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Review

Nuclear factor κB: a potential therapeutic target in atherosclerosis and thrombosis

Claudia Monaco, Ewa Paleolog*

Faculty of Medicine, Kennedy Institute of Rheumatology, Imperial College, Arthritis Research Campaign Building, 1, Aspenlea Road, London W6 8LH, UK

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Abstract

Cardiovascular diseases are the leading cause of morbidity and mortality in Western countries. Atherosclerosis, the background for many cardiovascular diseases, is characterized by the accumulation of lipid and fibrotic entities in large arteries and bears many similarities with chronic inflammatory diseases such as rheumatoid arthritis. Common features include extravasation of blood-derived leukocytes, as well as production of cytokines, chemokines and matrix-degrading enzymes. There are also many shared signaling pathways, including activation of the nuclear factor κB (NFκB) cascade. In the context of atherosclerosis, there are a range of candidate stimuli which can activate NFκB, including traditional risk factors, infectious agents, cytokines and cell–cell contact. Many inflammatory genes relevant to the pathogenesis of atherosclerosis are regulated by NFκB, the activated form of which is present in atherosclerotic plaques. Thus, it is essential to understand the role of this important signaling cascade in atherosclerosis, in a quest for more specific therapeutic targets.
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Keywords: Atherosclerosis; Thrombosis; Inflammation; NFκB

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1. Introduction

Cardiovascular diseases are the leading cause of death in developed countries and progressively increasing their impact on mortality in developing countries despite changes in lifestyle and the use of preventative pharmacological approaches. Atherosclerosis is the common pathological substrate underlying cardiovascular diseases. One of the first steps in atherogenesis is activation of vascular endothelium, which leads to recruitment of blood-borne leukocytes, such as monocytes and T lymphocytes. Once monocytes are recruited to the artery wall, these differentiate into macrophages and/or lipid-laden foam cells after lipid engulfment. This macrophage and T-cell infiltrate is a typical feature of the so-called 'fatty streaks,' proposed to represent an early stage of atherogenesis. This is followed by smooth muscle cell migration from the media to the intima, with subsequent proliferation and deposition of extracellular matrix, and organization of lesions into 'mature' plaques. At this stage, in humans plaques are typically characterized by a fibrous cap covering a raised lesion inside the internal elastic lamina composed of fibrous tissue, with or without a core filled with macrophage- and smooth muscle cell-derived foam cells and extracellular lipid deposits, causing a variable degree of stenosis. Thrombosis superimposed on an atherosclerotic plaque is responsible for acute disease complications, most frequently on non-stenotic lesions. Many pathological substrates have been described to underlie this event (plaque rupture, erosion, calcified nodule), and a complex array of mechanisms—platelet activation, tissue factor (TF) expression leading to activation of coagulation, matrix metalloproteinase (MMP) expression and activation leading to fibrous cap thinning and rupture, pro-inflammatory mediators—have been implicated [1].

In the past decade, a deeper understanding of the contribution of inflammation and immune responses to the pathogenesis of atherosclerosis has redefined atherosclerosis as an inflammatory disease [2]. Indeed, it is now apparent that atherosclerosis and chronic inflammatory diseases such

* Corresponding author. Tel.: +44-20-8383-4481; fax: +44-20-8383-4499.

E-mail address: e.paleolog@imperial.ac.uk (E. Paleolog).

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as rheumatoid arthritis (RA) display many common features, including extravasation of leukocytes and production of cytokines, chemokines and MMP. With relevance to this review, atherosclerosis and inflammatory diseases involve activation of nuclear factor κB (NFκB), which is now considered to be a major, if not the major, transcription factor regulating many functions of the vessel wall. Moreover, NFκB activation is thought to lie downstream of many of the stimuli proposed to be involved in atherosclerosis, such as modified lipoproteins (LDL), cytokines and infectious agents.

2. The NFκB signaling pathway

NFκB comprises a family of transcription factors first described as B-lymphocyte-specific nuclear proteins, essential for transcription of immunoglobulin kappa (κ) light chains. Mammalian cells contain five NFκB subunits—relA (p65), relB, c-rel, p50 and p52—which form homo- and heterodimers and are characterized by the conserved N-terminal 'rel homology' domain. NFκB is sequestered in the cytoplasm with members of the inhibitor of NFκB (IκB) family, which consists of IκBα, IκBβ, IκBε and Bcl-3 [3]. In the canonical activation pathway, liberation of NFκB from the inactive complex is initiated by phosphorylation of IκB on N-terminal serines. Phosphorylated IκBs are recognized by an E3 ubiquitin kinase complex and degraded by the 26S proteasome. Amino acid residues Ser-32 and Ser-36 of IκBα were identified as essential for phosphorylation whereas Lys-21 and Lys-22 for the ubiquitination process. IκB degradation leads to the exposure of a nuclear translocation sequence of the NFκB dimer, allowing its nuclear translocation and DNA binding [4].

Central to the NFκB cascade is the multi-subunit kinase IκB kinase (IKK) complex [5], which includes IKK-α (IKK-1) and -β (IKK-2) as well as regulatory subunits such as NEMO/IKK-γ and IKAP [6,7]. IKK-2 was shown to have a higher kinase activity for IκBα and to be the predominant kinase responsible for the phosphorylation of IκBα in response to tumor necrosis factor α (TNFα), interleukin (IL)-1, lipopolysaccharide (LPS) and double-stranded RNA [8–10]. IKK-2 knockout mice die as embryos and show massive liver degeneration due to hepatocyte apoptosis, a phenomenon similar to that of mice deficient in relA or IκBα. NFκB activation by IL-1 or TNFα is strongly impaired although not completely abolished. On the other hand, IKK-1 knockout mice have many morphogenetic abnormalities, including shorter limbs and skull, a fused tail, and die perinatally. They have hyperproliferative epidermal cells that do not differentiate, but IL-1- and TNFα-induced NFκB activation in embryonic fibroblasts is normal, as is IκB phosphorylation and degradation. This suggests that IKK-2 is crucial for NFκB activation upon inflammatory stimuli, but also that IKK-1 or presently unknown kinases may contribute to this action.

Activation of the IKK complex is thought to be mediated by phosphorylation of IKK-1 or IKK-2 by upstream kinases, including members of the mitogen-activated protein kinase kinase kinase family or NFκB inducing kinase (NIK) [11]. NIK, in particular, has reported to play a major role in NFκB activation [3]. However, recent studies in NIK-deficient mice and human primary cells have questioned its physiological role in NFκB activation and have suggested that its function may be restricted to signaling through the lymphotoxin B receptor [12].

3. Regulation of the NFκB pathway

Although many stimuli have the potential to activate the NFκB pathway, the responses elicited are both cell and stimulus specific, suggesting that not all activators utilize the same signaling components and cascades. There are several levels of control and diversification. For instance, the spectrum of adaptor proteins and kinases differs between different stimuli and receptors—for example, adaptors activated via Toll-like receptors (TLR) and IL-1 receptors are distinct from those recruited by TNF receptors. IκB kinases are also an important level of control, in that IKK-1 regulates mostly morphogenetic events, whereas IKK-2 is involved in inflammatory signaling. Moreover, there is heterogeneity of requirement of IKK-2 in different cell types and in response to stimuli [13]. Novel IκB kinase complexes have been recently identified, including IKK-i (IKK-ε) [14] which shares 30% overall identity with IKK-1 or IKK-2. Differential binding by NFκB dimers is another important level of control in this versatile pathway. NFκB consensus binding sites are decameric sequences of NFκB (5′-GGGRNNYYCC-3′, where R indicates A or G, Y indicates C or T and N indicates any nucleotide), or κB-like motifs (5′-HGGARNYYCC-3′ where H indicates A, C or T, R indicates A or G, Y indicates C or T and N indicates any nucleotide) [15]. Different NFκB dimers exhibit different binding affinities for NFκB or κB-like sites (reviewed in Refs. [16–18]). For example, the NFκB sequence contained in some MMP genes allows predominantly binding of p50/p65 [19], while other NFκB dimers (c-Rel/p50) are involved in regulation of other mediators (such as TF, whose promoter contains a κB-like site). In addition, while all five NFκB subunits contain the 'rel homology' domain, only relA and c-Rel contain a transactivation domain. Indeed, there is growing evidence that the p50/p50 homodimer, lacking transactivating potential, may inhibit gene transcription [20]. The major domain sensitive to phosphorylation is the transactivation domain located in the NFκB C-terminal region [21]. Both stimulatory and inhibitory phosphorylations of relA have been reported. Phosphorylation of Ser-927 within the p105 C-terminal PEST region by IKK has been reported to contribute to NFκB activation [22]. Several upstream kinases have been implicated in the transactivating event, including phosphatidyl inositol 3-kinase, p38 mito-

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gen-activated protein kinase (MAPK) and p42/44 MAPK [23]. Hence, it is the differential expression of NFκB components in tissues, cell types and possibly diseases, together with differential interactions with the transcription apparatus that contributes to coordinated regulation by NFκB of complex cellular responses.

Another mode of specificity in NFκB-dependent gene activation lies in its ability to orchestrate gene expression in concert with other transcription factors. For instance, the organization of the cytokine-inducible element in the E-selectin promoter is remarkably similar to that of the interferon-β gene, in that both require NFκB, ATF-2 and HMG-I(Y) [24], whereas another adhesion molecule, vascular cell adhesion molecule-1 (VCAM-1), is induced through interactions of NFκB with IRF-1 and HMG-I(Y) and also depends on constitutively present SP-1[25]. The ability of NFκB to interact with AP-1 is of particular importance, as many of the inflammatory genes require these two transcription factors working cooperatively, including VCAM-1, IL-8, cyclooxygenase (COX)-2, monocyte chemoattractant protein-1 (MCP-1) and MMP-13 [26–28].

A peculiarity of NFκB is the rapid nature of its activation and downregulation. NFκB activation induces IκBα, allowing switching off of the system. Hence, in physiological conditions, NFκB activation is a transient phenomenon, which allows appropriate expression of immune and 'stress' genes. In contrast, prolonged or inappropriate activation of the NFκB pathway is a feature of diseases such as RA, asthma and inflammatory bowel disease, where its disregulation may cause the enhanced inflammatory response associated with these conditions. NFκB is now also thought to play an important role in the pathogenesis of atherosclerosis and acute coronary syndromes.

4. NFκB activation in atherosclerosis

Activated NFκB has been identified in situ in human atherosclerotic plaques. Brand et al. detected nuclear translocation of NFκB subunit relA in the intima and media of atherosclerotic lesions, in smooth muscle cells, macrophages, endothelial cells and, to a lesser extent, T cells [29,30]. In situ analysis of p50 and relA in normal vessel walls revealed diffuse cytoplasmic expression, but no nuclear accumulation, suggesting that the system is quiescent. NFκB activation seems to be more prominent in acute complications of atherosclerosis, such as acute coronary syndromes. Nuclear NFκB binding activity has been found in peripheral blood mononuclear cells [31] and myocardial biopsies [32] of patients with unstable angina. Nuclear translocation of relA is higher in unstable coronary atherectomies, but also present in stable angina patients [33].

Despite these reports in human atherosclerosis, the majority of our knowledge has been gathered from studies using animal models of atherosclerosis. Activated NFκB was detected in coronary arteries of pigs fed a hypercholesterolemic diet [34] and in arterial smooth muscle cells after balloon injury in a rat model [35]. In LDL receptor (LDL-R) knockout mice, expression of relA, IκBα and IκBβ was 5- to 18-fold higher in a region of ascending aorta and arch highly predisposed to atherosclerotic lesion formation. However, nuclear translocation of relA was only found after initiation of an atherogenic diet or, more prominently, after systemic injection of LPS, and even then, only in regions predisposed to atherosclerosis [36].

As discussed previously, IKK-2 is the main component of the IKK complex. Bone marrow transplantation of LDL-R-deficient mice with macrophages lacking IKK-2 increased atherosclerotic lesion size, with more infiltrates in early lesions and more necrosis in advanced stages of disease [37]. The same group also studied mice targeted in NFκB p50, which lacks the transactivation domain and can heterodimerize with p65 to form the most common NFκB dimer, but can also form homodimers, which can inhibit signaling [20]. Studies using bone marrow transfers from p50-deficient mice to LDL-R-deficient mice showed a reduction in lesion size, but a surprising shift towards a different plaque phenotype, characterized by reduced foam cell numbers and an increase in macrophages and T cells, and the appearance of B cells (traditionally not present at the lesion site) [38].

These very recent targeted gene deletion studies have highlighted that the involvement of NFκB in atherogenesis is far more complex than expected. More research is therefore needed to identify the key stimuli leading to NFκB B activation and the signaling involved, in order to identify suitable therapeutic targets, less likely to interfere with the entirety of the pathway.

5. Candidate stimuli for NFκB activation in atherosclerosis

In the context of atherosclerosis, there are many stimuli with the potential to activate NFκB, including local factors such as vascular injury, as well as modified LDL, infectious agents and cytokines, reflecting the multifactorial pathogenesis of atherosclerosis. It is difficult to determine which of these stimuli are responsible for activation of NFκB in vivo, and indeed, NFκB may in fact be a convergence point, integrating these different stimuli throughout the lifetime of an individual (Fig. 1).

5.1. Local factors

The prototypical stimulus initiating atherogenesis is vascular injury [39,40]. Once endothelium is denuded, platelet adhesion and activation are thought to occur, leading to vascular smooth muscle cell migration and proliferation. These events may involve NFκB activation. Using a balloon catheter injury model in the rat carotid artery, low

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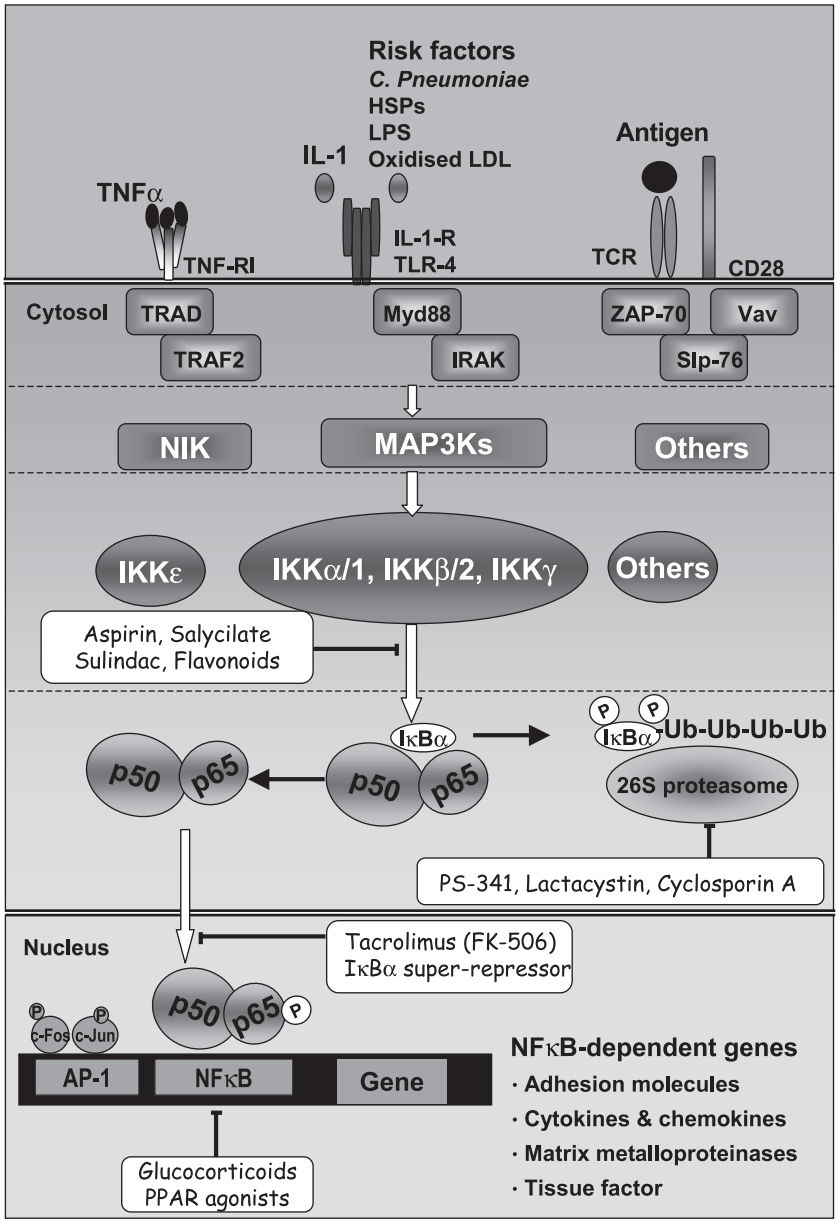

Fig. 1. NFκB signaling in atherosclerotic lesions—effect of available NFκB blocking agents. The NFκB pathway is a convergence point for many pro-atherogenic stimuli. In the model illustrated, oxidized LDL, LPS and HSPs from infectious agents, as well as cytokines, and activated T cells, interact with different receptors at the cell surface. These, in turn, activate intracellular signaling proteins via a set of adaptor proteins and kinases such as NIK, initiating a cascade of phosphorylations. Central to the NFκB cascade is the large multi-subunit IKK complex, which is the point of convergence of multiple signals. Genes regulated by NFκB include adhesion molecules, chemokine, MMP and cytokines, which play pivotal roles in atherosclerosis. The diagram also shows the NFκB inhibitors currently available. Other agents which might inhibit NFκB, such as statins, have not been included in this diagram as their mechanisms of action on the pathway have not been clarified.

levels of constitutively activated p50, relA and c-Rel were shown in normal carotid arteries, but immediately after injury, levels of IκBα and IκBβ were dramatically reduced and macrophage infiltration, expression of VCAM-1 and MCP-1 occurred [35]. More recently, IκBα adenovirus was applied in a rabbit iliac artery restenosis model and shown to reduce intercellular adhesion molecule-1 (ICAM-1) and MCP-1, as well as reducing recruitment of macrophages and lumen narrowing [41]. Similar results were obtained using NFκB decoy oligodeoxynucleotides [42]. It is now recognized that hypoxia may also contribute to development of cardiovascular disease. The adventitia and outer media of large- and medium-sized arteries are vascularized by vasa vasorum, which provide oxygen and nutrients to the external two-thirds of the vessel wall. In atherosclerosis, the augmented arterial wall thickness may lead to hypoxia, and indeed, there have been reports of adventitial neovascularisation of vasa vasorum in experimental models of athero-

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sclerosis, which could result from hypoxia-induced angiogenesis [43,44]. A key regulator of oxygen homeostasis is hypoxia inducible factor (HIF)-1, levels of which are regulated through a mechanism involving oxygen-dependent proteolysis of HIF-α [45]. In hypoxic cells, HIF-α degradation is suppressed, allowing HIF-α to accumulate within the nucleus, resulting in transcriptional activation of cytokines such as vascular endothelial growth factor (VEGF). However, hypoxia is also thought to activate NFκB, albeit through an atypical pathway, involving degradation-independent phosphorylation of Tyr-42 in the N-terminal domain of IκBα, which may prevent interactions with the IKK complex and inhibit phosphorylation of Ser-32 and Ser-36 [46].

5.2. Modified low-density lipoproteins

Lipids represent a key component of the atherosclerotic plaque. LDL retained in the intima undergo oxidative modifications resulting in the production of oxidized LDL. Minimally oxidized LDL stimulate endothelial cells to produce NFκB-dependent chemokines and adhesion molecules [47]. In a study of LDL oxidation in vivo [48] when human LDL particles were injected, these localized in the arterial wall and underwent oxidative modification accompanied by activation of endothelial NFκB and expression of NFκB-dependent genes. However, in vitro evidence has shown that the regulation of NFκB by LDL is complex and often dependent on incubation times and concentrations used. For example, short-term exposure of monocytes to oxidized LDL activates NFκB, but longer exposure may suppress NFκB-dependent responses [49]. Pretreatment with oxidized LDL results consistently in many in vitro systems in inhibition of LPS-induced NFκB activation [50,51]. Moreover, modified LDL contain multiple constituents whose identity and effects on signaling have been only partially unraveled. Some components of oxidized LDL such as lysophosphatidylcholine can activate NFκB in endothelial cells [52]. In contrast, an inhibitory effect on NFκB has been proposed for 4-hydroxynonenal, one of the most abundant aldehydes formed during oxidation of LDL, which has been reported to inhibit NFκB-dependent transcriptional activation of inducible nitric oxide (NO) synthase (iNOS) in smooth muscle cells [53]. Similarly, oxidized PAPC (oxidized 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphocholine, a component of minimally oxidized LDL) has been described as inhibiting NFκB binding to DNA [54].

5.3. Metabolic factors

Although the mechanisms are uncertain, it is thought that homocysteine creates oxidative stress by altering the redox thiol status of the cell [55]. A consequence of these effects is the activation of NFκB. In smooth muscle cells, homocysteine leads to both an increase in NO production and an NFκB-mediated increase in the expression of iNOS [56]. Advanced glycation end products (AGE), formed through non-enzymatic reactions of reducing sugars with the amino groups of proteins, nucleotides and lipids, have recently attracted an interest as candidate stimuli. Accumulation of AGE has been found in atherosclerotic plaques [57], and expression of RAGE (receptor for AGE) in endarterectomy specimens has been described [58]. AGE are thought to activate endothelial cells and monocytes through NFκB [59,60].

5.4. Microbial products and microbial agents

A pathogenic role for infectious agents in atherosclerosis has been suggested. For example, *Chlamydia pneumoniae* express molecules such as LPS and heat shock proteins (HSP), which potently activate the innate immune response. Initial recognition of microbial antigens is mediated by TLR, and currently, at least nine different TLR have been identified in mammals, each one with a certain degree of specificity. For example, TLR-4 is essential for signaling via LPS from Gram-negative bacteria, exceptions being *Leptospira interogans* and *Porphyromonas gingivalis*, recognized by TLR-2. LPS is thought to activate endothelial cells through TLR-4, and possibly TLR-2, to release IL-6 and IL-8 via an NFκB-dependent mechanism involving IKK-2 [13]. This is likely to involve an adaptor molecule, MyD88, the N-terminal region of which contains a death domain (DD), which recruits of IL-1 receptor-associated kinase (IRAK)-1 and -2. These kinases, in turn, recruit TNF receptor-associated factor (TRAF)-6, and signaling progresses through IKK to NFκB B activation [61].

In addition to LPS, however, there are other TLR ligands which could be relevant to the pathogenesis of atherosclerosis. *C. pneumoniae* HSP60 signals through TLR-4 [62], and endothelial and smooth muscle cells infected with *C. pneumoniae* express cytokines and TF in parallel with IκBα degradation and p50/p65 translocation [63]. TLR-4 also recognizes viral and plant products, including taxol (a plant product possessing LPS-mimetic effects on murine cells) and F protein from syncytial virus [64]. TLR-2 recognizes a variety of microbial products, such as peptidoglycan, zymosan (a yeast cell wall component) and glycosylphosphotidylinositol lipid anchor from *Trypanosoma cruzi*. Expression of TLR is low in normal arteries, but TLR-1, -2 and -4 are augmented in human atherosclerotic lesions. Human adventitial fibroblasts, which express TLR-4, respond to LPS by activating NFκB [65]. Both TLR-2 and TLR-4 frequently co-localize with p65 translocation, although the spatial correlation does not per se, indicate that TLR ligation caused NFκB activation.

The role of TLRs might be even more broad, as also host-derived products, such as fibrinogen and alternatively spliced fibronectin (EDA), as well as endogenous HSP60, could trigger TLR signaling independently from infectious

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agents [64]. Both fibronectin and HSP-60 could be expressed in situations of tissue injury or ischemia.

5.5. Cytokines

The concept that the atherosclerotic environment exhibits inflammatory and immune features is supported by the presence in plaques of pro-inflammatory cytokines, including TNFα [66] and IL-1 [67], both of which activate NFκB. Two types of TNF receptor have been described, p55 and p75 (TNF receptor 1 or CD120a and TNF receptor 2 or CD120b). The p55 TNF receptor has an intracellular DD and is required for TNFα-induced apoptosis. TNFα signaling through p55 can also induce NFκB activation. These signals have been shown to bifurcate at the level of TNF receptor-associated death domain protein (TRADD), where TRADD interacts with FADD to transduce the apoptotic signal and with TRAF-2 to induce NFκB activation. The p75 TNF receptor does not possess a DD but seems to form a heterodimeric complex with TRAF-1 and TRAF-2. There are two forms of the IL-1 receptor, which are members of the IL-1 receptor/TLR superfamily. The homology of the intracellular portion of TLR to the intracellular portion of IL-1 type I receptor suggested that these receptors might use an analogous framework for signaling, and indeed, the trimeric complex recruits MyD88 and then IRAK-1 and -2. These kinases, in turn, recruit TRAF-6 and activate NFκB [68]. The potent vasoconstrictor angiotensin II may play a role in atherosclerosis due to its effects on blood pressure and smooth muscle cell growth and has been proposed to act in the plaque as an 'honorary' cytokine. In fact, angiotensin II induces expression of adhesion molecules (VCAM-1, ICAM-1 and E-selectin) and IL-6 in smooth muscle cells at least in part through NFκB [69,70].

5.6. T-lymphocyte-dependent signaling

Analysis of T cells in atherosclerotic plaques reveals a memory CD45RO+ phenotype, expression of activation markers such as HLA-DR, VLA-1, CD69, CD40 ligand and CD25 and consistent enrichment of T-helper 1 cells. Both T-cell receptor (TCR)-dependent and -independent pathways are thought to be involved in the activation of lymphocytes in the atherosclerotic plaque [71]. Antigen-specific T-cell activation depends on TCR interaction with peptides presented by MHC, as well as interactions of co-stimulatory molecules with their ligands. NFκB is likely to play an important role both in T-cell activation and in T-cell-dependent signaling to neighboring cells. Most co-stimulatory molecules activate NFκB, and the first two inducible T-cell genes shown to be regulated by NFκB were those for IL-2 and the α chain of the IL-2 receptor. Moreover, NFκB regulates T-cell signaling to effector cells. Cell contact-mediated activation of endothelial cells and macrophages by T cells leads to expression of cytokines, MMP and TF [71,72]. Different pathways of T-cell activation result in differential responses of the target cells. Our own studies revealed that TCR-activated lymphocytes induce monocyte production of TNFα and the chiefly anti-inflammatory cytokine IL-10. In contrast, cytokine-activated lymphocytes selectively induced high levels of TNFα, but no detectable IL-10, in an NFκB-dependent manner [73]. In atherosclerosis, conditions for generating cytokine-activated T cells (production of TNFα, IL-6, IL-2) are likely to be present, suggesting that T cells may stimulate monocyte/macrophages to release TNFα through activation of NFκB. Molecules involved in contact mediated activation by T cells are likely to include membrane TNFα and CD40 ligand. In human primary cells, IKK-2 is essential for CD40 ligand-induced NFκB activation [13]. In atherosclerosis, T-cell-mediated contact-dependent activation via CD40 might be an important mechanism of perpetuating inflammation in vivo, via activation of NFκB in effector cells [74].

6. NFκB regulates the main features of atherosclerotic plaques

As discussed earlier, many stimuli relevant to the pathogenesis of atherosclerosis have the potential to activate the NFκB signaling pathway, inducing many downstream events (Fig. 1). In essence, all of the cells which can contribute to plaque initiation and progression—endothelial and smooth muscle cells, macrophages and immune cells—are likely to activate the NFκB pathway.

6.1. Activation of endothelium and recruitment of mononuclear cells: early stages of atherogenesis

Vascular endothelial activation is a prominent feature of atherosclerosis and is traditionally regarded as the initial step leading to atherosclerosis. Endothelial cells express transcripts encoding p50/p105, p65 and c-rel, steady-state levels of which are increased by TNFα. It was noted early on that most genes expressed in endothelial cells in response to LPS, IL-1 and TNFα contained NFκB binding sites in their promoter regions [75]. Sequence analysis of the 5′ flanking region of the E-selectin gene revealed at least three consensus DNA-binding sequences for NFκB [76]. Other examples of NFκB regulated genes include VCAM-1, E selectin, IL-1, IL-6, TF, plasminogen activator inhibitor-1, COX-2 and iNOS. For example, adenovirus-mediated overexpression of IκBα and a dominant negative form of IKK-2 inhibited TNFα-induced expression of E-selectin, VCAM-1 and ICAM-1 [77]. Induction of IL-6, MCP-1 and Groα, as well as TF, was also suppressed. This was associated with reduced arrest, spreading and transmigration of monocytes. Some of these inflammatory mediators, such as IL-1, can also per se activate NFκB, hence creating a positive autoregulatory loop that main-

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tains the activation of NFκB and amplifies the inflammatory response.

6.2. The initiation of the immune response in atherosclerosis: potential role of T cells

In atherosclerosis, candidate antigens for the immune response are thought to include oxidized LDL and HSP. T-cell clones obtained from the human atherosclerotic plaques recognize oxidized LDL as a classical, HLA DR-dependent antigen [78]. ApoE knockout mice exhibit a modified form of LDL in aortic lesions and elevated serum levels of antibodies against modified LDL [79]. Crucial to the presentation of antigens and the activation of antigen-specific T cells are dendritic cells (DC) that constantly circulate from the tissues to the secondary lymphoid organs, where they activate T cells and initiate adaptive immunity to the antigens recognized. DC have been found in human atherosclerotic lesions [80]. One of the major signals involved in the regulation of this process is likely to be CD40–CD40 ligand interactions between DC and T cells. CD40 ligand expressed by T cells activates DC and enhances DC T-cell stimulatory ability, by inducing cytokine production and upregulating antigen-presenting and co-stimulatory molecules [81] in a process that requires IKK-2 but not NIK. In contrast, activation of DC by LPS does not require either IKK-2 or NIK [82].

6.3. Monocyte/macrophages in atherosclerosis: key players in the inflammatory process

Macrophages resident in atherosclerotic lesions are derived from blood-borne monocytes [83] and express cytokines, chemokines and growth factors, such as TNFα, IL-6, IL-8 and MCP-1 [66,67,84]. The requirement for NFκB is complex and varies according to the stimulus and the species studied. For example, we showed that overexpression of IκBα in human macrophages inhibited LPS-induced production of TNFα, IL-1, IL-6 and IL-8, but not of anti-inflammatory cytokines IL-10 and IL-11. Interestingly, not all stimuli use NFκB to upregulate pro-inflammatory cytokines, in that responses to the yeast cell product zymosan were unaffected [85]. More recently, we showed that dominant negative IKK-2 adenovirus did not inhibit LPS-induced cytokine (TNFα, IL-6 and IL-8) production [13], although VEGF expression was reduced. Release of cytokines and VEGF from CD40 ligand-activated macrophages did, however, require IKK-2 [13,86].

In contrast to human monocyte/macrophages, in vitro studies showed that p50-deficient bone marrow-derived macrophages show reduced LPS-induced endocytosis of modified lipoproteins and prolonged TNFα secretion, as well as an increase in IL-10 and IFNγ, but a reduction in MCP-1, IL-6 and IL-12. The deletion of p50, which has been proposed to have an anti-inflammatory role, is associated with smaller atherosclerotic lesions, but a more inflammatory phenotype [38]. In addition, in contrast to human cells, deletion of IKK-2 in murine macrophages was associated with reduced TNFα, IL-6 and IL-10 production, in the absence of any modification of LPS-induced modified LDL uptake [37]. IKK-2 deletion in this mouse model increased atherosclerotic lesions, with more prominent necrosis. It is possible to speculate that NFκB may be involved in vivo in the resolution of inflammation. In a model of acute lung inflammation, NFκB activation in leukocytes during the onset of inflammation was associated with pro-inflammatory gene expression, whereas such activation during the resolution of inflammation was associated with the expression of anti-inflammatory genes and induction of apoptosis, that per se has a less pro-inflammatory impact than necrosis [87]. The consequences of NFκB blockade seem at this stage far more complex than can be foreseen, due to the central role of NFκB in several key cellular functions. Further studies in human cells are needed to assess the best therapeutic targets within the pathway. Critically, different features of atherosclerotic lesions between humans and animal models, together with differences in regulation of pro-inflammatory and anti-inflammatory cytokines upon NFκB blockade between human and animal models, point towards the need for more evidence to be gathered in the setting of human disease.

6.4. Smooth muscle cell responses in atherosclerosis and restenosis: life and death in the atherosclerotic plaque

Smooth muscle cell migration from the media to the intima and proliferation is a trademark of atherosclerotic plaques. Furthermore, neointimal hyperplasia characterized by smooth muscle cell proliferation is the main feature of restenosis after percutaneous interventions. As discussed earlier, vascular injury is a major stimulus for NFκB activation and smooth muscle cell proliferation. NFκB is central to smooth muscle cell proliferation and survival [88,89] via induction of genes with survival and protective functions. Cell survival is regulated by the balance of caspases and members of the inhibitors of apoptosis (IAP) and Bcl-2 families. When caspases are activated, these cleave a variety of proteins, including certain key substrates in the cell, and it is the changes in function of the latter that kill the cell via apoptosis. The functions of the caspases are modulated by the IAPs, including NAIP, c-IAP-1 and -2, XIAP and survivin. Death receptors, such as Fas/CD95 or TNF receptor 1, can trigger caspase activation. The DD of TNF receptor 1 interacts with TRADD and thereby with FADD that, in turn, binds pro-caspase-8 and -10. By being brought into proximity with one another, these pro-caspases cleave their nearest neighbors to form active, mature caspases which now efficiently cleave pro-caspase-3 (and other executioner caspases) to allow apoptosis to proceed. Additionally, however, TRAF-1/TRAF-2 heterocomplexes can interact with c-IAP-1 and -2. Signaling in response to TNFα can therefore trigger either the extrinsic apoptotic pathway

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via FADD and caspases, or cell survival through TRAFs/IAPs. There is a close interaction between the IAP and NFκB pathways. As discussed previously, TRAFs are upstream of NFκB activation. Activation by TNFα of a fibrosarcoma line expressing a form of IκBα that cannot be phosphorylated by IKK inhibits caspase-8 activation [90]. Several studies have also demonstrated that overexpression of IκBα in endothelial cells results in TNFα-induced cell death through suppression of IAPs [91]. In smooth muscle cells induction of c-IAP-2 has also been found to be NFκB dependent [92]. Finally, smooth muscle cells are key players in the production and degradation of extracellular matrix within the plaque. The role of NFκB in the induction of transcription of matrix metalloproteinases will be discussed in the next section.

6.5. NFκB activation regulates plaque thrombotic potential: pathophysiological events leading to clinical manifestation of the disease

Thrombosis associated with plaque rupture or erosion underlies most acute complications of atherosclerosis, such as unstable angina and acute myocardial infarction. Several molecules have emerged as leading pathophysiological contributors, including TF, the major initiator of the coagulation cascade in vivo, MMP, which degrade collagen fibrils leading to loss of fibrous cap integrity, and pro-inflammatory cytokines, which promote infiltration and activation of inflammatory cells, as well as inducing TF and MMP. Expression of these mediators is under the control of transcription factors such as NFκB.

TF, a member of the cytokine receptor superfamily, acts as essential co-factor for factor VII/VIIa to form a complex which cleaves factors IX and X, thereby activating the coagulation cascade [93]. Several studies have demonstrated that endarterectomy specimens from patients with unstable angina contain more TF than those from patients with chronic stable angina [94]. In human atherosclerosis, TF is expressed mainly by macrophages, smooth muscle cells and endothelial cells that cover the plaque and in the extracellular matrix. The human TF promoter contains a non-consensus NFκB binding site, with a one-base difference to the consensus NFκB binding site [95]. Protease inhibitors of the chloromethylketone class prevent LPS activation of TF transcription in human monocytic cells, possibly preventing degradation of IκB [96]. Pyrrolidine dithiocarbamate, a more specific inhibitor of the NFκB pathway, has the same effect on TF synthesis in endothelial cells induced by LPS, PMA, TNFα and IL-1β [97]. Overexpression of IκBα or a dominant negative form of IKK-2 inhibits TF in endothelial cells [98]. In monocytes/macrophages, the intracellular signaling pathways regulating TF have not yet been explored, but NFκB is a good candidate.

A key event leading to loss of fibrous cap integrity is overexpression of matrix-degrading enzymes and, thus, reductions in proteins such as collagen. Macrophages in human plaques constitutively express MMP-1, -3 and -9 [99]. As discussed previously, release of MMP is regulated by NFκB, but this may depend on the cell type and stimulus involved. In fibroblasts, IκBα overexpression has been shown to inhibit MMP-1, 3 and -13, but not the inhibitor TIMP-1 [85,100]. In human and rabbit smooth muscle cells IL-1 activates NFκB to upregulate expression of MMP-1, -3 and -9 [101]. However, spontaneous secretion of MMP-9 by human macrophages does not appear to involve NFκB, although MMP-1 secretion in response to CD40 ligand was inhibited by IκBα [102]. Another stimulus relevant to atherosclerosis, namely, oxidized LDL, has been found to increase macrophage MMP-9, associated with increased nuclear binding of NFκB and AP-1 [103].

7. Uncharted territory: therapeutic strategies for NFκB inhibition

Several agents already safely used in clinical practice have been recently shown to have properties which go beyond their traditional pharmacological action. These 'pleiotropic' properties include NFκB inhibition, at least in the in vitro setting (Fig. 1).

For example, statins, a group of drugs that act by inhibition of 3-hydroxy-3-methylglutaryl coenzyme A reductase, a rate-limiting enzyme in the cholesterol synthesis pathway, reduce the incidence of coronary events and stroke. Surprisingly, statins reduce mortality with a very modest or null improvement in the percentage stenosis of lesions in humans, suggesting that properties other than lipid lowering may contribute to their benefit. In experimental atherosclerosis, statins reduce macrophage numbers, MMP and TF expression, cytokines and leukocyte adhesion molecules. Furthermore, clinical trials have showed that statins may have a benefit even in patients with relatively low lipid levels but with high C-reactive protein levels [104]. These effects might involve inhibition of NFκB, as in vitro statins were shown to upregulate IκBα in smooth muscle cells and endothelial cells [105,106]. A recent study also reported that cerivastatin can inhibit NFκB activation and induction of MCP-1 and RANTES in smooth muscle cells infected with *C. pneumoniae* [107].

Recent work has also highlighted the role of peroxisome proliferator-activated receptors (PPAR), a subset of the nuclear hormone receptor superfamily. So far, three PPAR isoforms, α, β/δ and γ, have been identified. Fatty acids, leukotrienes and prostaglandins can act as natural ligand agonists for PPAR isoforms. Of interest to this review, agonist-activated PPARα can antagonize the NFκB pathway by interaction with relA [108], whereas PPARγ-specific ligands inhibits expression of cytokines and adhesion molecules through repression of NFκB. Agonist-induced PPARγ can also influence DC maturation through NFκB downregulation [109]. Other drugs which may act through attenuation of the NFκB pathway include anti-hypertensive

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angiotensin-converting enzyme inhibitors such as ramipril and quinapril. Administration of quinapril was found to reduce cytokine levels, macrophage infiltration and NFκB activation in rabbit models of atherosclerosis [110].

Similarly, many of the effects of glucocorticoids, widely used in clinical therapy for their anti-inflammatory and immunosuppressive properties, are thought to be mediated by interactions with the NFκB pathway. One mechanism is induction of IκBα leading to cytosolic retention of NFκB. However, other mechanisms are likely to be involved, such as repression of relA-dependent transactivation [111]. The most common mechanism thought to account for the effects of non-steroidal anti-inflammatory drugs is COX-1 and -2 inhibition. However, additional mechanisms involving NFκB have been suggested. Aspirin and sodium salycilates inhibit activation of NFκB at concentrations found in the sera of patients treated with these agents for chronic inflammatory diseases, although their effect is unknown at lower concentrations, such as those used for platelet inhibition. The effect on NFκB may be mediated by inhibition of IKK-2-dependent phosphorylation of IκB. Studies regarding the FANS in experimental studies on atherosclerosis have reached contrasting results. In a study in LDL-R knockout mice, low-dose aspirin induced a significant reduction of NFκB activity in the aorta [112]. In contrast, concentrations of indomethacin that inhibit COX activity do not prevent activation of the NFκB pathway. Sulindac, a dual COX-1/COX-2 inhibitor, can also inhibit IKK-2 activity [113]. Finally, the immunosuppressive drugs cyclosporin A and tacrolimus (FK-506) inhibit the NFκB pathway through at least two distinct mechanisms. Cyclosporin A inhibits proteasome activity, while tacrolimus blocks c-Rel nuclear translocation [114].

In the future, NFκB signaling could be more selectively interfered at the levels of upstream receptors or adaptors, once signaling pathways specific for atherosclerosis have been identified, minimizing the possible harmful effects of complete NFκB blockade.

8. Conclusion

Atherosclerosis, and, in particular, its acute complications, is a major cause of mortality and morbidity around the world and is likely to continue to increase as a cause of death. Prevention and treatment of cardiovascular disease is still a clinical challenge. Several lines of research have converged to suggest that the biology of the plaque is the real ‘culprit’ of clinical manifestations of atherosclerosis. Inflammatory signaling pathways are implicated in early atherogenesis, in the progression of lesions and finally in the acute complications of the disease. The increased understanding of the role of signaling pathways such as NFκB will lead to the identification of therapeutic targets able to specifically downregulate pro-inflammatory and pro-thrombotic responses in atherosclerotic plaques, increasing the therapeutic options of patients and doctors beyond control of risk factors and treatment of thrombosis and yielding further improvements in outcomes.

While blockade of NFκB could be beneficial in atherosclerosis and thrombosis, there are obvious questions regarding the balance between efficacy and safety, as maintenance of appropriate levels of NFκB activity is critical for immune and inflammatory responses and maintenance of homeostasis. Furthermore, recent studies in mice have suggested that chronic, complete blockade of NFκB could even be harmful and interfere with resolution of inflammation and survival responses in atherosclerotic lesions. As a result, the best strategy to inhibit NFκB activation in atherosclerosis remains to be ascertained. One concern about the use of some NFκB inhibitors is the lack of specificity. For example, the proteasome, which is responsible for IκB degradation, has many other vital functions, and its inhibition causes several side effects. However, growing understanding about the heterogeneity of the proteasome could lead to development of inhibitors specific for the NFκB pathway. Because available inhibitors lack the necessary specificity for tackling NFκB activation avoiding major side effects, there is a need to identify appropriate therapeutic targets within the pathway in order to achieve specific inhibition. Moreover, it may not be feasible to block NFκB for prolonged periods. The best timing for intervention, early vs. advanced stages and/or acute versus chronic phases of disease, needs to be considered, in an attempt to maximize benefits and minimize side effects. Transient NFκB inhibition might be beneficial in acute coronary syndromes [31,33]. Another possibility is local targeting of NFκB during interventional procedures by drug-eluting stents using small molecules inhibitors, RNA interference or gene therapy [41,42]. Finally, further understanding of the signaling events preceding NFκB activation in atherosclerotic plaques will allow the targeting of specific receptors or adaptors within the NFκB pathway.

However, such approaches are still in the realm of the future, and the therapeutic potential of inhibition of the NFκB pathway in atherosclerosis is unknown, with further research needed to clarify clinical benefit. More questions than answers are arising from current research on the feasibility of NFκB blockade in atherosclerosis, and more research is warranted in this fast-moving field before definite conclusions can be reached. Nevertheless, the possibilities offered by a deeper understanding of regulation of inflammatory signaling, including not just NFκB but also other pathways, open up the promise of specific inhibition of disregulated inflammatory mechanisms causing disease.

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