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The Nuclear Factor κB Signal Transduction Pathway

Its role in atherogenesis and intimal hyperplasia

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To my famliy

ABSTRACT

Vascular inflammation is a hallmark of major cardiovascular diseases such as atherosclerosis, and also suggested as a critical component implicated in intimal hyperplasia. Given its central role in regulating expression of inflammatory genes, the nuclear factor κB (NF-κB) signal transduction pathway was postulated to play an important role in these pathological processes.

The aim of the thesis was to elucidate the role of the NF-κB pathway in intimal hyperplasia using a rodent model of carotid artery balloon injury. Specific objectives were to characterize NF-κB activation upon vascular injury and to explore the molecular basis of the inflammatory response, specifically expression and functional relevance of neutrophil gelatinase-associated lipocalin (NGAL), telomerase (TERT) and leukotriene B4 (LTB4) in the vascular repair process.

The present study demonstrates that angioplastic injury to the carotid artery elicited two phases of NF-κB activation characterized by an early activation in the arterial media and a late activation coupled with high levels of inhibitor of NF-κB kinase (IKK) activity in the intima. The early NF-κB activation is crucially involved in the acute inflammatory response in the media, but appears to be dispensable for intimal formation. The late NF-κB activation is critical to the development of intimal lesions, as interruption of IKKβ by overexpressing an adenoviral-mediated dominant negative mutant of IKKβ (dnIKKβ) in the injured artery inhibited the late phase of NF-κB activation, resulting in down-regulation of inflammatory gene expression combined with a reduction of intimal size.

To elucidate the molecular mechanisms linking NF-κB mediated inflammation with vascular repair, NGAL and TERT expression were investigated. NGAL is a member of the lipocalin family, and was recently found associated with increased proteolytic activity in atherosclerotic lesions. TERT, an RNA reverse transcriptase plays a critical role in maintaining cell replication capacity and was shown involved in smooth muscle cell (SMC) proliferation in a genetic hypertension model. Therefore, NGAL and TERT might contribute to intimal hyperplasia by modulating proteolytic activity and maintaining the proliferative potential of intimal SMC, respectively. We demonstrate that both NGAL and TERT are highly induced in conjunction with IKKβ/NF-κB activation in the intima post-angioplasty and in the isolated intimal SMC *in vitro*, but suppressed by dnIKKβ. These results for first time reveal that NF-κB regulates NGAL and TERT expression in intimal SMC following vascular injury. Furthermore, in addition to mono- and homomeric forms, SMC produced NGAL could form a heterodimer with matrix metalloproteinase-9, and is associated with increased proteolytic activity in the intimal SMC, indicating that NGAL could be important in the regulation of proteolytic activity involved in vascular repair. Additionally, pharmacological inhibition of telomerase led to a dose-dependent growth arrest of intimal SMC due to replicative senescence, suggesting that telomerase activity is indispensable for the proliferative capability of intimal cells.

Finally, we assessed the direct effects of LTB4 on vascular SMC. LTB4 is derived from the 5-lipoxygenase metabolism of arachidonic acid, and exerts its action via cell surface receptors denoted BLT1 and BLT2. Our data reveal that SMC express functional BLT1, activation of which induces SMC migration and proliferation, and that up-regulation of BLT1 in SMC by proinflammatory cytokines occurs via activation of an IKKβ/NF-κB dependent pathway. BLT1 was also expressed in human atherosclerotic lesions. Blockade of BLT1 significantly suppressed intimal hyperplasia after angioplastic injury in the rat as well as SMC migration *in vitro*. These results suggest that targeting BLT1 on SMC may represent a novel therapeutic strategy for restenosis after angioplasty.

In summary, the present study demonstrates that NF-κB is an important transcription factor in the vascular inflammatory response and intimal hyperplasia, and that NGAL, TERT and BLT1 in vascular SMC are regulated by NF-κB in these pathological processes. Therefore, NF-κB could be a potential therapeutic target.

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LIST OF PUBLICATIONS

This thesis is based on the following original papers, which will be referred to by their Roman numerals:

I. **Bu DX**, Erl W, de Martin R, Hansson GK, Yan ZQ: IKKβ-dependent NF-κB pathway controls vascular inflammation and intimal hyperplasia, Faseb J 2005, 19:1293-1295.

II. **Bu DX,** Hamdahl AL, Gabrielsen A, Fuxe J, Zhu C, Eriksson P, Yan ZQ: Induction of Neutrophil Gelatinase-Associated Lipocalin in vascular Injury via activation of NF-κB, Manuscript under revision of American Journal of Pathology

III. **Bu DX**, Xu DW, Hou M, Takakura, M, Edelfedt K, Yan ZQ: NF-κB mediated transcriptional activation of telomerase in vascular smooth muscle cells post angioplastic injury, Manuscript

IV. Back M, **Bu DX**, Branstrom R, Sheikine Y, Yan ZQ, Hansson GK: Leukotriene B4 signaling through NF-κB-dependent BLT1 receptors on vascular smooth muscle cells in atherosclerosis and intimal hyperplasia, Proc Natl Acad Sci U S A 2005, 102:17501-17506

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LIST OF ABBREVIATIONS

Adv-β-Gal	adenoviral vectors encoding E. *coli* β-galactosidase
Adv-dnIKKβ	adenoviral-recombinant dominant negative IKKβ
ALT	alternative telomerase-independent pathways
AP-1	activating protein-1
apoE	apolipoprotein E
bFGF	basic fibroblast growth factor
BLT	leukotriene B4 receptor
C-IAP	cellular-inhibitors of apoptosis
COX-2	cyclooxygenase-2
cPLA2	cytosolic phospholipase A2
DKC	dyskeratosis congenital
ECM	extracellular matrix
EDTA	ethylenediaminetetraacetic acid
EMSA	electrophoretic mobility shift assay
5-FLAP	5-lipoxygenase-activating protein
GPCR	G-protein-coupled cell surface receptor
HDAC	histone deacetylation
hnRNP	heterogeneous nuclear ribonucleoprotein
HSP	heat shock protein
hTERT	human TERT
ICAM-1	intracellular adhesion molecule-1
IFN-γ	interferon-γ
IκB	inhibitor of NF-κB
IKK	IκB kinase
IL	interleukin
iNOS	inducible nitric oxide synthase
IP	immunoprecipitation
Jak	Janus family of kinase
LDL	low-density lipoprotein
5-LO	5-lipoxygenase
LPS	lipopolysaccharide
LTs	leukotrienes
MAPK	mitogen-activated protein kinase
MCP-1	monocyte chemoattractant protein-1
MMPs	matrix metalloproteinases
MOI	multiplicity of infection
MTT	3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide

NF-κB	nuclear factor κB
NGAL	neutrophil gelatinase-associated lipocalin
PCNA	proliferating cell nuclear antigen
PDGF	platelet-derived growth factor
PDTC	pyrrolidine dithiocarbamate
PI3K	phosphoinositide 3-kinase
PPAR	peroxisome proliferator activated receptor
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SMC	smooth muscle cells
SRS-A	slow reacting substance of anaphylaxis
STAT	signal transducer and activator of transcription
TEP1	telomerase-associated protein 1
Terc	telomerase RNA component
TERT	telomerase reverse transcriptase
TGF-β	transforming growth factor-β
TIMPs	tissue inhibitors of matrix metalloproteinase
TLR	toll-like receptor
TNF-α	tumor necrosis factor-α
TRF1	telomeric-repeat binding factor 1
TUNEL	terminal deoxynucleotidyl transferase mediated-deoxyuridine triphosphate nick-end labeling
VCAM-1	vascular cell adhesion molecule-1

INTRODUCTION

The normal vessel wall comprises three layers, the intima, media and adventitia (figure 1). The intima consists of a continuous monolayer of endothelial cells seated on a basement membrane. Underneath, separating the intima and media is the internal elastic lamina. The normal media is the muscular layer containing mainly vascular smooth muscle cells (SMC). The adventitia contains mostly connective tissue, fibroblasts capillaries and fat, separated from the outer media by an external elastic lamina. The intima can become thickened naturally with aging, a process called intimal hyperplasia, which is implicated in the pathogenesis of major cardiovascular diseases such as atherosclerosis and restenosis after angioplasty. The underlying causes of intimal hyperplasia are activation, migration and proliferation of SMC, accompanied by accumulation of extracellular matrix (ECM) as an adaptive or repair response to a variety of stimuli acting on the vessel wall. Accelerated proliferation of SMC is now known to play an important role in atherosclerotic lesion progression and in post-angioplasty restenosis[1].

ATHEROSCLEROSIS

Atherosclerosis is the major source of morbidity and mortality in the developed world. It is a multifactorial, slowly progressing disease that typically starts in early life and develops over decades, characterized by lipid accumulation and chronic inflammation in the inner wall of large and medium-sized arteries, especially in areas of arterial branching and non-laminar blood flow[2]. Epidemiological studies have revealed several important genetic and environmental risk factors such as hypercholesterolemia, hypertension, diabetes and cigarette smoking associated with atherosclerosis[3]. It is now clear that atherosclerosis is not simply an inevitably degenerative consequence of aging, but rather a chronic inflammatory condition that can be converted into an acute clinical event by plaque rupture and thrombus formation, giving rise to myocardial infarction, stroke, and vascular occlusive disease of the extremities. Atherosclerotic lesions (atheroma) are asymmetric focal intimal thickenings (figure 1), often develop in a characteristic fashion in three different stages:

1. Fatty streak formation. The formation of fatty streak represents the first microscopically detectable alteration, characterized by lipid-rich macrophages (foam cells), T cells and SMC in varying proportions within the arterial intima[4]. The fatty streak is initiated by the trapping and oxidation of low-density lipoproteins (LDL) into the subendothelial layer, conceivably owing to endothelial damage/dysfunction[5]. It was well recognized that several families of enzymes including cyclooxgenases

(COX), lipoxygenases (LO), NADPH oxidases, nitric oxide synthases (NOS), and peroxidases facilitate LDL oxidation[6], although it remains largely unknown where, how, and to what extent, LDL becomes oxidized during atherogenesis. Nevertheless, this signals a cascade of leukocyte recruitment, further lipoprotein oxidation, and foam cell formation[4]. The fatty streak may be reversible, either never cause symptoms, or progress to atheromatous plaque.

2. Fibrous plaque formation. The fatty streak may progress to form a fibrous plaque, resulting from progressive lipid accumulation and migration, proliferation of SMC. The latter is responsible for the deposition of ECM and form a fibrous cap that overlies a core of lipid-laden foam cells, extracellular lipid, and necrotic cellular debris. Proinflammatory cytokines and potent mitogens produced by activated platelets, macrophages, T cells and dysfunctional endothelial cells characterize early atherogenesis and vascular inflammation[4, 7]. Growth of the fibrous plaque results in intimal thickening, lumen narrowing, and target organ ischemia.

3. Advanced lesions and plaque rupture. At the advanced stage, plaques can become increasingly complex and vulnerable with a large 'necrotic core' of more accumulated lipids, necrotic debris, immune cells such as macrophages, T cells and mast cells[4, 8], but less SMC, together with the calcification and endothelial erosion, may lead to plaque rupture and thrombosis, resulting in acute clinical syndromes.

Recent studies have emphasized the involvement of inflammation in mediating all stages of atherosclerosis[4, 5, 7, 9-13]. Accompanied by the inflammation, a key process of atherosclerosis involves the proliferation of vascular SMC[1, 14]. The exact function of SMC in atherosclerosis is, however, still under debate[1]. In early stages, SMC may contribute to the development of the atheroma via producing pro-inflammatory mediators such as monocyte chemoattractant protein 1 (MCP-1) and vascular cell adhesion molecule-1 (VCAM-1), and via synthesizing matrix molecules required for the retention of lipoproteins[15]. However, SMC may be responsible for a fibrous cap formation. The proliferation and production of ECM, especially collagen by SMC accounts for most of the tensile strength of the plaque's fibrous cap. Increased inflammation in the lesion and release of factors that induce apoptosis of SMC and matrix metalloproteinases (MMPs), which degrade collagen, weaken the plaque and predispose for rupture, especially at the 'shoulder' region[4, 8].

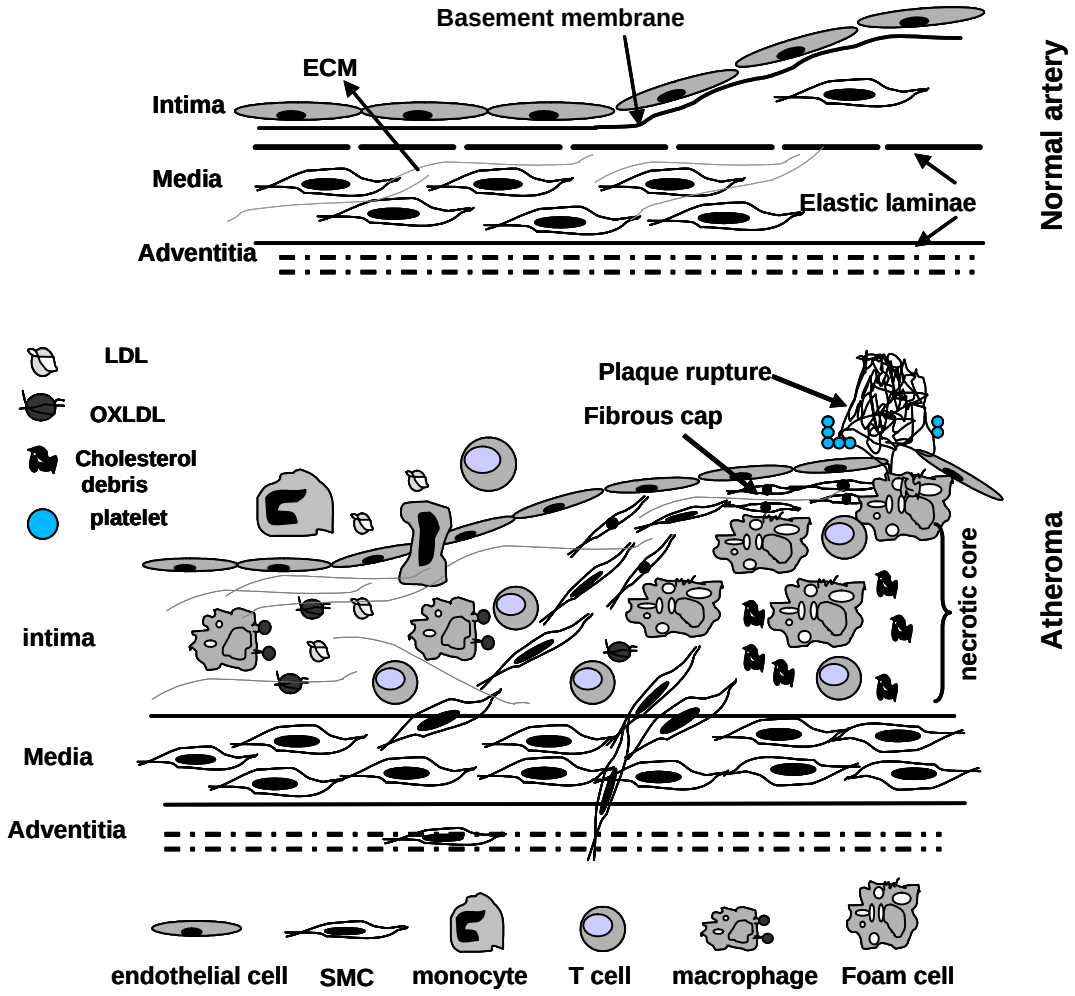

Figure 1. Simplified scheme of the normal artery and an atheroma (abbreviations: ECM, extracellular matrix; SMC: smooth muscle cell; LDL, low-density lipoproteins; oxLDL, oxidized LDL).

RESTENOSIS

Restenosis is defined as the re-narrowing of the artery after initial angioplastic treatment, identifiable by a lumen diameter diagnosis of <50% at follow-up. It comprises two major processes—intimal hyperplasia and vessel remodeling and was proposed to be the arterial wall's healing response to mechanical injury. Platelet aggregation, inflammatory cell infiltration, release of growth factors, SMC proliferation, collagen deposition and ECM remodeling were identified as the major events of this response. However, the underlying molecular basis of restenosis remains unclear. Experimental observations support a causal correlation between inflammation and experimental restenosis[16]. Leukocyte recruitment and infiltration in conjunction with the deposition of platelets occur at sites of vascular injury where the

lining endothelial cells have been denuded[17, 18], and subsequent interaction with vascular cells via releasing chemokines, growth factors and proinflammatory cytokines trigger SMC modulation, migration and proliferation. These molecular mechanisms eventually result in persistent intimal formation[19].

BIOLOGY OF VASCULAR SMC

Phenotypic diversity

Normally, vascular SMC in the media reside in a quiescent, differentiated state, and express a unique repertoire of contractile proteins to keep vascular tone by contraction or relaxation thus being referred to contractile phenotype. However, SMC also maintain considerable plasticity[20]. After disruption of the normal steady state by vascular injury or disease, SMC undergo a process often referred to as phenotypic modulation/switching characterized by dramatic increases in the rates of proliferation, migration, and synthesis of ECM proteins, along with decreased expression of SMC-specific/-selective marker genes such as smooth muscle(SM) α-actin, SM-myosin heavy chain (MHC), SM22α, h1-calponin, smoothelin, caldesmon, and telokin[20]. SMC also exhibit phenotypic diversity in other aspects, such as various functions in different blood vessels and in diverse embryological origins[21, 22]. However, the precise molecular mechanisms determining the phenotype plasticity and diversity are poorly understood.

Morphologically distinct populations of cultured SMC have been observed in many species, including humans. Until now, the most-studied species has been the rat. Two SMC populations were identified using the rat carotid artery injury model (figure 2): 1) a spindle-shaped phenotype, with the classic "hill-and-valley" growth pattern, and the cells were obtained from the normal media, and 2) an epithelioid phenotype, in which cells grow as a monolayer and exhibit a cobblestone morphology at confluence, the cells were isolated from the intima 14 days after balloon injury[23-26]. For further information on SMC diversity, the reader is referred to recent reviews[22, 27].

Intimal SMC share several characteristics with a subpopulation of medial SMC, but are dramatically different from the majority of medial SMC. They play a critical role in vascular repair and several lines of studies have revealed different gene profiles between these two types of cells[28-31]. Due to these special properties, intimal SMC have been proposed to originate from diverse sources including medial SMC[20], transdifferentiation of endothelial cells[32, 33], adventitial fibroblasts[34], or circulating progenitor cells[35-37]. However, the contribution of each source of intimal SMC to intimal hyperplasia is controversial[20, 38]

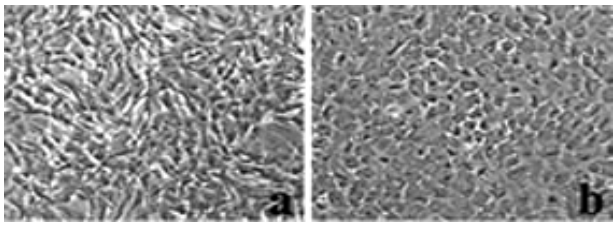

Figure 2. Morphological features of SMC subpopulations. Phase-contrast microphotographs show spindle-shaped (a) and epithelioid (b) phenotypes, SMC were isolated from the media and intima of rat carotid artery, respectively.

Response to injury and inflammation

Activation and Migration

SMC are normally surrounded by a basement membrane, densely packed into an interstitial matrix that supports their quiescent and contractile state. In response to inflammation or mechanical injury, multiple factors lead to the activation of SMC. Early markers of SMC activation such as expression of nuclear oncogenes are detectable as soon as 30 minutes after injury[39, 40]. This expression of nuclear oncogenes in the hours after angioplasty is associated with the early G1 events preceding DNA synthesis in SMC. Activated SMC undergo a modulation into a synthetic and proliferative phenotype, preferentially in the innermost layer of the media[41]. Although it is still unclear what actually triggers the process of phenotypic modulation *in vivo*, injurious stimuli are known to alter the environment of the vascular wall by affecting endothelial function and inducing platelet adhesion and activation, migration of immune cells and changes in the ECM. These environmental changes subsequently induce sequential gene expression events in SMC, leading to production of growth factors and cytokines, which in turn activate autocrine/paracrine pathways that lead to further phenotypic modulation[20]. For example, in atherogenesis, platelet-derived growth factor (PDGF) was shown to down regulate SMC differentiation markers such as SM α-actin and SM myosin heavy chain[42]; matrix-degrading proteases produced by macrophages may contribute to the degradation of the basement membrane[43, 44], and in restenosis, proteases may also derive from the injured SMC in the lesion[45]. This activation renders the SMC responsive to chemoattractants and mitogens resulting in subsequent migration and proliferation. Growth factors such as basic fibroblast growth factor (bFGF) and PDGF[46-49] as well as angiotensin II, epidermal growth factor and insulin-like growth factor 1[50, 51], stimulate SMC migration from the media and promote proliferation.

Proliferation and Apoptosis

DNA synthesis is detectable in SMC of the arterial media, already 24 hours after balloon injury[52]. The replicative SMC produce more collagen, contributing to intimal expansion, in addition to stabilizing the plaque. However, SMC are not isolated, but rather interact with other cells in the lesions. Inflammatory cells such as macrophages and T cells produce cytokines modulating SMC proliferation. For example, activated T cells in atheroma produce interferon-γ (IFN-γ) which inhibits SMC proliferation[53] and collagen deposition[54]. Macrophage can directly promote SMC proliferation by producing PDGF and the key fibrogenic cytokine, transforming growth factor-β (TGF-β), which promotes matrix formation but inhibits SMC proliferation[55, 56]. Thus, SMC proliferation is crucially linked with inflammation in the context of intimal hyperplasia.

SMC in the normal vessel wall demonstrate little, if any, basal cell proliferation or apoptosis. However, in the presence of inflammatory cells, cytokines, modified LDL-cholesterol, and under the systemic effects of altered blood pressure and flow, may dysregulate the delicate balance between proliferation and cell death[1, 57]. For example, SMC derived from plaque tissue exhibit both reduced proliferative capacity[58] and an increased sensitivity to apoptosis[59] compared with cells from normal vessels. Moreover, intimal SMC show increased apoptosis compared with medial cells[60]. *In vitro* studies suggest that SMC death can be triggered by interaction with inflammatory cells that express cell surface death ligands or secrete pro-apoptotic cytokines such as tumor necrosis factor α (TNF-α)[61]. However, the triggers for SMC apoptosis *in vivo* are mostly unknown. A main finding in apoptosis regulation is the simultanous induction of apoptosis and control of cell proliferation. For example, activation of the angiotensin II type 2 receptor exerts anti-proliferative and pro-apoptotic effects on vascular SMC during intimal formation after vascular injury in mice[62], supporting an important concept that the local induction of apoptosis may reduce intimal formation after injury. However, SMC apoptosis may also promote plaque rupture in advanced atherosclerosis, hence, leading to detrimental clinical complications.

TELOMERASE

Fundamentally, the replicative capability of most somatic cells is correlated with its telomerase activity (TERT). It has been observed that the induced TERT underlies increased proliferation rate of vascular SMC and inhibition of TERT diminished cell growth in a genetic hypertension model[63] and an *in vitro* hypoxia model[64, 65]. A more recent study also showed that PDGF and interleukin- (IL-) 6 could induce TERT

expression in human SMC[66]. These initial findings indicated its potential role in regulating SMC growth.

Identification, Structure and Expression

Telomerase is a specific multi-subunit ribonucleoprotein that synthesizes TTAGGG telomere DNA onto chromosomal ends, thereby compensating for telomere loss that normally occurs with each cell division[67, 68]. It was originally identified in *Tetrahymena*[69] and later in HeLa cells[70]. It contains two core components, a catalytic telomerase reverse transcriptase (TERT) and a telomerase RNA component (Terc) that serves as a template for the synthesis of new telomeric DNA repeats. In addition to these core components, biochemical and genetic studies have identified several other potential components of the telomerase holoenzyme complex such as telomerase-associated protein 1 (TEP1)[71, 72], and heterogeneous nuclear ribonucleoprotein (hnRNP) A1[73].

Most adult somatic cells exhibit low or absent telomerase activity and thus undergo progressive telomere attrition with each mitotic cycle, both in cell culture as a function of population doublings and during aging of the whole organism[74, 75]. In contrast, germ cells, stem cells and most tumor cells maintain high telomerase activity and long telomeres and thus have an extended proliferative potential[76-78]. Of note, telomerase can be reactivated in some adult organs possessing self-renewal capacity such as liver and spleen[79-81]. Moreover, telomerase activity was found in inflamed lungs[82], injured liver[83] and hypertensive blood vessels[63], indicating a potential role for telomerase in tissue repair and remodeling processes. However, not all normal, immortal, or tumor cells re-express detectable levels of telomerase, so alternative telomerase-independent pathways for telomere length maintenance, termed ALT, have been proposed[84-90], although their mechanism(s) remain currently unknown. Recent evidence, however, suggests telomere stabilization by way of the ALT pathway may occur through non-reciprocal recombination of telomeres[91].

Regulation of Telomerase Activity

"Switching on and off" of telomerase is controlled in a cell-specific manner. Recent investigations indicate that this regulation occurs at transcriptional and post-transcriptional levels.

Transcriptional regulation

Transcriptional regulation is the primary mechanism controlling TERT activity. Expression of TERT gene is regulated by yet defined interaction of both positive and negative regulators (figure 3). The human TERT (hTERT) promoter is a GC-rich, TATA-less promoter[92, 93]. Deletion analyses in reporter assays demonstrated the 200-bp proximal region of the hTERT promoter responsible for most of the transcriptional activity and designated this region as the hTERT core promoter[94] containing multiple E-boxes elements (5′-CACGTG-3′) and Sp1 binding sites. c-Myc, a transcription factor encoded by a proto-oncogene binds to these E-boxes through heterodimer formation with Max proteins and activates transcription of hTERT[95, 96]. Mad proteins are antagonists of c-Myc and switching from Myc/Max binding to Mad/Max binding decreases promoter activity of the hTERT[97-99]. Sp1 is also a key molecule that binds to GC-rich sites on the core promoter and activates hTERT transcription[100]. Cooperative action of c-Myc and Sp1 is required for full activation of hTERT promoter. There are also several putative binding sites for known transcription factors, including nuclear factor κB (NF-κB), activating protein-1 (AP-1) and signal transducer and activator of transcription (STAT3)[66, 101] in upstream sequences of the hTERT promoter and mutations of these sites decrease promoter activity. NF-κB was shown to activate hTERT expression directly or indirectly by stimulating expression of c-Myc[102, 103]. In addition, hormones such as estrogen and progesterone can function as activators of this gene[104]. However, the tumor suppressor, p53 and modification of DNA by histone deacetylation (HDAC) can decrease hTERT expression[105, 106].

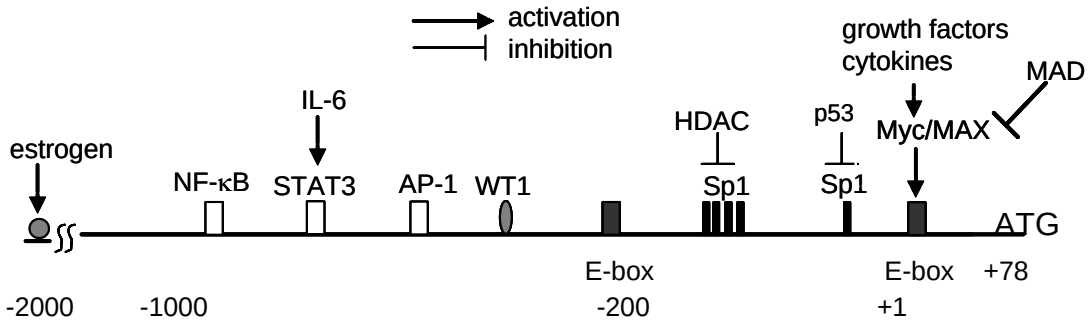

Figure 3. Simplified scheme of the possible transcriptional regulatory mechanisms of telomerase activity. Potential cis-acting elements in the hTERT promoter as well as factors that interact with them are shown. The +1 indicates the start site of transcription. Abbreviations: WT1: Wilms' tumor 1 tumor suppressor gene product; HDAC: histone deacetylation.

Post-transcriptional regulation

Telomerase activity is also controlled by post-transcriptional mechanisms. After translation, the assembly, maintenance, and disassembly of functionally active telomerase holoenzyme presumably require interaction with other proteins. Studies have shown that hTEP1[71] and telomeric-repeat binding factor 1 (TRF1)[107] play crucial roles in telomerase activity. Moreover, phosphorylation and the nuclear translocation of hTERT are additional mechanisms regulating telomerase activity[64, 108-111]. Activation of signaling pathways that result in serine/threonine and/or tyrosine phosphorylation of the telomerase protein may function in this regulation[112].

Telomerase and Disease

Cancer

Research on the connection between cancer and telomerase is a vibrant area today. Telomerase re-activation is thought to be a critical step in cellular immortalization and carcinogenesis. Most cancer cells have high telomerase activity, and inhibition of TERT was postulated as a potential for cancer therapy. A mouse knockout model[113] has demonstrated that telomerase is essential for telomere length maintenance, but it is not required for establishment of cell lines, oncogenic transformation, or tumor formation in mice, indicating the differences between the genomic organization of mouse and human telomeres.

Immune dysfunction

A definitive role for telomerase or telomere-length change has not been established during normal human immune responses. Nevertheless, there are studies indicating that abnormalities in telomerase and telomere-length maintenance can affect immune function[114]. Such evidence has been provided by a mouse model of telomerase deficiency[113] and by a recently characterized human genetic syndrome, dyskeratosis congenital (DKC), which is marked by abnormal telomerase function with immunodeficiency[115].

Atherosclerosis

Age-related diseases such as congestive heart failure[116], ulcerative colitis[117], liver cirrhosis[118, 119] and atherosclerosis[120], as well as several premature aging syndromes (i.e., Werner syndrome, ataxia telangectasia and DKC)[121] are characterized by an accelerated rate of telomere attrition. Progressive telomere shortening in cell culture and during aging of the whole organism is a characteristic of most adult somatic cells, which exhibit low or absent telomerase activity.

Aging is a major risk factor for atherosclerosis. Senescence has been associated with endothelial dysfunction and increased risk for atherosclerosis[122], including decreased TERT expression in advanced human atherosclerotic plaques[123]. Data from animal studies are controversial, old mice doubly deficient for Terc and apolipoprotein E (Terc/apoE$^{-/-}$) have shorter telomeres are protected from diet-induced atherogenesis compared to apoE$^{-/-}$ control with a complete Terc gene and longer telomeres, suggesting that telomere shortening protects against atherosclerosis[124]. These data probably reflect profound differences in the pathogenesis of atherosclerosis between humans and mice but also the recognized complexity of TERT function in the disease.

MATRIX METALLOPROTEINASES

Collagen modulation contributes to intimal hyperplasia and the progression of atherosclerosis, since a prerequisite for SMC proliferation and migration *in vivo* is degradation of the basement membrane, which is mediated by MMPs such as the gelatinases MMP-2 and MMP-9[125-127]. MMPs, a family of zinc-containing endopeptidases, are usually secreted in a latent proform and require activation to exert their proteolytic activity.

In 1989, MMP expression was reported in cultured vascular SMC[128]. Subsequent studies suggested that MMPs contributed to early migration and proliferation of medial SMC[125, 127]. Inflammatory cells such as macrophages and neutrophils are important sources of MMPs and secrete cytokines that upregulate MMP gene expression in SMC. MMP-9 overexpression enhanced SMC migration *in vitro*, increased SMC migration into an arterial matrix *in vivo*, and altered vessel remodeling[129]. Furthermore, MMP-9 gene deficiency impairs SMC migration and geometrical arterial remodeling[130]. Recent studies have identified enhanced expression of MMPs in the atherosclerotic lesion and their contribution to weakening of the vascular wall by degrading the ECM[15, 131, 132].

Regulation of MMP activity occurs at three levels: 1) induction and suppression of gene expression, 2) activation of the latent forms, and 3) inhibition by their endogenous binding proteins such as the tissue inhibitors of matrix metalloproteinase (TIMPs). More recent studies also identified that a protein called neutrophil gelatinase-associated lipocalin (NGAL) interacts with MMP-9[133-136].

NEUTROPHIL GELATINASE-ASSOCIATED LIPOCALIN

Identification, Structure and Ligands

NGAL, also known as lipocalin 2, is a 25-kDa glycoprotein, belonging to the lipocalin family, and originally purified from 92-kDa gelatinase/MMP-9 of activated human neutrophils[137, 138]. Homologous proteins have been identified in mouse (24p3/uterocalin) and rat (alpha(2)-microglobulin-related protein/neu-related lipocalin)[137-142]. NGAL exists as a 25 kDa monomer, a 46 kDa disulfide-linked homodimer, and a disulfide-linked heterodimer with MMP-9[138, 143].

Analysis of its primary structure indicates that the protein backbone is 20 542 Da consisting 178 amino acids, in agreement with the observed molecular mass of the deglycosylated protein of 21 kDa. Tertiary structure is a typical lipocalin fold with an eight-stranded beta-barrel with an unusually large internal cup shaped hydrophobic cavity, where a lipophilic ligand is bound[139, 144].

Chemotactic formylpeptides from bacteria have been proposed as ligands of NGAL[145, 146], a similar low affinity for NGAL was found for platelet-activating factor and leukotriene B4 (LTB4), however, binding experiments and the structure of NGAL do not support these hypotheses[145, 147, 148]. Hence, the true ligands remain to be determined.

Expression and Regulation

NGAL is expressed in immature neutrophil precursors[137, 149, 150], but not in mature blood neutrophils[151]. In humans, NGAL reveals a tissue-specific expression pattern[152]. Firstly, it is expressed in most tissues normally exposed to microorganisms and can be induced in epithelial cells during inflammation[151], as well as macrophages, endothelial cells and SMC in human atherosclerotic plaques[136]. Secondly, NGAL was observed during neoplastic transformation in some cancers, accompanied with inflammation[151-153].

Analysis of human NGAL gene promoter has identified several *cis*-acting elements of potential importance for the expression of NGAL, including binding sites for CTF/CBP, the hematopoietic transcription factors GATA-1 and PU.1, and NF-κB[154]. An important role for NF-κB in the control of NGAL expression is sustained by the recent study showing that IL-1β-induced up-regulation of NGAL is strongly dependent upon NF-κB activation in human epithelial cells[155]. Additionally, activation of the NF-κB is also linked with the expression of NGAL in many of the nonhematopoietic tissues and in the epithelial cells of the colon in areas of inflammation[151].

Biological functions

1) ***A disease activity marker in body fluids***: Elevated plasma NGAL levels, possibly due to activation of blood leukocytes, are associated with atherosclerosis and implicated as a predictor for cardiovascular mortality after cerebrovascular ischemia[156-158], renal failure following ischemic injury, cisplatin nephrotoxicity**,** or infection[159-162]. In inflammatory bowel diseases, serum NGAL did not correlate with disease activity, but the content measured in feces and rectal dialysis fluid did[163]. NGAL concentrations are elevated in the sputum of subjects with asthma or chronic obstructive pulmonary disease[164], and in the bronchial fluid from the emphysematous lung[165]. Irrespective of its origin, serum NGAL was found to be a more sensitive and specific marker than C-reactive protein in the discrimination between bacterial and viral infections[166].

2) ***Iron-transporting protein***: it has been shown that NGAL can be an iron-transporting protein during nephrogenesis[142, 167], and may limit bacterial growth by sequestrating the iron-laden siderophore through Toll-like receptors (TLRs) mediated innate immune response[168]. Mori K et al[169] showed that endocytic delivery of lipocalin-siderophore-iron complex rescues the kidney from ischemia-reperfusion injury.

3) ***A modulator of the inflammatory response***: NGAL can be induced in murine macrophages by LPS[170], hepatocytes by IL-1β[171], fibroblasts by serum, bFGF, and prostaglandin F2α[172, 173], and it could also be intensively synthesized in inflamed colon[151], ischemic tubule cells during tubular repair with supposedly rapid epithelial turnover[160], and in various malignant tumors. The manifold involvement of NGAL in inflammatory conditions may indicate either a microbicidal activity of NGAL or a role in regulation of inflammation or cellular growth. NGAL knockout mice have normal litters and phenotype when housed in specific pathogen-free conditions. However, intraperitoneal challenge with a sublethal dose of bacteria results in sepsis[168].

4) ***A modulator of MMP-9 activation***: The partial association of NGAL with MMP-9 suggests that NGAL may exert modulatory actions on MMP-9 by protecting MMP-9 from degradation[134, 143], and preserving its enzymatic activity. A recent study demonstrated MMP-9 and NGAL co-localized in macrophages, SMC in human atherosclerotic plaques. *In situ* zymphography showed higher MMP-9 activity where NGAL are more expressed, indicating the potential role of NGAL in modulating MMP-9 activity and destabilizing plaque[136]. However, little is known about the underlying mechanisms of NGAL expression in vascular cells.

LEUKOTRIENES

The 5-LO and its products are well established inflammatory modulators and have recently been implicated in initiation and progression of atherosclerosis and vascular remodeling by participating LDL oxidation and by biosynthesizing of proinflammatory leukotrienes (LTs)[174, 175], indicating its significance for the lesion development. Therefore, a better understanding the biological process of the 5-LO pathway is critical for its contribution to atherogenesis.

History and Names

The overall term, LTs, was coined because the substances were first characterized from leucocytes and contain a conjugated triene structure. The LTs were discovered as bioactive lipids derived from arachidonic acid[176] and found to be smooth muscle contracting agents carrying a biological activity previously referred to "slow reacting substance of anaphylaxis" (SRS-A). LTs, together with prostaglandins, thromboxanes, and lipoxins, are the major constituents of a group of biologically active oxygenated fatty acids known as eicosanoids[177].

LTB4 Structure and Biosynthesis

LTs biosynthesis can be initiated in a variety of cell types by either immunological or chemical stimuli through activating the linear 5-LO pathway[178, 179](figure 4). The analysis of the products of this pathway led to the structural elucidation of three major compounds, one of which was LTB4[178, 180]. The complete structure of LTB4 was described as 5S,12R-dihydroxy-6,14-cis-8,10- trans-icosatetraenoic acid in the early 1980s[181-184]. LTB4 is synthesized from arachidonic acid released from membrane phospholipids by the action of cytosolic phospholipase A2 (cPLA2). First, 5-LO in conjunction with 5-LO-activating protein (FLAP) catalyzes arachidonic acid to LTA4, the unstable LTA4 then can be transformed into LTB4 by LTA4 hydrolase or LTC4, LTD4 and LTE4 by transferases[178, 179, 185]. Collectively, the latter three are known as the cysteinyl LTs, the constituents of SRS-A.

LTB4 Receptors and down-stream signaling pathway

LTB4 exerts biological effects through binding to and activation of distinct G-protein-coupled receptors (GPCRs). Two GPCRS have been cloned and characterized in the last few years[186-190]. The two LTB4 receptors, also called BLT1 and BLT2 are high- and low-affinity LTB4 receptors respectively, have 45% amino acid identity and different cellular expression (figure 4). In contrast to the BLT2, which is ubiquitously expressed in various tissues, BLT1 receptor is predominantly found in

leukocytes. However, a recent study showed that BLT1 is expressed on effector CD4+ T cells and mediates early effector T cell recruitment[191], indicating its potential effect on other cell types. In general, BLTs enhance the production of inositol phosphates, mobilize intracellular calcium, and activate several kinases including phosphoinositide 3-kinase (PI3K), mitogen-activated protein kinase (MAPK) and Akt[192, 193]. Additionally, the sequence of the human BLT1 receptor promoter suggests that it contains elements responsive to the transcription factor NF-κB[194].

Although the classical LTB4 receptors are BLTs, the first molecule identified as an LTB4 receptor was a nuclear receptor, peroxisome proliferator activated receptor (PPAR) α. It has been reported that LTB4 binding to PPARα activates transcription of genes that terminate inflammation[195]. Thus, LTB4 is one of the few ligands that utilize a dual-receptor system.

Biological functions

LTB4 is known as one of the most potent chemoattractants and activators of multiple leukocyte subsets during inflammation including neutrophils, monocytes, eosinophils[196], B cell[197] and $CD4^+$, $CD8^+$ T cells[191, 198-200], linking the innate and adaptive responses through acting on BLTs.

Stimulation of leukocyte with LTB4 causes a number of prominent alterations in function such as adhesion to vascular endothelial cells[201], transendothelial migration[202], chemotaxis[203], release of lysosomal enzymes[204], production of reactive oxygen species[205], and induction of gene expression[206]. LTB4 plays important roles in the host defense system against infection of pathogens[207, 208]. Mice lacking LT production are insensitive to some inflammatory stimuli. However, an overproduction of LTB4 is implicated in several inflammatory diseases including bronchial asthma[209], inflammatory bowel disease[210], and atherosclerosis (see below). The role of the BLT1 receptor has been studied by targeted gene disruption. Two lines of BLT1 deficient ($BLT1^{-/-}$) mice were generated[211, 212]. $BLT1^{-/-}$ mice were protected from the lethal effects of platelet-activating factor induced anaphylaxis[211]. Conversely, mice overexpressing BLT1 exhibit enhanced responses to infections[213], which confirmed the importance of the LTB4–BLT1 interaction in inflammation *in vivo*. Studies on $BLT1^{-/-}$ mice have also greatly contributed to the understanding of BLT1 function in the recruitment of T cells[191, 198]. Development of mice with BLT2 deficiency will allow a better understanding of the role of this receptor in inflammation.

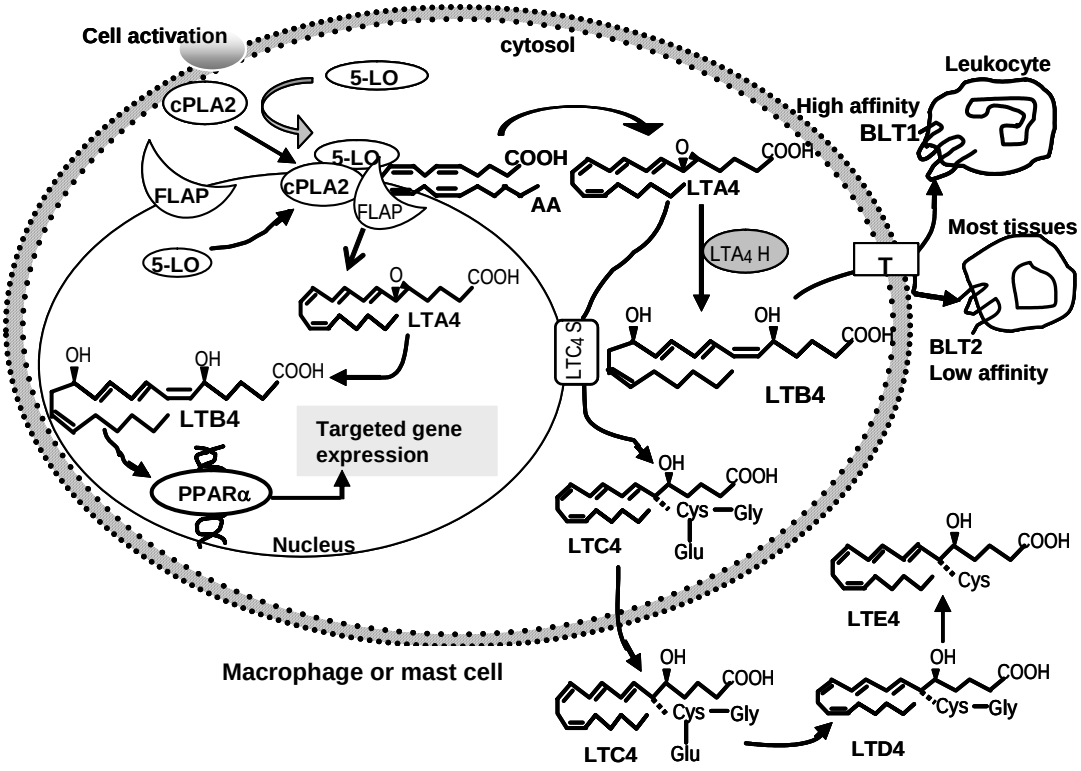

Figure 4. Leukotriene biosynthesis and actions. During cell activation, $cPLA_2$ and 5-LO translocate to the nuclear membrane. FLAP, acts as an arachidonic acid transfer protein and present it to 5-LO for conversion to LTA4. LTA4 and LTB4 can potentially be formed on either side of the nuclear membrane by nuclear or cytosolic 5-LO and LTA4 hydrolase. LTB4 can be transported out of the cell by a transporter (T), and then will bind to the BLT1 receptor to induce chemotaxis on neutrophils or to the BLT2 receptor to induce the currently unknown functions. LTB4 may also bind intracellularly to the nuclear transcription factor PPARα to induce target genes which cause a negative feedback loop causing LTB4 degradation and terminating its proinflammatory actions. LTA4 can also be converted to LTC4 by LTC4 synthase (LTC4 S). LTC4 is transferred out of the cell, where it is metabolized to LTD4 and LTE4 (See the text for abbreviations; the drawing is modified after Funk, CD, 2001[179]).

LTB4 in atherosclerosis

Multiple lines of investigation have implicated the LTB_4/BLT1 axis in atherogenesis in both mouse and man. $ApoE^{-/-}/BLT1^{-/-}$ mice fed a western-type diet had a marked reduction in plaque formation compared with $apoE^{-/-}$ controls with a significant decrease in SMC, macrophages, and T cells in the early lesions[214]. Studies of a congenic mouse strain demonstrated resistance to atherosclerosis linked to a locus on chromosome 6, mapping closely to the gene for 5-LO[215], and genetic studies have revealed association of a polymorphism in the 5-LO promoter with an increased carotid artery intima thickness[216]. Furhtermore, the gene encoding FLAP is associated

with an increased risk of stroke and myocardial infarction[217]. Also, products of the 5-LO pathway, LTB4 and LTE4, are detectable in atherosclerotic lesions[218-220] and the increased LTB4 production is closely associated with plaque instability in humans[221]. However, little is known about BLT receptors in the human normal and diseased arterial wall. Although studies have shown that treatment of the atherosclerosis-susceptible LDL receptor knockout ($LDLR^{-/-}$) mice and $apoE^{-/-}$ mice with a small-molecule BLT1 antagonist reduced plaque burden[222], suggesting this pathway as a potential therapeutic target, the precise role of the LTB4/BLT1 pathway in atherogenesis remains largely to be explored. For instance, its functional roles in regulating SMC biology and the mechanism behind are unknown, which become one of our focuses in this thesis.

As reviewed in the preceding sections, the underlying molecular basis and regulatory mechanism linking the biology of SMC and vascular inflammation is complex, however, numerous studies have demonstrated that the NF-κB signal transduction pathway play a central role in regulating SMC biology through modulating the expression of multiple inflammatory genes in the aforementioned pathological processes.

NF-κB SIGNAL TRANSDUCTION PATHWAY

NF-κB is a ubiquitous, inducible transcription factor, first described in 1986[223, 224] as a nuclear factor necessary for immunoglobulin κ light chain transcription in B cells, now known to exist in virtually all cell types[225] and organelles such as mitochondria[226]. It regulates the transcription of a large number of genes involved in immune, inflammatory, and acute phase responses[227-230], as well as cell proliferation and apoptosis[231-234].

NF-κB family

NF-κB is a family of transcription factors containing 5 members: p65 (RelA), c-Rel, RelB, NF-κB1 (p50 and its precursor p105), and NF-κB2 (p52 and its precursor p100) (figure 5A), all of which have a structurally conserved N-terminal 'rel homology' domain responsible for dimerisation, nuclear translocation and DNA binding, whereas only the first 3 contain a transactivation domain. The proteins can form either homodimers or heterodimers. The most widely expressed complex, often referred to as being "NF-κB," is p65/p50. In most cells, NF-κB is sequestered in the cytoplasm and associated with members of the inhibitor of NF-κB (IκB) family, which consists

of IκBα, IκBβ, IκBγ, IκBε and Bcl-3 (figure 5A), with IκBα being the most predominant. The NF-κB-IκBα interaction inhibits NF-κB-DNA binding and results in the NF-κB complex being primarily in the cytoplasm due to a stronger nuclear export signal in IκBα, compared to the the import signaling on NF-κB. Thus, the complex is actually continuously shuttling between the nucleus and the cytoplasm. In contrast, the IκBβ interaction with the NF-κB complex does not undergo nucleo-cytoplasmic shuttling, keeping the complex is retained in the cytoplasm[225].

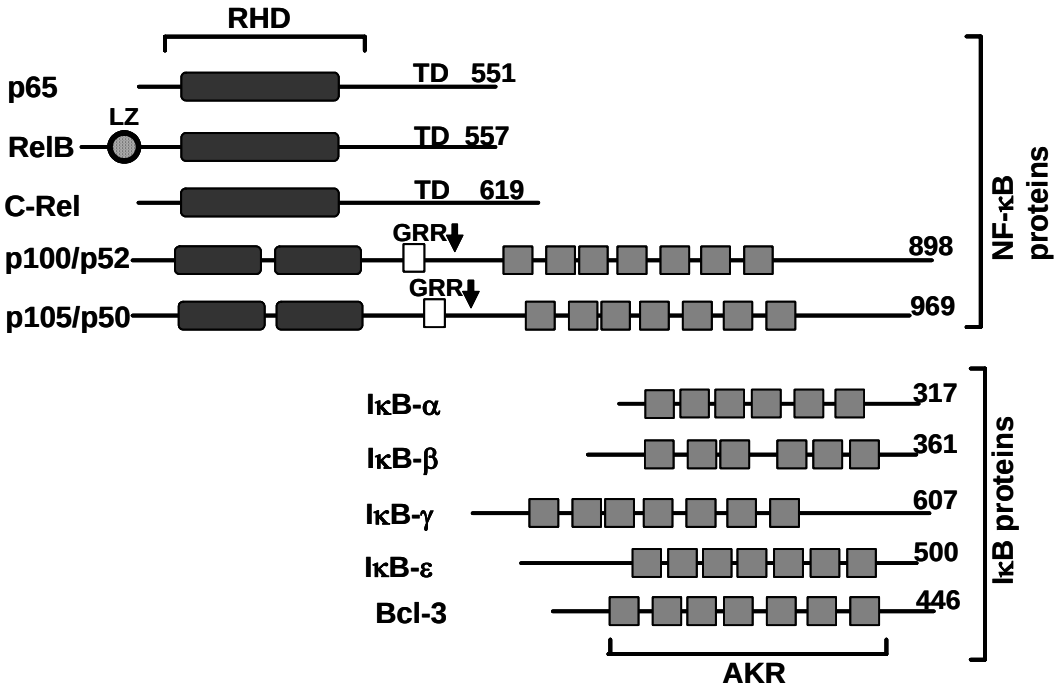

Figure 5A. The Rel/NF-κB/IκB family of proteins. Members of the NF-κB/rel and IκB families of proteins are shown. The number of amino acids in each protein is shown on the right. The *arrows* point to the endoproteolytic cleavage sites of p100/p52 and p105/p50. Abbreviations: RHD, rel homology domain; TD, transactivation domain; LZ, leucine zipper domain of rel-B; GRR, glycine-rich region; AKR: ankyrin repeats (the drawing is modified after Karin, M[230]).

NF-κB activation cascades

NF-κB could be activated via two pathways, a canonical pathway and an alternative pathway[235] (figure 5B): Activation of the canonical NF-κB pathway is initiated by a variety of extracellular stimuli, including cytokines such as TNF-α and IL-1β, viral products, bacterial components such as LPS, and yeast products signaling through different TLRs. These agents activate the cells through their receptors, leading to the activation of different signaling cascades, which will activate the IκB kinase (IKK)

complex. This complex will phosphorylate IκB on its N-terminal serines, resulting in its ubiquitination by an E3 ubiquitin kinase complex, degradation by the 26S proteasome, and translocation of the NF-κB dimer to the nucleus, where it binds to the NF-κB consensus sequence GGGRNNYYCC (R=purine; Y=pyrimidine), leading to the transcription of many genes such as cytokines, adhesion molecules, antiapoptotic genes and the IκBα.

The phosphorylation of IκB by the IKK complex is a convergent point in the activation of this classical cascade. The complex comprises several subunits, including two highly homologous catalytic kinases, IKKα (IKK1) and IKKβ (IKK2), and an essential NF-κB modulator (NEMO; IKKγ), IKK Associated Protein 1 (IKKAP1), and FIP-3 (type 2 adenovirus E3–14.7-kD interacting protein)[236-241]. IKKβ is probably the most dominant kinase[242], whereas IKKα reveals partial redundancy for the activation of the classical NF-κB pathway[243]. NEMO is necessary for NF-κB activation[240, 242], despite lack of kinase activity. IKKβ deficient mice die as embryos and show massive liver degeneration due to hepatocyte apoptosis, a phenotype similar to that of mice deficient in p65 or IκBα, and the NF-κB activation by IL-1 or TNF-α is largely impaired. This suggests that IKKβ is crucial for NF-κB activation upon inflammatory stimuli. Moreover, a newly identified protein called ELKS is also an essential regulatory subunit of the IKK complex as silencing ELKS expression blocked expression of NF-κB target genes, including IκBα, and COX-2 and IL-8[244]. The name of ELKS is derived from the relative abundance of its constitutive amino acids: glutamic acid (E), leucine (L), lysine (K), and serine (S).

IKKα was shown to be responsible for an alternative NF-κB activation pathway via the processing of NF-κB2/p100[245, 246] by activating lymphotoxin-β[247, 248], B-cell–activating factor (BcAF)[249], CD40 ligand (CD40L)[250], and LPS[248], some of which can induce the classical pathway. The relB-p52 dimers, not associated with IκB proteins, transfer to the nucleus, where they mediate transcription of genes involved in skin and skeletal development, as well as in B cell maturation[243, 245, 251, 252]. IKKα knockout mice have many morphogenetic abnormalities, including shorter limbs and skull, a fused tail, and die perinatally. They exhibit a normal NF-κB activation after induction by IL-1β and TNF-α in embryonic fibroblasts.

However, a recent study has revealed a new role for IKKα, which contributes to suppression of NF-κB activity and the resolution of inflammation in macrophages as a negative modulator. Inactivation of IKKα in mice enhances inflammation and bacterial clearance[253]. Therefore, the two IKK catalytic subunits seem to have opposing but complimentary roles needed for controlling inflammation and innate immunity.

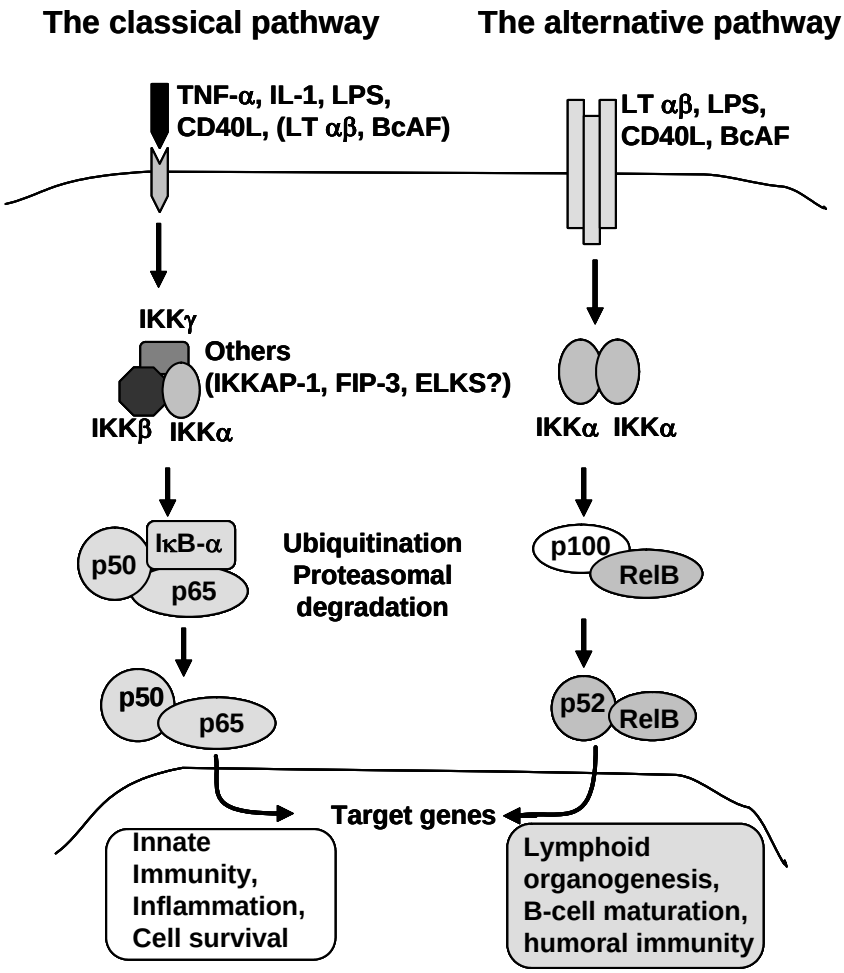

Figure 5B. Schematic representation of the two NF-κB signaling pathways. The classical pathway (left) is activated by TNF-α, IL-1, LPS, CD40L and to a lesser extent by LT αβ and BcAF and involves the activation of the three-subunit IKK holocomplex, which phosphorylates IκBs to induce their degradation and nuclear translocation of p65/p50. This pathway is crucial for the activation of innate immunity and inflammation, and for inhibition of apoptosis and the increased cell survival. The alternative pathway (right) is activated by LT α/β, CD40L, BcAF and to less extent, LPS. Activation of this pathway depends on IKKα homodimers, which induce processing of p100 and nuclear translocation of RelB–p52 dimers. This pathway is crucial for secondary lymphoid organ development, maturation of B cells, and adaptive humoral immunity (See text for abbreviations, the drawing is modified after Karin, M[230]).

Regulation of NF-κB pathway

NF-κB is subjected to multilevel control mechanisms. **1)** the response is cell and stimulus specific, indicating that inducers use different signaling components and cascades. For instance, the different adaptor proteins and upstream kinases differ

between different stimuli and receptors[254]. **2)** IKK is an important operator, as described above. Moreover, recent reports suggested that A20, a cytoplasmic protein can modulate the ubiquitin process as a negative-feedback regulation of IKK[255, 256]. **3)** auto feedback regulation of NF-κB by IκBα. The rapid transcription of the IκBα upon NF-κB activation is important in termination of NF-κB activation[257]. **4)** differential binding by NF-κB dimers is another important level of control[258]. **5)** different NF-κB dimers exhibit different binding affinities for NF-κB or κB-like sites and may affect the transcriptional response[259-262]. **6)** phosphorylation and acetylation regulate the transcriptional activity of the NF-κB in the nucleus[225, 263, 264]. **7)** cross-talk between NF-κB and other transcription factors such as PPARs[265, 266], enhancer binding proteins-β[267, 268], SP-1[269], AP-1[270] and Janus family of kinase (Jak)-STAT[271] also affects the NF-κB signaling pathway. Taken together, the complicated regulatory mechanisms contribute to the complex cellular responses of NF-κB activation.

The peculiarity of NF-κB is its rapid activation and termination. Transient activation allows appropriate expression of immune genes in physiological conditions. However, prolonged or inappropriate activation of NF-κB, particularly IKKβ/NF-κB pathway is involved in diseases such as arthritis[272], asthma[273], inflammatory bowel disease[274], a variety of cancers[275-278], and virus infections[279-281]. IKKβ/NF-κB is now also thought to be crucial in atherogenesis; whereas, the relative contribution of the alternative NF-κB pathway in atherogenesis is unknown.

IKKβ/NF-κB pathway in atherogenesis and intimal hyperplasia

The evidence suggesting a role for NF-κB in atherosclerosis is mainly based on its characterization. NF-κB activation was observed in SMC, macrophages, endothelial cells, and to a lesser extent, T cells of human atherosclerotic plaques[282, 283]. Furhermore, it is enhanced in unstable coronary plaques[30, 284, 285] and colocalizes with the expression of target genes[286].

Despite these reports in man, studies on animal models provided us with a deep insight into the involvement of the NF-κB pathway. Activated NF-κB was detected in coronary arteries of pigs fed a hypercholesterolemic diet[287], and in atherosclerotic lesions of $LDLR^{-/-}$ mice[288]. Transplantation of bone marrow from mice with macrophages specific deficiency in IKKβ into $LDLR^{-/-}$ mice increased atherosclerotic lesion size, with enhanced inflammation in lesions[289]. Studies using bone marrow transfers from p50-deficient mice to $LDLR^{-/-}$ mice showed a reduction in lesion size, but with a distinct plaque phenotype, characterized by reduced foam cell numbers and an increase in immune cells[290]. Hence, these studies highlight the complex role of NF-κB in atherogensis.

A number of *in vitro* studies further confirmed the notion that NF-κB involves in atherogensis by regulating different gene expression in a cell specific manner. Of note, many stimuli such as modified LDL[291], cytokines such as TNF-α[292] and IL-1[293], microbial products like LPS and heat shock proteins (HSP)[294-296], have the potential to activate NF-κB in the initiation of atherogenesis. NF-κB regulated genes include TNF-α, IL-1β, IL-6, IL-10, VCAM-1, E selectin, COX-2 and inducible NOS (iNOS) expressed in SMC, endothelial cells and macrophages and are involved in the progression process[297], even if some of them (e.g, IL-10, COX-2) can have anti-inflammatory roles[253, 289, 298, 299]; NF-κB also mediate genes with plaque thrombotic potentials like tissue factor and MMPs[300-302].

It has been shown that NF-κB is critical for SMC proliferation and viability by inducing genes with survival functions[303, 304]. Many experiments on the role of NF-κB in proliferation have been conducted. In cultured SMC, induction of cellular inhibitors of apoptosis (c-IAP) and Bcl-2 family members was found to be NF-κB dependent[233, 305]. Using a balloon injury model in the rat carotid artery, low levels of constitutively activated p50, relA and c-Rel were shown in normal vessels, however, immediately after injury, levels of IκBα and IκBβ were dramatically reduced, expression of VCAM-1 and MCP-1 was observed[306]. Using NF-κB decoy oligodeoxynucleotides[307] or antisense oligos against p65 could inhibit SMC proliferation and initimal formation in rat carotid arteries[308]. More recently, IκBα adenovirus was applied in a rabbit iliac artery restenosis model, showing a reduction of intracellular adhesion molecule-1 (ICAM-1) and MCP-1 expression, as well as limiting recruitment of macrophages and lumen narrowing without affecting intimal thickness[309]. Taken together, data from different models are inconsistent, indicating that the mechanisms by which NF-κB modulates the complex process of atherogenesis and intimal formation require further investigation.

AIMS

This study was initiated to characterize NF-κB activation and its role in intimal hyperplasia and atherogenesis with the following specific aims:

- To characterize NF-κB activation and its role in injury-induced intimal hyperplasia.
- To evaluate the expression, regulation and functional role of NGAL in vascular repair.
- To assess the expression, regulation and role of TERT in intimal formation.
- To explore the effects of LTB4/BLT1 on vascular SMC in atherogenesis and intimal hyperplasia, and the potential link between NF-κB and LTB4/BLT1 pathways.

METHODOLOGICAL CONSIDERATIONS

Rat model of carotid artery balloon injury

The arterial response to balloon angioplasty in humans has mainly been endpoint studies, providing limited understanding about pathological processes. Research has benefited from various experimental vascular remodeling models with new insights into the process of intimal hyperplasia[310-316].

The rat balloon injury model of the common carotid artery is a well established and the most widely used animal model for studying vascular injury and intimal hyperplasia[41]. Briefly, the common carotid artery, and the internal and external carotid arteries were exposed by a midline incision under general anesthesia. The common carotid artery was injured by an inflated 2F Fogarty balloon catheter introduced via the external carotid artery and advanced to the proximal end of the common carotid artery with forwarding and withdrawing for three times. This method has several advantages, including simple performance, cost-effectiveness, and remarkable safety. It generates prominent and reproducible intimal lesions, and there is a slow re-endothelialization of the artery due to lack of side branches in the common carotid artery which makes the model superior to aortic injury. Other advantages of this model include allowing delivery of experimental materials to both the adventitial side and/or the lumen for local incubation. In the present study, this model was employed for characterization of NF-κB activation and for exploring the role of IKKβ in the injured artery. All animal experiments were approved by the Regional Ethics Committee for Animal Research.

Although it is a well-established and characterized model, it is noteworthy that the injury to normal arteries differs considerably from angioplasty to human atherosclerotic lesions which already contain abundant intimal SMC, a variety of leukocytes, and prominent inflammation. Therefore, although the rat carotid injury preparation has provided insight into the biology of the response to injury, it has its limitation in mimicking human restenosis. However, some real experimental retenosis models based on pre-existing plaques have been set up in pigs, monkeys and mice[317-319], which might well serve to elucidate the mechanisms underlying restenosis and to evaluate potential antirestenotic therapies.

Adenoviral-recombinant dominant negative IKKβ transfection *in vivo* and *in vitro*

To intervene with NF-κB activation *in vivo*, two recombinant, replication-deficient, adenoviral vectors encoding E. *coli* β-Galactosidase (Adv-β-Gal) as a reporter gene

or encoding the Flag tagged dominant negative form of human IKKβ (Adv-dnIKKβ) as a specific inhibitor of NF-κB were used. For gene transfection, 50 μL Adv-dnIKKβ or Adv-β-Gal solution at 4 $x10^{10}$ plaque forming units (pfu)/mL was instilled into the common carotid artery via the external carotid and incubated for 40 minutes following the deendothelialization. Thereafter, the Adv-dnIKKβ or Adv-β-Gal solution was withdrawn and blood circulation in common carotid artery was restored.

To intervene with NF-κB activation *in vitro,* SMC originally isolated from the media and intima of adult Sprague-Dawley rats were cultured as described previously in detail by our laboratory[26]. Passage 5 to 9 of SMC were used through the whole study, the cells were grown in Dulbecco's Modified Eagles medium (DMEM) supplemented with 10% fetal calf serum (FCS) and 100 unit/mL penicillin/streptomycin. Cells were starved in DMEM-F12, supplemented with 1% FCS for 24h, and subsequently exposed to infection with Adv-β-Gal or AdV-dnIKKβ at a multiplicity of infection (MOI) from 50:1 to 200:1 for 1h.

The advantages of using recombinant, replication deficient, adenoviral vectors are that: **1)** high rate of transfection, **2)** transfection and infection of genes can be achieved in both non-replicative and replicative cells with low pathogenicity in most mammalian cells, **3)** there is no insertional mutagenesis, remaining epichromosomal (especially compared with retrovirus), **4)** efficient replication to high titers makes it the best suited vector system for gene therapy applications. The transfection efficiency was evaluated by both X-Gal staining and anti-Flag immunohisto- or cyto-chemistry in this study. The disadvantages are that it can induce host cellular immune responses and transient expression of the therapeutic gene.

In our pilot study, we tested different doses of vectors from 5 $x10^9$, $1x10^{10}$ up to 4 $x10^{10}$ pfu/ml and the latter proved to efficiently inhibit NF-κB activation without inducing toxic effects such as weight loss, abnormal behavior and distress. For *in vitro* studies, we also tried different MOI from 50:1 to 200:1, and found that 200:1 is most efficient without affecting SMC viability. To increase the infection efficiency, we minimized the volume of medium used so the viral particles were as close as possible to the cells for the 1h of infection while occasionally slowly rocking the plates, then adding media to cover the cells.

Assessment of NF-κB activation

NF-κB activation after vascular injury *in vivo* and in SMC *in vitro* was assessed by three different approaches in this study:

IKK activity assay

Central to NF-κB activation is the IKK complex, therefore, we assessed IKK activity by IKK activity assay. In brief, IKK protein was pulled down with IKKα antibody by immunoprecipitation (IP), then the IKK activity assay was performed by incubating the immunoprecipitates with GST-IκBα (1-54), the substrate of IKK in kinase buffer and in the presence of ATP-^{32}P, then the samples were resolved on 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). The IKK activity as reflected by the phosphorylated GST-IκBα was assessed by autoradiography analysis of ^{32}P. Immunoblots for IKK protein were subsequently performed to assure equal protein loading.

Electrophoretic Mobility Shift Assay (EMSA)

EMSA is one of the most sensitive methods for evaluating DNA-protein interactions, and can be used to identify and semi-quantify sequence-specific DNA-binding proteins such as transcription factors. Despite its high sensitivity, EMSA has its disadvantages, including the fact that the DNA-protein binding is not assessed in intact tissues or cells, and the probe used is artificial. In our study, the transactivation of NF-κB either in carotd artery tissue or in SMC was determined by this method. Briefly, nuclear extracts were prepared from vessel or cell homogenates and incubated with ^{32}P labelled NF-κB probe containing a κB binding site: 5'-AGT TGA GGG GAC TTT CCC AGG C-3', except that we used the ^{32}P-labeled NF-κB consensus oligonucleotide in the human NGAL promoter region for NGAL study (paper II), the sequences are 5'-ACTCCGGGAATGTCCCTCAC-3' + 5'-GTGAGGGACATTCC-CGGAGT-3'. Then the samples were resolved using 4% or 7% polyacrylamide gel. Dried gels were analyzed by autoradiography. Specificity of the NF-κB signal was verified by addition of excess (25 or 50-fold) nonradioactive AP-1 and NF-κB oligonucleotide. The intensity of DNA-nuclear protein binding complex was semi-quantitatively analyzed using Fuji Film Image Gauge V3.12.

Immunohistochemistry

We detected activated NF-κB by immunostaining for its subunit p65 in the injured vessels with or without transfection. This method provided us not only the rough abundance of protein (not exactly quantitative), but more importantly, its localization in whole tissues or cells. Although our antibody used here is not originally raised against the phosphorylated p65, this antibody recognizes nuclear translocated p65 staining which can be detected at high magnification.

We also used immunohistochemistry to detect other proteins (paper I to IV) in injured vessels, human atherosclerotic plaques and normal mammary arteries. A standard

protocol was used including testing the specificity of the antibody involved by omitting the primary antibody, using blocking peptides or staining with an isotype control, generated from the same species as the one recognizing the protein of interest.

In short, we used three different approaches to detect NF-κB activation in order to compensate for the shortcomings of each method, gaining a comprehensive and understanding of NF-κB activation *in vivo* and *in vitro*.

Gene expression analysis

RT-PCR

The RT-PCR technique is sensitive enough to enable semi-quantification of mRNA from a single cell. This method was used in paper III to detect TERT by using human TERT primers in rat tissues. All experiments were preceded by titration experiment on PCR products amplified at different cycles to ensure detection in the linear range of the PCR reaction. The relative expression was normalized to the house-keeping gene β-actin, and afterwards, semi-quantitative analysis was carried out using Quantity One® 1-D software. However, quantitative real-time RT-PCR, used in papers I, II and IV, provides many advantages over this conventional RT-PCR.

Quantitative Real-Time RT-PCR

Real-time PCR monitors the fluorescence emitted during the reaction as an indicator of amplicon production during each PCR cycle. It quantitates the initial amount of the template specifically, sensitively and reproducibly and avoids problems associated with endpoint detection used in other forms of quantitative RT-PCR. The 5' nuclease assay (TaqMan® chemistry) and two types of TaqMan® probes were used in our study, TaqMan® probes with TAMRA as the quencher dye were used in Paper I and II, while TaqMan® MGB probes used in paper II and IV. The latter was supplied by Applied Biosystems Assay-On-Demand with primers and probe mixture. The transcripts were normalized to house-keeping genes and are presented as relative units.

Protein analysis

In addition to immunohistochemistry, we also used the following approaches to analyze proteins and protein interactions.

Western Blotting and IP

For semi-quantitative *in vitro* determination of a specific protein, we performed western blotting (WB). WB provides high specificity for analysis of protein, and the resulting data can be semi-quantitatively analyzed using available software; we used Fuji Film Image Gauge V3.12 software in our study. In paper II, we performed this method under reducing and non-reducing conditions respectively for different purposes. Reducing condition is conventional and generally used to detect a single protein; non-reducing condition allows detecting disulfide-linked proteins. The sample buffer used for non-reducing WB contains 4% SDS, 0.15 M Tris, pH 6.8, 20% v/v glycerol, and 0.5% w/v bromphenol blue. Results of non-reducing WB show existence of NGAL as a monomer, homodimer, trimer and heterodimer with MMP-9 in SMC. To further support this finding, we subsequently performed IP to detect interaction between these two molecules.

IP is the technique of precipitating an antigen out of solution by specific antigen-antibody interaction. This process can be used to identify protein complexes present in cell extracts by targeting any one of the proteins assumed to be in the complex. The antibody brings the entire complex out of solution, from where it can be analyzed using western blotting for identifying constituents in the complex. Based on this principle, we characterized NGAL-MMP-9 complex in rat SMC.

Gelatinase activity assay-gel and *in situ* zymography

In Paper II, to assess MMP-9 proteolytic activity, we performed gel and *in situ* zymography, respectively. These are commonly used methods to detect MMP activity utilizing gelatin (or casein) as a substrate. Protease activity can be visualized as clear bands against a dark background following the renaturing developing and staining protocol. Analysis of MMP-9 activity in tissue was achieved *in situ* in tissue by incubating fluorescein-labeled gelatin-agarose on unfixed fresh cryostat sections. To test the signal specificity, the MMP-2 inhibitor, OA-Hy cis-9-Octadeconoyl-N-hydroxylamide was mixed with the agarose. Generation of proteolytic activity was prevented in controls by the addition of phenanthroline and ethylenediamine-tetraacetic acid (EDTA).

SMC migration, proliferation and apoptosis

SMC migration assay *in vitro* was performed in Paper IV to evaluate the effect of LTB4/BLT1 signaling. Briefly, SMC were seeded in chemotaxis chambers with a gelatin-coated membrane with pores. LTB4 was added to the lower chamber. To interrupt LTB4, either BLT1 agonist or antagonist was preincubated with SMC before the cells addition to the upper chamber. After 6 h, cells that had migrated to the lower

membrane surface were fixed and stained by eosin/hematoxylin. Five randomly chosen visual fields per membrane were counted, and the mean value was used as a measure of SMC migration. Recombinant PDGF-BB was used as a positive control; negative controls were SMC without LTB4 in the lower chamber or in both upper and lower chambers.

In paper I, we performed immunohistochemistry for both proliferating cell nuclear antigen (PCNA) and cyclin D1 (both are associated with regulating G1/S phase of the cell cycle) to evaluate SMC proliferation during intimal formation. PCNA is present in the nuclear regions where DNA synthesis is occurring and cyclin D1 is seen in the cytoplasm. A terminal deoxynucleotidyl transferase mediated-deoxyuridine triphosphate nick-end labeling (TUNEL) assay was performed to detect cell apoptosis in the tissue.

To measure SMC proliferation *in vitro*, we used the WST-1 reagent that quantitatively monitors the metabolic activity of cells. This kit provides a fast and sensitive approach for quantification of cell proliferation and viability by detecting mitochondrial dehydrogenases using a dye and can be quantified by a microplate reader. This method is simple, requiring no washing, no harvesting and no solubilization steps, and it is faster and more sensitive than the MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) -based assay.

Analysis of transcriptional activity

To determine the transcriptional activity of TERT in Paper III, luciferase-reporter constructs driven by shortened (181bp), full-length (1.4kb) or E-box mutated shortened human TERT promoters were used. The constructs were transfected into SMC by using Lipofectamin. In some experiments, cells were pre-transfected with other constructs. Reporter levels were normalized to co-transfected *Renilla reniformis* luciferase expression using the Dual luciferase assay kit (DLR™) and an MLX luminometer. In the DLR™ assay, the activities of firefly and *Renilla reniformis* luciferases are measured sequentially from a single sample. It provides rapid assessment of both reporters either in transfected cells or in cell-free transcription/translation reactions.

RESULTS AND DISCUSSION

Activation of NF-κB represents a prominent feature of human atherosclerotic lesion. However, the role of NF-κB in the disease progression remains controversial. Recent studies have shown that NF-κB is activated in the models of experimental atherosclerosis and arterial angioplastic injury[286, 306, 307, 320, 321]. To clarify the role of NF-κB signaling in the process of vascular repair, we characterized the IKKβ/NF-κB activation and investigated its functional relevance to vascular healing process, including regulation of NGAL and TERT gene expression and its effect on LTB4/BLT1 axis in the present study using the rat model of carotid artery angioplastic injury.

Angioplastic injury elicits biphasic activation of NF-κB in the media and intima (Paper I).

Progression of NF-κB activation was investigated in the rat carotid artery subjected to angioplastic injury. Immunostaining for nuclear NF-κB p65 showed that the activated NF-κB signal was present in medial cells at day 3 post-injury. Later, the signal was located in most intimal cells in conjunction with high levels of IKK activity at day 14. In contrast, a few p65 positive cells and weak IKK activity were detected in the media, indicating that NF-κB activation was resolved in the media at this stage. These results suggest that the angioplastic injury elicits two phases of NF-κB activation: an early activation in the media and a later one in the intima. These data, for the first time, depict a dynamic course of NF-κB activation in the injured artery. We speculate that the two phases of NF-κB activation are driven by different activation and resolution mechanisms, which may also contribute to the phenotypic difference between the medial and the intimal SMC. Indeed, the intimal cells exhibit a distinct NF-κB signaling, characterized by a high IKK activity and marked constitutive NF-κB activity (Paper II and other submitted data), and these two types of SMC display a difference in gene expression patterns, for instance, regarding NGAL and TERT (see the following sections).

Early NF-κB activation underlies the early vascular inflammation in the media (Paper I).

To clarify the functional relevance of the early phase of NF-κB activation, pyrrolidine dithiocarbamate (PDTC) was applied perivascularly to the injured carotid artery via Pluronic F-127 gel which was rapidly absorbed *in vivo* by day 3. Treatment with PDTC suppressed the early NF-κB activation as assessed by EMSA, and resulted in reduced expression of iNOS and TNF-α at day 3. The short term PDTC

treatment, however, did not affect intimal formation. To interrupt NF-κB specifically, the injured arteries were transfected with dnIKKβ. Although the expression of dnIKKβ protein was detectable in the artery at day 3, neither NF-κB nor the vascular inflammatory response was suppressed by this time point. One explanation for the failure of dnIKKβ to block the early NF-κB activation and the associated inflammatory responses could be that the expressed dnIKKβ does not attain a sufficient level required for interruption of endogenous IKKβ. Alternatively, one can not exclude the possibility that the early NF-κB activation in the media could be elicited by an IKKβ-independent mechanism, for instance, by an IKKα dominant NF-κB pathway since IKKα was shown to be a negative modulator in inflammation in macrophage from mice[253], or by the newly identified protein ELKS, an essential regulatory subunit of the IKK complex. In line with this, silencing ELKS expression blocked expression of NF-κB target genes, including IκBα, and COX-2 and IL-8 in Hela cells[244]. However, these possibilities need to be tested in future studies.

Late NF-κB activation is associated with intimal hyperplasia (Paper I).

Given the potent high IKK activity in the intima and role for IKKβ-mediated NF-κB signaling in inflammation, we speculated that IKKβ is the predominant kinase involved in the late NF-κB activation as well as inflammatory responses in the intima. Our results demonstrated that overexpression of dnIKKβ effectively inhibited the late NF-κB activation in the intima, and resulted in downregulation of iNOS, TNF-α and MCP-1 expression along with a significant reduction in intimal size at day 14, supporting previous findings that blockade of NF-κB suppressed vascular inflammatory responses accompanying with various degrees of impact on intimal hyperplasia[307-309, 322].

Additionally, the late IKKβ/NF-κB activation possesses a potent mitogenic activity via induction of cyclin D1 and PCNA, and confers protection against apoptosis since inhibition of NF-κB by dnIKKβ causes massive apoptosis in the intima. IKKβ confers protection against apoptosis by inducing antiapoptotic molecules such as XIAP, c-IAP2 and IAP-1 as described by prior studies[305, 323, 324]. Therefore, these findings highlight that IKKβ-dependent NF-κB signaling is essential for intimal hyperplasia by regulating vascular inflammation and the simultaneous promotion of proliferation and reduction of apoptosis. However, transfer of macrophages deficient in IKKβ to the $LDLR^{-/-}$ mice even accelerates atherogenesis, as shown by increased lesion size with severe vascular inflammatory response[289]. Another study using bone marrow transfers from p50-deficient mice to $LDLR^{-/-}$ mice showed a reduction in lesion size, but with a distinct plaque phenotype, characterized by reduced foam cell numbers and an increase in immune cells[290]. Taken together, these discrepancies are likely related to three crucial factors, i.e, the targeted cell type, the time point of

inactivation of NF-κB and the targeted molecule in the NF-κB signaling pathway. Nevertheless, our study provides new insights into the role of NF-κB activation in vascular inflammation and repair, which render IKKβ in intimal SMC a promising therapeutic target for atherosclerosis and restenosis.

NGAL expression, regulation and functional roles in vascular repair (Paper II)

Gene array data of a recent study in our lab show that 24p3/NGAL is one of the most highly unregulated genes in the hypoxic stress-induced infarcted myocardium and in the atherosclerotic plaque in mice[136]. Further analysis indicates that NGAL is also highly expressed in human plaque, co-localized with endothelial cells, SMC and macrophages[136]. Importantly, marked proteolytic activity of MMP-9 co-localized where NGAL was expressed in atherosclerotic plaques. Taken together, these data suggest a potential role for NGAL in atherosclerosis. However, the underlying mechanisms of NGAL expression in vascular cells are largely unknown. Therefore, we used the same animal model and gene transfer strategy to evaluate its expression and regulation in vascular SMC.

Our data demonstrated that NGAL expression was highly induced post-angioplasty and exclusively expressed in the intima of rat carotid arteries, in conjunction with IKKβ/NF-κB activation, albeit undetectable in normal vessels both at transcriptional and protein levels. Data from *in vitro* studies showed that the intimal SMC exhibited much higher basal and inducible expression of NGAL, compared to the medial SMC. Expression of NGAL can also be induced in human vascular SMC. In line with the previous report from our lab[136], the present finding suggests that vascular SMC is an important source of NGAL in the diseased vessels.

There is evidence of NF-κB binding site in the human NGAL promoter region, although the regulatory mechanism is unclear. We hypothesized that NGAL expression might be modulated by the NF-κB signaling pathway in intimal hyperplasia. Our data showed that the upregulated NGAL expression was attenuated by dnIKKβ post-angioplasty at day 14, indicating its expression is NF-κB dependent. This notion was further confirmed by *in vitro* studies using SMC isolated from the intima and media of the rat artery and exposed to IL-1β stimulation. In this experiment, blockade of NF-κB by dnIKKβ transfection resulted in suppression of NGAL expression in SMC, suggesting that NF-κB activation serves as a regulatory mechanism for the expression of NGAL.

The ability to form a complex with MMP-9 makes NGAL a potential modulator of plaque stability, as suggested in our recent study[136], and further endows NGAL with a potential role in vascular remodeling. In the present study, we demonstrated the presence of NGAL/MMP-9 in rat SMC lysates which can be secreted into supernatants by western blotting and IP analysis. Thus our data contrast with previous findings that NGAL does not bind with MMP-9 in murine cells. This finding also highlights the need for clarification of the structure of rodent NGAL/24p3.

The precise model of action that depicts the modulation of MMP-9 activity by SMC produced NGAL is lacking, and the relevance to vascular remodeling remains to be defined. Thus, a further study using an animal model with tissue-specific knockout or overexpression of NGAL is necessary. Nevertheless, *in vitro* gelatinase zymography of cell supernatants suggests a marked increase in MMP-9 activity in intimal SMC upon IL-1β stimulation albeit no apparent alteration in the quantity of NGAL/MMP-9 complex, in agreement with a previous study[133]. To validate these *in vitro* findings and to assess the functional relevance of vascular cell expressed NGAL to MMP-9 activity in injured vessels, the proteolytic activity was also examined by *in situ* zymography. Proteolytic activity of MMP-9 was markedly increased in the intima where NGAL was abundantly expressed, but the proteolytic activity reduced in conjunction with the attenuation of NGAL expression in the vessels transduced with dnIKKβ. Taken together, these data support a plausible but yet-to-be-proven hypothesis that NGAL may be a novel mechanism regulating the vascular repair-remodeling process by enhancing MMP-9 activity.

NGAL itself may have other biological functions as well, since we found that cytosolic NGAL exists as a monomer, homodimer, and homotrimer in rat SMC. For example, NGAL has been proposed to play a role in cellular growth and re-epithelialization post tubular injury[160]. Its co-localization with PCNA and cyclin D-1 in the intima suggests that NGAL is implicated in SMC proliferation. Since NGAL is a secreted protein, increases in serum NGAL resulting from activation of neutrophils, have been proposed to reflect an acute systemic inflammatory response to events such as stroke, renal failure, or upon infection[156, 159, 160, 162]. Therefore, it is plausible that NGAL produced by vascular cells is secreted into the circulation, thus affecting its serum level, suggesting its potential as a marker of vascular dysfunctions/diseases. However, these speculations need to be tested.

During the exploration of NGAL, we have also noticed that expression of MMP-9 is dependent on IKKβ/NF-κB activation in SMC as well as in the injured artery, although in a different pattern. First, compared with slowly induced NGAL expression, MMP-9 was rapidly upregulated in the media in the early phase after vessel injury and remained highly expressed in the developing intima, suggesting that

MMP-9 and NGAL are co-expressed during intimal formation. However, intimal SMC produce much less MMP-9 both at the transcriptional and protein level, compared with medial SMC, and this does not correspond to its proteolytic activity, further confirming the regulatory complexity of MMP-9 activity.

Telomerase expression, regulation and potential roles in intimal hyperplasia (Paper III)

Functional TERT is essential for maintenance of cell proliferative capability and was shown to be involved in SMC proliferation in the genetic hypertension model[63]. We therefore hypothesized that TERT might contribute to the proliferative potential of the intimal SMC. Using the same arterial injury model, expression of TERT was examined in normal and injured vessels. Our results showed that TERT protein was abundant and co-localized with the activated NF-κB in the intima, despite its absence in normal vessels. *In vitro* data showed that a low level of basal TERT activity was detected in both the intimal and medial SMC. It was further enhanced by both TNF-α and bFGF in the medial SMC and to even greater levels in the intimal SMC, further suggesting a phenotypic difference between these two types of SMC.

We also found that activation of NF-κB is involved in regulation of TERT expression. Blockade of NF-κB using the same dnIKKβ transfection approach markedly suppressed TERT expression in the intima.

With the knowledge of the complexity of TERT regulation (See INTRODUCTION), we investigated the mechanism by which NF-κB modulates TERT expression in SMC. Analysis of the promoter activity suggests that the TERT core promoter conveys the essential transcription activity in the intimal SMC upon stimulation with bFGF and TNF-α. Furthermore, the transcriptional activation is partly mediated by c-Myc, since deletion of the E-Box in the gene promoter or over-expression of MAD1, a c-Myc competitor, abrogated TERT transcriptional activity. Therefore, TERT can be regulated by IKKβ/NF-κB and c-Myc. To elucidate the interaction between these two transcription factors in regulating TERT, c-Myc expression was evaluated in the injured vessels and in the isolated SMC. Blockade of NF-κB signaling resulted in inhibition of the up-regulated c-Myc expression. This was accompanied by reduced TERT expression in the intimal lesion and in SMC isolated from the intima, suggesting that vascular injury induces TERT expression in intimal SMC, probably via activation of NF-κB and up-regulation of c-Myc. However, we still cannot exclude the possibility of a direct effect of NF-κB on TERT expression, further study is needed to address this regulatory mechanism.

The functional role of telomerase reactivation in the intimal SMC was also evaluated by pharmacological inhibition of TERT in intimal SMC. It resulted in a dose-dependent impairment of cell proliferation along with increased cell senescence and death, suggesting a critical role for telomerase in intimal SMC proliferation. Thus, TERT could be *de novo* activated upon injury to enhance the proliferative capability of intimal SMC in the vascular repair process.

Expression and regulation of BLT1 in SMC and its role in intimal hyperplasia (Paper IV)

It has been suggested that the LTB4/BLT1 axis is implicated in atherogenesis both in mouse and man. However, direct effects of LTB4 on vascular SMC were not previously studied. Therefore, we aimed to identify LTB4 signaling on SMC, and eventually to assign functional relevance using the same rodent model of vascular remodeling.

Initially, we found that human vascular SMC express BLT1 receptors *in vivo* and *in vitro*, as demonstrated by immunohistochemical staining of human atherosclerotic plaques, and in cultured human coronary SMC by Western blotting and RT-PCR. BLT1 was also present in endothelial cells and macrophages in the lesion, suggesting that LTB4 may play multiple roles in atherosclerosis. Endothelial cells stained positive for the BLT1 receptor only in atherosclerotic but not in normal vessels, indicating an up-regulation of endothelial BLT1 receptors during atherogenesis. Consistent with a recent report that LTB4 induced endothelium-dependent formation of histamine and thromboxane A_2[325], our findings suggest a possible mechanism linking LTB4 to atherosclerosis and thrombus formation. Furthermore, monocytes expressed BLT receptors, in agreement with LTB4 being a potent trigger of monocyte adhesion[326] and MCP-1 production[327]. Interestingly, all these cells stained positive for both the BLT1 and BLT2 receptor, implicating that also the low-affinity BLT2 receptor may be involved in vascular dysregulation induced by LTB4. Of note, it has been reported that macrophages from $BLT1^{-/-}$ mice show chemotaxis toward LTB4 after activation of the BLT2 receptor[214].

Secondly, functional expression of BLT1 receptors on SMC was demonstrated by patch-clamp analysis showing an increase of whole-cell currents after challenging of human SMC with either LTB4 or U75302, a partial agonist for the BLT1 receptor. Furthermore, we also found that LTB4 induced migration and proliferation of SMC *in vitro*. Importantly, treatment with the BLT receptor antagonist BIIL 284 subsequent to carotid artery balloon injury *in vivo* inhibited intimal hyperplasia in rats. Together, these observations provide a molecular explanation for the previously reported chemotactic action of LTB4 on rat vascular SMC[328], demonstrated importance of

LTB4/BLT1 in intimal hyperplasia and identified an additional mechanism linking the 5-LO pathway to vascular pathology.

Thirdly, LTB4/BLT1 exerts its pathophysiological role on SMC via activation of IKKβ/NF-κB pathway, as demonstrated by our *in vitro* study on rat intimal and medial SMC. **1)** IL-1β or LPS induced BLT1 was suppressed by overexpression of dnIKKβ in the SMC. **2)** Intimal SMC exhibited higher levels of BLT1, compared with medial SMC, and this expression was also suppressed by dnIKKβ.

Of note, after the submission of this manuscript, Heller *et al*[329] reported that hypercholesterolemic apoE$^{-/-}$/BLT1$^{-/-}$ mice developed smaller atherosclerotic lesions with less SMC content than their apoE$^{-/-}$/BLT1$^{+/+}$ counterparts, and they showed that LTB4 stimulated migration and proliferation of murine vascular SMC, expanding the initial recognition on LTB4-induced effects on rat SMC[328, 330]. Taken together, these findings extend the role of inducible BLT1 in vascular pathology from leukocytes to the resident vascular cells.

CONCLUSIONS AND IMPLICATIONS

By using a rodent vascular injury model, the present study for the first time demonstrated that angioplastic injury induces biphasic NF-κB activation with distinct spatiotemporal features, characterized by an early activation in the arterial media and a late activation coupled with high levels of IKK activity in the intima. The early NF-κB activation is strictly involved in the acute inflammatory response in the media, but appears to be dispensable for intimal formation. The late NF-κB activation mediated by IKKβ in the intimal SMC is critical in controlling intimal hyperplasia and the associated vascular inflammation (**Paper I**). The intimal SMC reveal higher basal and inducible NF-κB activity, compared with the medial SMC, potentially contributing to the differences in gene expression profile between these two types of cells. Based on this hypothesis, we identified and characterized expression of three previously unappreciated genes, namely NGAL, TERT, and BLT1 in SMC, and studied their role in intimal hyperplasia. Our data demonstrated that NGAL, TERT, and BLT1 are highly induced after arterial injury and preferentially expressed by the proliferating intimal SMC. Induction of these genes involves IKKβ mediated NF-κB activation and is associated with vascular inflammation and intimal hyperplasisa. These findings further support the concept that NF-κB orchestrates expression of a broad array of genes, involved in intimal hyperplasia (**Paper II to IV**). In addition to mono- and homomeric forms, SMC produced NGAL could form a heterodimer with MMP-9 and secreted from the cells. Proteolytic activity was markedly increased in the intimal SMC in parallel with NGAL expression but reduced in the vessels transduced with dnIKKβ, implying that NGAL could be an unrecognized component in the regulation of proteolytic activity involved in vascular repair and remodeling (**Paper II**). In **Paper III**, we demonstrate that vascular injury induces TERT expression in intimal SMC, probably via activation of NF-κB and up-regulation of c-Myc. TERT, in turn, was involved in regulation of intimal cell proliferative capability during the vascular repair process. Finally, in **Paper IV,** we show that vascular SMC express functional BLT1 receptors, activation of which induces SMC migration and proliferation. Up-regulation of BLT1 in the intimal and medial SMC by proinflammatory cytokines occurs through an IKKβ/NF-κB dependent pathway. BLT1 receptors are expressed in human atherosclerotic lesions and promote intimal hyperplasia after angioplastic injury in the rat, suggesting that targeting BLT1 receptor on SMC may represent a therapeutic strategy in the treatment of atherosclerosis and the prevention of restenosis after coronary angioplasty.

In vivo studies on NF-κB in the scenario of atherosclerosis are limited, despite a body of *in vitro* data. Most evidence derives from animal models; however, results are controversial. The general conclusion from those findings is that NF-κB regulation is

complex and targeting therapy must be specific for cell type and for disease stage. Our present study further illustrates this complexity by characterizing the pattern of NF-κB activation using a rodent artery injury model and expands the knowledge by identification of NGAL, TERT, BLT1 expression and their regulation in SMC. They imply a central role of NF-κB in regulating vascular SMC functions in atherogenesis and intimal hyperplasia. Therefore, NF-κB may represent a promising therapeutic target, although the target cell and tissue type must be considered. Furthermore, the identification of NGAL, TERT and LTB4/BLT1 in SMC also extends this field and these initial findings bring new areas of interest for future research.

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