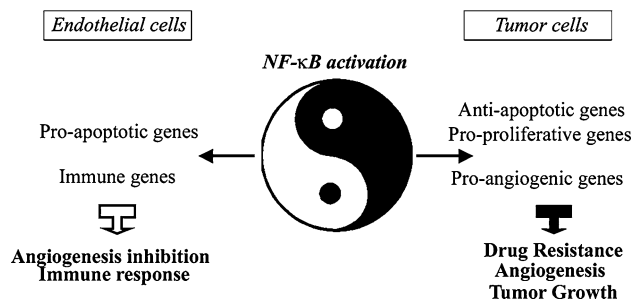


Fig. 2 Model of the dual role of NF- κ B in tumorigenesis. In tumor cells, activation of NF- κ B leads to tumor growth by both direct and indirect mechanisms. The direct mechanisms involve expression of anti-apoptotic and pro-proliferative molecules while the indirect mechanism involves promotion of angiogenesis. In endothelial cells, activation of NF- κ B can 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

angiogenesis and stimulation of anti-tumor immunity. While these data raised a cautionary note about the pharmaceutical agents that block NF- κ B, they also suggest that a targeted activation of NF- κ B, specifically in endothelial cells, could represent a new and promising strategy in cancer treatment (Fig. 2). Further studies remain necessary to fully understand the molecular mechanisms induced by angiostatic agents and the role of NF- κ B in endothelial cells.

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