Toll-like receptor 4 signaling in atherosclerotic disease

atherosclerosis, arterial remodeling, cross-talk

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Contents

Chapter 1

Introduction

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Introduction

Atherosclerosis

Atherosclerosis is the underlying cause of major cardiovascular events such as myocardial infarction and stroke, two of the most common causes of illness and mortality in the Western world. During atherosclerosis, the arterial wall gradually thickens to form an atherosclerotic plaque, which leads to narrowing of the arterial lumen. The onset of atherosclerotic disease can be triggered by risk factors like hypertension, diabetes mellitus, smoking, high fat diet, stress and high plasma concentrations of low-density lipoprotein (LDL) cholesterol. These risk factors induce endothelial dysfunction that leads to monocyte adherence and infiltration into the endothelial layer. Fatty streaks develop already during early childhood [1] and may progress into advanced atherosclerotic plaques. Plaque development is characterized by migration and proliferation of smooth muscle cells and accumulation of lipids in the sub-endothelial layer. Dependent on the composition of the advanced atherosclerotic lesion, stable and unstable lesions can be discriminated. Unstable lesions or rupture-prone lesions are characterized by a thin fibrous cap, a large atheroma, infiltrated and activated inflammatory cells (predominantly macrophages), and low smooth muscle cell content. Clinical symptoms appear in a later stage of life, when the lesions have developed into unstable atherosclerotic plaques. When the plaque ruptures, the lipidrich plaque content comes in contact with the blood and immediately initiates thrombus formation, which often results in an acute occlusion of the artery (Figure 1). In addition to plaque formation, the lumen of the atherosclerotic artery is also determined by arterial remodeling (Figure 1). Inward remodeling accelerates the process of luminal narrowing, while outward remodeling compensates for lumen loss by lesion formation in the early phase of atherosclerosis. This seems a favorable process; however, outward remodeling is considered to be a hallmark of vulnerable, rupture-prone plaques, which cause clinical symptoms [2].

Arterial remodeling

An artery is not a rigid tube, only responsible for blood supply. In response to hemodynamic, mechanical and biological stimuli the artery and the extracellular matrix supporting the artery are capable to adapt its size and structure. Three different arterial layers can be distinguished; the tunica intima, existing of connective tissue and a single layer of endothelial cells lining up the artery at the luminal site. The endothelial cells are able to sense differences in stretch or shear stress. The medial layer is the tunica media,

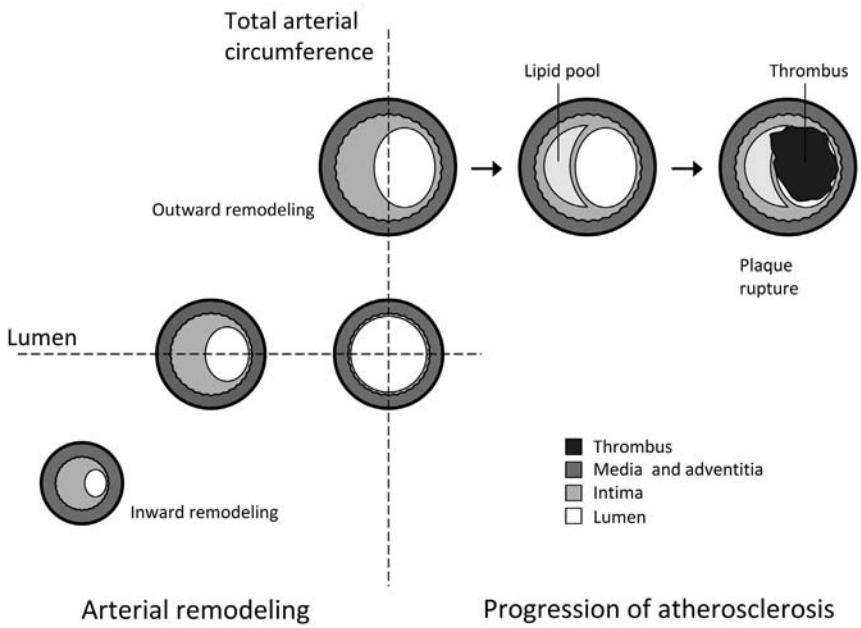

Figure 1. Schematic representation of arterial remodeling together with atherosclerotic plaque formation

Inward arterial remodeling accelerates luminal narrowing, while outward remodeling compensates for intima formation. Outward remodeling is associated with unstable plaques, vulnerable for rupture, and when the plaque ruptures the thrombogenic plaque contents come in contact with the blood and induce thrombus formation.

which mainly consist of smooth muscle cells embedded in extracellular matrix molecules like collagen, fibronectins and other proteoglycans and is involved in adaptations to changes in blood pressure. The outer layer is called the tunica adventitia, consisting of fibroblasts and extracellular matrix.

A change in shear stress by an acute change in blood flow leads to dilation or constriction of the artery. If these changes in blood flow become chronic, structural adaptations will be made. This process is called arterial remodeling and is defined as a structural change in arterial size compared to a control or reference artery. An increase in size is referred to as outward remodeling, while a decrease size is inward remodeling. During arterial remodeling, the structural changes of the arterial wall involve matrix turnover [3-6], cell migration [7] and inflammation [8].

Inflammation and immunity in atherosclerosis and arterial remodeling

The immune system responds effectively to harmful agents by close interplay between the innate and the adaptive immune recognition systems. The innate immune system is limited to the recognition of evolutionary highly conserved pathogen motifs and is considered to be the first line of defense. The adaptive immune system involves dynamic adaptation to unique epitopes present on pathogens in the environment.

Atherosclerosis

Atherosclerosis is a chronic inflammatory disease of the vascular wall [9]. The immune response is evident during initiation and progression of atherosclerotic disease [9,10]. Susceptibility to plaque formation is the greatest in vascular regions where haemodynamic stress is changed [1], such as bifurcations and curvatures. At these low-shear rate sites, the endothelial cells become activated and permeability, adhesion molecule expression on the endothelial surface and chemokine production increases.
Atherosclerotic lesions mostly hide cells that play an important role in the first line of defense against exogenous ligands, particularly macrophages and T-lymphocytes.
The monocytes and T-cells attach to the endothelial layer and home-in on the arterial wall during the initiation of atherosclerosis. Oxidative processes and subsequent endothelial dysfunction are recognized as aetiological factors in monocyte adherence to the endothelial layer. In addition, monocytes/macrophages can be activated by infectious agents, which have been suggested to play a role in atherosclerotic plaque formation, but results are conflicting and causality is difficult to prove in humans [11-13].

Arterial remodeling

Arterial remodeling is a 'wound healing'-like response of the arterial wall to sustained changes in bloodflow, arterial injury after balloon angioplasty or de novo atherosclerosis. During restructuring of the artery, inflammation and subsequent matrix turnover and cell migration are involved. Matrix degradation in the arterial wall involves matrix metalloproteinases (MMPs) [6,14-16]. MMPs are proteolytic enzymes capable of degrading molecules in the extracellular matrix. MMP activity levels are increased during remodeling [15] and without MMP-9, no structural adaptation can be induced [6]. Next to matrix breakdown, also cell migration is hampered in absence of MMPs. Genetic mouse models lacking MMP expression and studies using MMP inhibitors both show a reduction in cell migration, and a decrease in arterial remodeling [6,17,18].
During arterial remodeling not only collagen breakdown, but also collagen synthesis is increased. Collagen synthesis is essential for the maintenance of structure of an artery

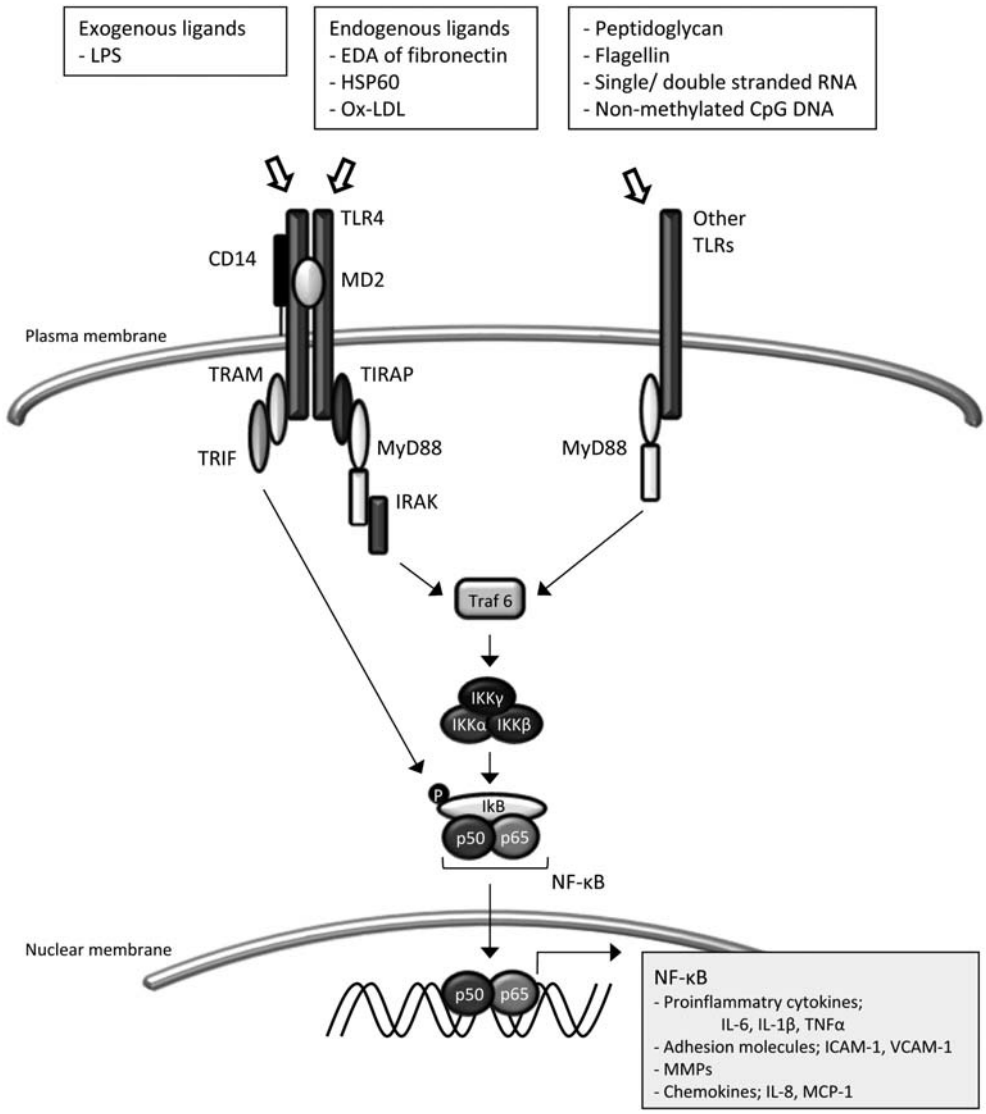

Figure 2. Schematic representation of Toll-like receptor signaling

TLRs can be activated by either exogenous or endogenous ligands. Upon ligand binding TLRs recruit intracellular signaling adaptors to their receptor-signaling domain, resulting in activation and nuclear translocation of NF-kB. NF-kB activates pro-inflammatory cytokines, like IL-6, IL-1b and TNF-a, increases expression of adhesion molecules such as ICAM-1 and VCAM-1, and increases expression of metalloproteinases and release of chemokines, e.g. MCP-1 and IL-8.

and is related to cell migration [19]. Active collagen synthesis is associated with cell migration towards the neointima, and intact collagen can promote cell migration as a chemotactic factor [20,21] or by providing a substrate for cell migration [22]. Collagen synthesis and breakdown are well-balanced during outward remodeling, collagen turnover is increased, while total collagen content in the arterial wall does not change [23], to keep the integrity of the vessel wall but also allow cell migration.
The presence of macrophages in the atherosclerotic wall is associated with outward arterial remodeling. These cells produce the MMPs capable of degrading the molecules in the extracellular matrix.

Toll-like receptors

The innate immune system is the first line of defense against pathogens. Toll-like receptors (TLRs) recognize pathogen-associated molecular patterns (PAMPs) [24] and activate the inflammatory cell via the NF-κB pathway (Figure 2) [25-27]. Not only macrophages express these TLRs: cells commonly found in the vessel wall express TLR proteins, such as endothelial cells [28], adventitial fibroblasts [29], and vascular dendritic cells [30,31].

Toll-like receptor signaling and Nuclear Factor-kappa B

Ligand binding to TLRs transmits a transmembrane signal that activates nuclear translocation of Nuclear Factor-kappa B (NF-kB) (Figure 2) [25,26,32]. NF-kB controls the transcription of inflammatory genes and is composed of homo- and heterodimers of Rel family proteins (p65 (RelA), RelB, c-Rel, p50 (NF-kB1) and p52 (NF-kB2)) [33]. In non-activated state the NF-kB dimers are captured in the cytoplasm bound to inhibitoryprotein B (IkB). NF-kB activation via Toll-like receptors or cytokines results in translocation of predominantly p50/p65 heterodimers to the nucleus. The NF-kB p50 subunit lacks a transactivation domain [34] and forms heterodimers with subunits that are transcriptionally active. Homodimers of p50 are capable to bind to the DNA and thereby inhibit transcription activity by other NF-kB dimers. NF-kB p50 homodimers therefore can act as a brake on NF-kB signaling [35,36]. Indeed, several studies report that p50 has an inhibitory effect on TLR4 signaling. Upregulation of p50 homodimers attenuates lipopolysaccharide (LPS) response and is essential for LPS tolerance [37], and NF-kB p50 inhibits a LPS-induced shock [38]. The exact role and regulation of the individual components of the NF-kB complex are not well-understood and are under intense investigation.

Toll-like receptor 4

TLR4 was identified as the first human homologue of the Drosophila Toll [39]. TLR4 is well known as the receptor for lipopolysaccharide (LPS), a product of the outer membrane of Gram-negative bacteria [39-41]. TLR4 is the only TLR for which endogenous ligands have been identified. Furthermore, genetic variation in the human TLR4 gene is associated with carotid intima thickness, and therefore an interesting receptor to study in relation to atherosclerotic disease.

Arbour *et al.* [42] reported that a point mutation in the TLR4 gene cause hyporesponsiveness to LPS in humans. There is increasing evidence supporting the role of TLR4 in atherosclerotic lesion development. Toll-like receptor 4 expression has been described in human atherosclerotic lesions [28]. Activation of TLR4 stimulates mononuclear phagocytes to secrete chemokines which are recognized for their involvement in the recruitment of monocytes and T lymphocytes in the arterial wall [43]. The production of cytokines induced after TLR4 activation influences proliferation and migration of vascular smooth muscle cells [11]. In addition, TLR4 activation leads to higher expression levels of MMP-2, -9 and cathepsins; proteins involved in matrix break down [44,45].

Toll-like receptor 4 and atherosclerosis

In human atherosclerotic plaques, elevated mRNA and protein levels of TLR1, TLR2 and TLR4 have been found [10,46]. In those atherosclerotic plaques, expression of TLR2 and TLR4 has been described in macrophages and endothelial cells. TLR2 and TLR4 expression by endothelial cells in normal arteries is low, but significantly increased in endothelial cells lining atherosclerotic lesions [28]. Xu *et al.* showed that TLR4 is expressed in lipid-rich, macrophage-infiltrated atherosclerotic lesions of mice and humans [47].

Bone marrow transplantation of the atherosclerotic ApoE-/- or ApoE+/+ to a C3H/HeJ host, which has a critical point mutation in the TLR4 molecule, revealed no differences in atherosclerotic lesion formation [48]. However, a lower number of macrophages was observed in the atherosclerotic lesions of the bone marrow recipient C3H/HeJ mice compared with control mice [48].
It could be suggested that the recruitment of monocytes into the arterial wall is affected in TLR4-deficient C3H/HeJ mice. Macrophages and endothelial cells in human atherosclerotic plaques express TLR4. Stimulation with ox-LDL, a trigger for atherogenesis, induces an up-regulation of TLR4 expression in macrophages in vitro, suggesting a role for TLR4 in lipid-mediated proinflammatory signaling [47].

Injections of LPS in rabbits on hypercholesterolemic diets and in ApoE-/- mice increased the rapid development of atherosclerotic disease [49,50]. Michelsen *et al.* demonstrated that TLR4-/- mice crossed onto the ApoE-/- background show significantly reduced atherosclerotic lesion development [51] with lesions containing less lipids and macrophages. Furthermore, in hyperlipidemic mice lacking expression of MyD88, a downstream adaptor molecule in TLR signaling, early atherosclerosis is significantly reduced [51,52]. In those mice macrophage recruitment to the arterial wall is decreased resulting in reduced chemokine levels.
The inflammatory response in the atherosclerotic artery is not limited to the luminal side of the vessel wall. Inflammatory infiltrates are frequently present in the adventitia of atherosclerotic vessels as well [53]. In the adventitial layer of the atherosclerotic coronary artery, not only macrophages but also adventitial fibroblasts express the TLR4 [29]. Activation of adventitial fibroblasts by LPS in mice, using a cuff model, induces formation of intimal lesions. In C3H/HeJ mice with the TLR4 point mutation, this intima formation was reduced by 60% compared with wild-type mice [29].

Toll-like receptor 4 polymorphism and cardiovascular disease
Recently, also the first human studies confirmed the role of TLR4 in the progression of atherosclerotic disease. Kiechl *et al.* [54] showed that humans with the Asp299Gly TLR4 polymorphism had a lower risk of carotid atherosclerosis and less intima media thickness in the common carotid artery. Their results were confirmed in a case–control study in which acute coronary syndromes was the primary endpoint [55]. However, also conflicting data have recently been published. In a large cohort of 1400 participants, the presence of the Asp299Gly polymorphism was not associated with predisposition or progression of atherosclerotic disease [56]. Moreover, longitudinal progression of intima media thickness was not affected by this polymorphism in patients suffering from familiar hypercholesterolemia [57]. The inclusion criteria and thereby the domain of these studies merit careful consideration, as they differed substantially.
The TLR4 polymorphism Asp299Gly is also found to be associated with an increased incidence of cardiovascular events. Boekholdt *et al.* showed that the Asp299Gly polymorphism is also associated with a higher efficacy of statin therapy [58]. Statin therapy resulted in an average reduction of cardiovascular events by 50%. Strikingly, the risk on cardiovascular events in noncarriers was reduced from 18.1% to 11.5%, while in carriers of the Asp299Gly allele, the risk was reduced from 29.6% to 2% [58]. Thus, an impressive reduction of cardiovascular events by statin therapy was mainly observed when the TLR4 polymorphism was present. Edfeldt *et al.* studied the

Asp299Gly genotype in relation to myocardial infarction and found that among 852 cases and 1054 controls the Asp299Gly genotype was associated with an increased risk of myocardial infarction in men [59]. Other groups did not find significant associations between Asp299Gly and atherosclerosis. Zee *et al.* reported no association with risk of atherothrombosis, myocardial infarction or stroke [60].

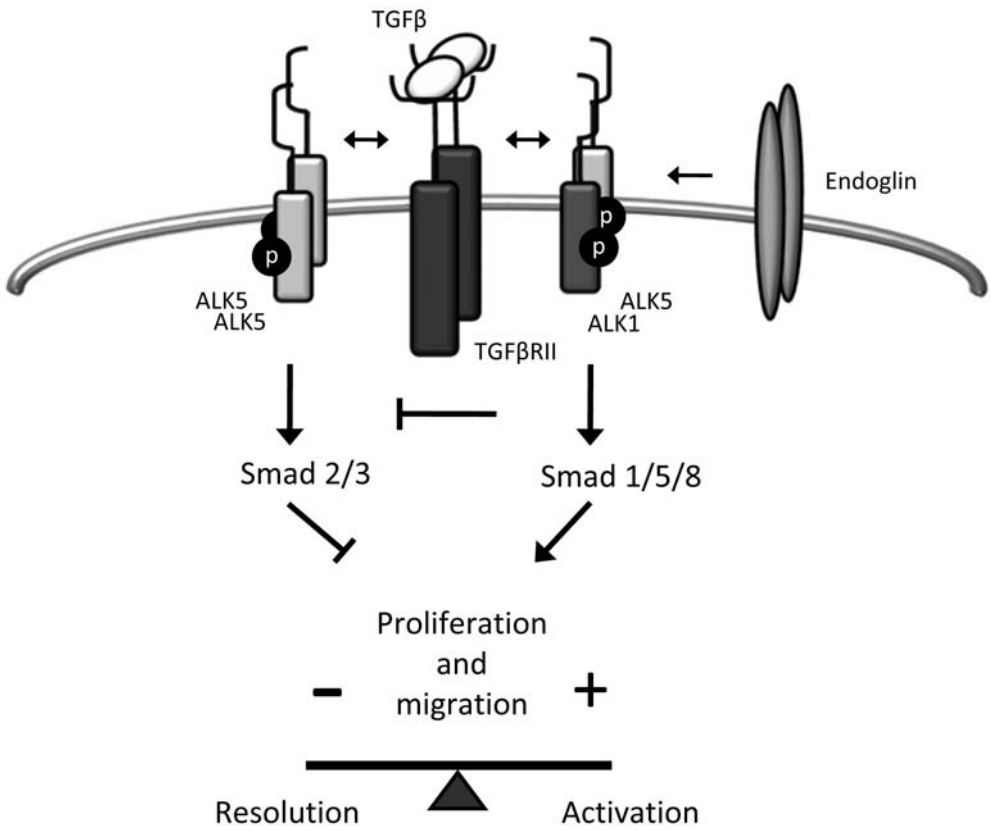

Figure 3. TGFß signaling pathway

The TGFß/ALK5 activation via SMAD2/3 leads to inhibition of cell proliferation and migration, whereas the TGFß/ALK1 via SMAD1/5 induces cell proliferation and migration.

Endoglin, the accessory receptor for TGFß is required for ALK1 signaling. In absence of Endoglin TGFß/ALK5 signaling is predomininant, while high Endoglin expression stimulates ALK1 signaling and leads to cell activation.

Endogenous Toll-like receptor 4 ligands

Evidence is accumulating that not only exogenous ligands are able to activate TLRs but that they can also be stimulated by endogenous ligands that are mostly produced during injury. The best described endogenous ligands are the ligands for TLR4 that are produced during stress or cell damage. Among others, HSP60 [61] and EDA [44] have been described to activate TLR4. These ligands have been associated previously with arthritic [62] and oncological disease [63,64]. These pathologies have, like atherosclerotic disease, local inflammation and matrix turn-over in common.

Heat-shock proteins are highly conserved molecules which participate in protein folding and assembly, and are crucial for correct transportation of proteins through the cell. Heat-shock protein synthesis is dramatically increased during cellular stress. The C3H/HeJ mice strain has a nonfunctional TLR4 due to a point mutation in the TLR4 gene and is resistant to human HSP60-induced macrophage activation, which suggests a role for TLR4 in cellular activation by human HSP60 [61]. It should be recognized, however, that publications dealing with endogenous TLR ligands are based on in vitro and artificial in vivo data. This becomes more relevant, as recent publications have revealed that these experimentally used potential TLR ligands often suffer from LPS contaminations [65-67].

EDA is an extra domain of fibronectin. This domain is incorporated by alternative splicing of fibronectin mRNA, and is expressed during cell injury. EDA is able to induce the production of interleukins [68]. Okamura *et al*. [44] showed that EDA is capable of activating NF-kB via TLR4. Similar to LPS and HSP60, the response to EDA is blunted in C3H/HeJ cells. Evidence for a role in atherosclerosis is accumulating; EDA levels are elevated in plasma of ApoE-/- mice and EDA mRNA levels are increased during outward remodeling. Furthermore, in ApoE-/- mice lacking EDA, atherosclerotic lesion areas are reduced, lesions are thinner and lipid content of foam cells is lowered compared to lesions in animals that do express EDA fibronectin [69].

Toll-like receptor and Transforming Growth Factor crosstalk

Transforming Growth Factor-beta (TGFß) is generally considered as an anti-inflammatory factor involved in regulation of fibrosis. TGFß regulates cell proliferation, migration and production of collagen [70]. Although involvement of TGFß in arterial remodeling has been shown, results have been ambiguous. Inward arterial remodeling can be inhibited using a TGFß signaling inhibitor [71] while adenoviral overexpression of TGFß (which would stimulate TGFß signaling) also inhibits inward arterial remodeling [72].

This might be due to the existence of two distinct TGFß receptor pathways [73] depending on TGFß concentration and/or duration of TGFß stimulation to mediate downstream events. These pathways are the Activin receptor-like kinase 5 (ALK5) pathway stimulating collagen production but inhibits cell proliferation, migration and MMP production via SMAD-2 and -3 while the ALK1 pathway stimulates cell proliferation, migration and MMP production but inhibits collagen production via SMAD-1 and -5 (Figure 3). It is not known whether both or only one of these pathways is involved in the regulation of atherosclerotic plaque stabilization but distinct signaling pathways activated by the same ligand may reconcile the apparent contradictory outcomes described above.

Although not in cardiovascular disease, evidence is emerging that the TGFß and TLR4 pathways communicate. Blocking serum TGFß in the mouse increased the severity of malaria infection suggesting that TGFß inhibits the (TLR) innate immunity response [74]. This is supported by Jono *et al.* [75] showing that the TGFß pathway cooperates with NF-kB after an influenza infection. Recently, it was shown that Ecsit, an obligatory protein in the Toll-like signaling pathway, is also essential for signaling of the TGFß superfamily [76] establishing a novel molecular link between the TLR and TGFß family [77]. Next to this, deficiency of TGFß in mice leads to extensive inflammation ad death within 3 weeks. In these young TGFß null mice, TLR4 expression was increased and associated with the inflammatory sequelae while a defective TLR4 increased the life span [78].

Aim and outline of this thesis

The last decade the role of TLRs and in particular TLR4 during intima formation and in atherosclerotic disease is subjected to intensive research. In this thesis, we focus on the role of TLR4 and downstream NF-kB components in outward arterial remodeling. In chapter 2 of this thesis the role of TLR4 in outward arterial remodeling is investigated and chapter 3 describes how the p50 subunit of NF-kB is involved in outward remodeling. A role for endogenous TLR4 ligand EDA in arterial remodeling has been postulated, and its potential as a marker for atherosclerotic disease is suggested. In chapter 4 the role of EDA in arterial remodeling is assessed in a mouse model, and the relation between EDA expression and plaque phenotype in human atherosclerotic lesions is determined in chapter 5.

Evidence is accumulating for communication between Toll and TGF signaling cascades. Also in atherosclerosis a role for both signals separately is described. However, whether components of TLR4 and TGF pathways are associated in human atherosclerotic lesions is not known. In this thesis the relation between endogenous TLR4 ligand EDA and TGFß accessory receptor Endoglin is investigated in chapter 6.

As a result of TLR and TGF communication both stimulatory and inhibitory effects are described. In chapter 7 in vitro experiments are performed to identify genes regulated by an interaction of TLR and TGF signals. These genes are of interest to study in human atherosclerotic lesions as well. In chapter 8 the data of preceding chapters are summarized and discussed.

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Chapter 2

Toll-like receptor 4 is involved in outward arterial remodeling

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Abstract

Background

Toll-like receptor 4 (TLR4) is the receptor for exogenous lipopolysaccharides (LPS). Expression of endogenous TLR4 ligands, heat shock protein 60 (Hsp60) and extra domain A of fibronectin, has been observed in arthritic and oncological specimens in which matrix turnover is an important feature. In atherosclerosis, outward remodeling is characterized by matrix turnover and a structural change in arterial circumference and is associated with a vulnerable plaque phenotype. Since TLR4 ligands are expressed during matrix turnover, we hypothesized that TLR4 is involved in arterial remodeling.

Methods and results

In a femoral artery cuff model in the atherosclerotic ApoE3 (Leiden) transgenic mouse, TLR4 activation by LPS stimulated plaque formation and subsequent outward arterial remodeling. With the use of the same model in wild-type mice, neointima formation and outward remodeling occurred. In TLR4-deficient mice, however, no outward arterial remodeling was observed independent of neointima formation. Carotid artery ligation in wild-type mice resulted in outward remodeling without neointima formation in the contralateral artery. This was associated with an increase in TLR4 expression and EDA and Hsp60 mRNA levels. In contrast, outward remodeling was not observed after carotid ligation in TLR4-deficient mice.

Conclusions

These findings provide genetic evidence that TLR4 is involved in outward arterial remodeling, probably through upregulation of TLR4 and TLR4 ligands.

Key Words: remodeling • arteries • plaque • atherosclerosis • proteins

Introduction

Toll-like receptor 4 (TLR4) is the receptor for exogenous lipopolysaccharide (LPS) [1], endogenous heat shock protein 60 (Hsp60) [2], and extra domain A of fibronectin (EDA) [3]. TLR4 expression has recently been described in atherosclerotic arteries in endothelial cells, macrophages [4,5], and adventitial fibroblasts [6]. Using a mouse femoral cuff model, we recently demonstrated that TLR4 is involved in neointima formation [6]. Moreover, TLR4 polymorphism is associated with carotid intima thickness in humans [7]. However, a function for TLR4 in arterial geometrical remodeling, the other determinant for arterial lumen loss, is unknown.

Expression of endogenous TLR4 ligands, Hsp60 and EDA, has been observed in arthritic [8] and oncological specimens [9,10] in which matrix turnover is an important feature.

Matrix turnover in arteries occurs during arterial remodeling in response to sustained blood flow changes [11,12], balloon injury [13,14], and atherosclerotic plaque formation [15]. Arterial remodeling, which can be outward to reduce lumen loss and inward to increase lumen loss, is characterized by structural changes of the arterial wall involving cell migration and collagen (matrix) breakdown [16] by matrix metalloproteases (MMPs), including MMP9, which can be stimulated by TLR4 activation [3].

Outward remodeling in arteries is an important determinant for lumen loss because it can compensate for plaque accumulation in the arterial lumen [17]. However, although the luminal area is preserved, the plaque beneath the surface of the lumen often has a vulnerable plaque phenotype [18]. Next to a vulnerable plaque phenotype, outward remodeling is associated with aneurysm formation [19] and shear stress–induced arteriogenesis [20].

Previous observations revealed enhanced expression of TLR4 ligands in non–vascular tissue remodeling. In the current study, we hypothesized that TLR4 is involved in arterial remodeling. We studied arterial plaque formation and subsequent arterial remodeling after TLR4 activation in the atherosclerotic ApoE3 Leiden mouse. We investigated the involvement of TLR4 in femoral artery remodeling with neointima formation and carotid artery remodeling without neointima formation by using wild-type and TLR4-deficient mice. Furthermore, expression and localization of carotid TLR4 was determined and Hsp60 and EDA mRNA levels were measured. This revealed that TLR4 is involved in the outward arterial remodeling process and shows that TLR4 and its endogenous ligands are upregulated during remodeling.

Methods

Animal experiments

Femoral cuff placement was performed in male ApoE3 Leiden mice that were crossbred for 18 generations with C57BL/6 mice (8 to 12 weeks old) and received a high-fat, cholesterol-rich (HFC) diet throughout the experimental period starting 4 weeks before surgery, as described before [21]. LPS (1 µg/µL) or PBS (control) dissolved in gelatin was administered inside the cuff [6]; mice were killed 3 weeks after cuff placement (+LPS, n=10; control mice, n=10). Femoral cuff placement with or without LPS (1 µg/µL) was also performed in female BALB/c (n=18) and C.C3H-*Tlr4*$^{LPS\text{-}d}$ mice (TLR4-deficient mouse on a BALB/c background, Jackson Laboratory, n=18; age, 12 to 20 weeks) and killed 3 weeks after cuff placement [6].

Carotid artery ligation [22] was performed as described by de Kleijn *et al.* [23] in 14 BALB/c and 17 C.C3H-*Tlr4*$^{LPS\text{-}d}$ mice by using the carotid artery contralateral to the ligation as a model for outward remodeling. Nonligated female BALB/c and C.C3H-*Tlr4*$^{LPS\text{-}d}$ mice served as control (BALB/c n=5; C.C3H-*Tlr4*$^{LPS\text{-}d}$ n=5). All arteries used for morphometry were perfusion-fixed first for 3 minutes through the left ventricle with PBS plus 10 to 4 mol/L nitroprusside at 99 mL/h to get maximal dilation of the arteries and normal intraventricular pressure for nitroprusside-treated mice (50 to 60 mmHg [24]). Intraventricular pressure was measured with a pressure sensor with data recorder (Spacelabs) connected between the pump and the needle in the left ventricle. This was followed by a 3-minute perfusion with 4% paraformaldehyde in PBS plus 10 to 4 mol/L nitroprusside at 99 mL/h to fix the arteries at maximal dilation and normal pressure.

Carotid ligation was also performed in another group of 60 female BALB/c mice to measure carotid RNA and protein levels at 0, 3, 5, 8, 20, and 28 days after ligation (n=10/time point), as described previously by de Kleijn *et al.* [23].

The ethics committee on animal welfare of TNO (The organization for Applied Scientific research) and the Utrecht University approved the animal experiments.

Definition of arterial remodeling

Arterial remodeling is defined as a change in arterial size compared with a control or reference artery. An increase in size is defined as outward remodeling, whereas a decrease in size is defined as inward remodeling. As a measure of arterial size, we used the external elastic lamina (EEL) area calculated from the EEL length including corrugations. For the cuff models, the EEL areas are compared between the cuff with

and without LPS (reference). For the ligation model, EEL areas are compared between the carotid artery contralateral to the ligated carotid artery and unligated carotids (reference).

Morphological quantification in sections of cuffed femoral artery and contralateral unligated carotid artery

Paraffin sections were stained with either elastin–van Gieson or HPS. Ten equally spaced (200 µm) cross sections were used in ApoE3 Leiden mice and 3 to 6 in the BALB/c and C.C3H-*Tlr4*$^{LPS\text{-}d}$ mice to quantify intimal lesions and EEL. Using image analysis software (Leica or Analysis), total cross-sectional medial area was measured between the external and internal elastic lamina; total cross-sectional intimal area was measured between the endothelial cell monolayer and the internal elastic lamina.

Quantitative RT-PCR

Tripure (Roche) was used for isolation of RNA and protein according to the manufacturer's protocol. Mouse TLR4 (forward: 5'-tcacctctgccttcactac-3'; reverse: 5'-caaagatacaccaacggctc3') EDA (forward: 5'-acgtggttagtgtttatgctc-3'; reverse: 5'-tggaatcgacatcca-catcag-3'), Hsp60 (forward: 5'-accgtctattgccaaggag-3'; reverse: 5'-cagcaattacagcatcaacag-3'), and 18S (forward: 5'-tcaacacggg-aaacctcac-3'; reverse: 5'-accagacaaatcgctccac-3') primers were designed with the use of the Prime Program at CMBI (Nijmegen). cDNA synthesis, and quantitative PCR was performed as described before [23].

Western blotting

Denatured samples (6 µg/lane) with ß-mercaptoethanol were separated on a 10% SDS polyacrylamide gel and blotted to a Hybond-C membrane (Amersham Pharmacia). Blocking and incubation steps were done in 5% defatted dry milk in PBS/0.1% Tween 20. Blots were incubated with rabbit anti-human TLR4 (1:500, Santa Cruz Biotechnology), biotin-labeled swine anti-rabbit (1:2000, DAKO) followed by streptavidin horseradish peroxidase (1:1000, Vector Laboratory). Bands were visualized with the use of the ECL kit (Amersham). Optical density of TLR4-positive bands was measured with the use of the GelDoc system (Biorad) and expressed in arbitrary units.

Immunohistochemistry

Mouse femoral or carotid arteries were embedded in paraffin. Four-micrometer sections were cut and deparaffinized. Endogenous peroxidase was quenched in MeOH

containing 1.5% H_2O_2 for 30 minutes at room temperature. Antigen retrieval was performed by boiling sections for 20 minutes in 10 mmol/L citrate buffer, pH 6.0. Sections were blocked with 10% normal swine serum and incubated overnight at 4°C with rabbit antihuman TLR4 immune serum (1:50, Santa Cruz Biotechnology, Inc). Sections were then incubated 1 hour. at room temperature with biotin labeled swine anti-rabbit (1:1000, DAKO) followed by streptavidin horseradish peroxidase (1:1000, Vector Laboratory). As a negative control, the immune serum was replaced with nonimmune rabbit IgG (4 µg/mL, Vector Laboratory). TLR4 was visualized with AEC staining (red), and nuclear staining was performed with hematoxylin.

Statistics

All data are presented as mean ± SEM. The Mann-Whitney test was used for all experiments except for the mRNA and protein measurements. To determine whether there was an upregulation in mRNA or protein level over time, the logarithm was taken and ANOVA with a Bonferroni post hoc test was performed. A value of $P<0.05$ was regarded as significant.

Results

TLR4 stimulation in an atherosclerotic mouse model

After 3 weeks, stimulation with LPS of the cuffed femoral artery in ApoE3 Leiden mice on a HFC diet resulted a larger EEL area compared with the group without LPS application (Table, P=0.009, Figure 1). The group with LPS application also showed a larger plaque area, mainly consisting of accumulated smooth muscle cells and foam cells as described before [21], compared with the group without LPS application (Table, P=0.011, Figure 1). The cholesterol levels are comparable in both groups (control group, 17.7 ± 7.9 mmol/L versus LPS group, 16.3 ± 4.6 mmol/L).

TLR4 and outward arterial remodeling in a femoral cuff model with neointima formation

The femoral cuff model in BALB/c and C.C3H-*Tlr4*$^{LPS\text{-}d}$ mice (a TLR4-deficient mouse on a BALB/c background) showed that after LPS stimulation, wild-type mice revealed a larger EEL area in comparison to the nonstimulated wild-type mice (Table, P=0.019, Figure 2A). The C.C3H-*Tlr4*$^{LPS\text{-}d}$ mice, however, did not show an increased EEL after

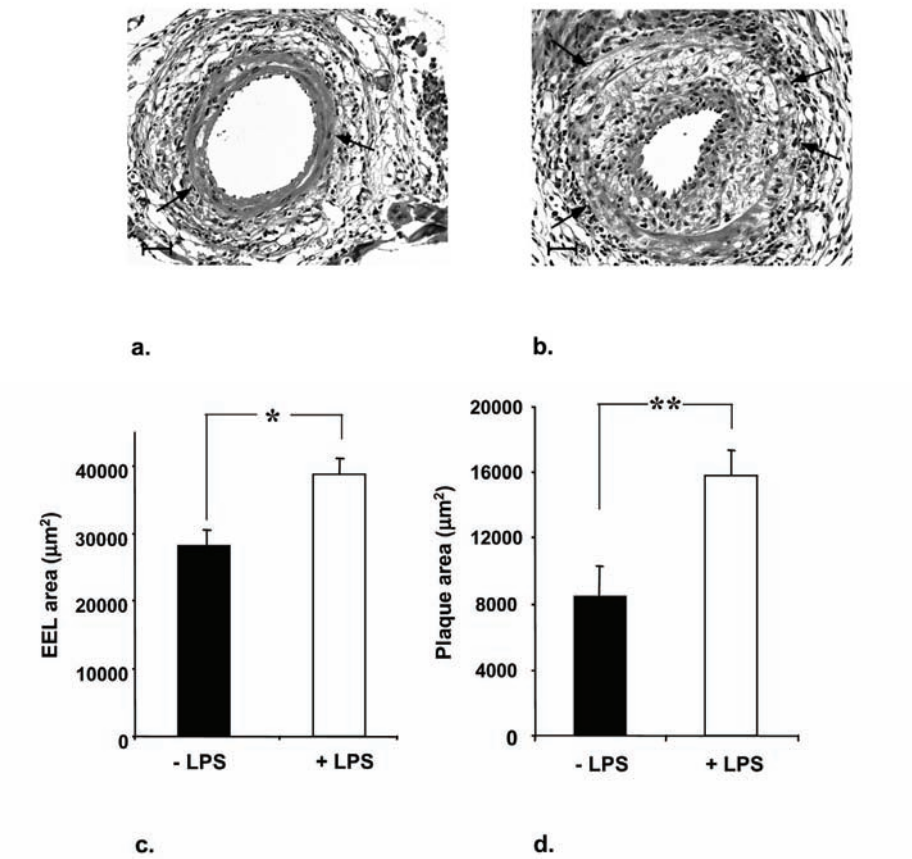

Figure 1.
HPS staining of femoral artery cross sections of APOE3 Leiden mouse treated with periadventitial cuff and gelatin (A) and with cuff and gelatin containing LPS (B). Arrows indicate EEL. EEL area (C) and plaque area (D) of the femoral artery of APOE3 Leiden mice after cuff placement with or without stimulation of LPS. n=10 mice per group. *P=0.009, **P=0.011. Bar =50µm.

LPS stimulation compared with the nonstimulated C.C3H-*Tlr4*$^{LPS\text{-}d}$ mice (Table and Figure 2A). Neointima formation was less in the TLR4-deficient mice compared with the wild-type mice but is increased in the LPS-stimulated TLR4-deficient mice compared with the nonstimulated TLR4-deficient mice (Figure 2B). The wild-type mice showed a correlation between the EEL area and intima area (r=0.525, P=0.024, Figure 2C), which was absent in the C.C3H-*Tlr4*$^{LPS\text{-}d}$ mice (Figure 2D). These data suggest that TLR4 is involved in the outward remodeling process in a model with neointima formation.

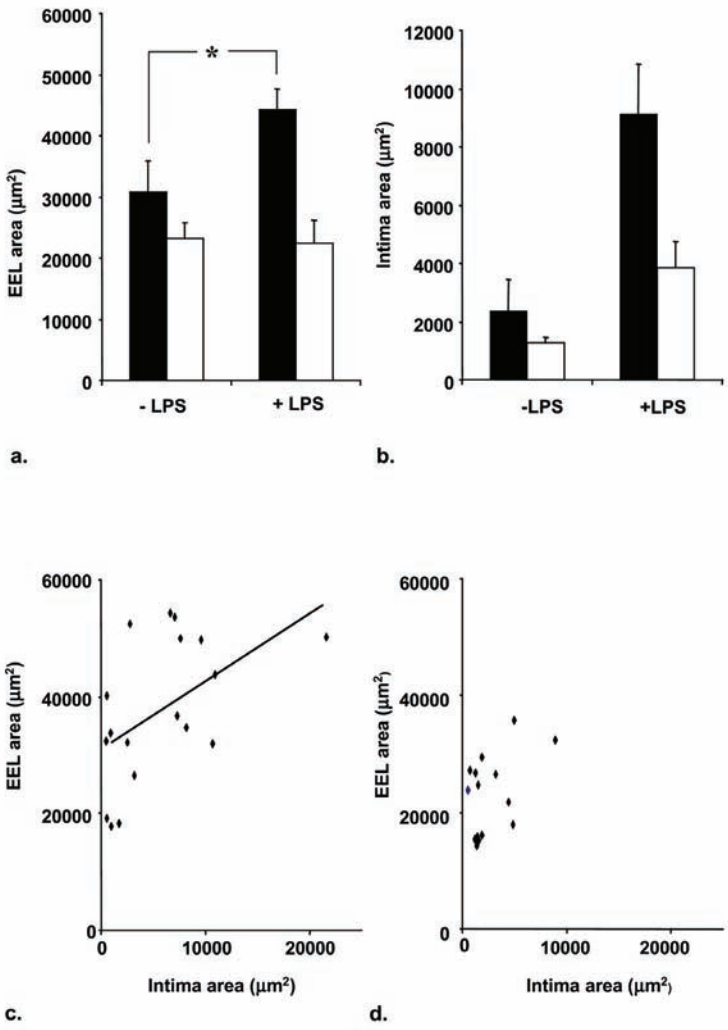

Figure 2.
EEL area (A) and intima area (B) of wild-type (Wt) BALB/c (black bar) and C.C3H-Tlr4$^{LPS\text{-}d}$ (white bar) mice treated with periadventitial cuff containing gelatin with or without LPS. n=7 to 9 mice per group, *P=0.019. Intima area versus EEL area plot in wild-type mice (C) (P=0.024) and in C.C3H-Tlr4 LPS-d mice (D).

TLR4 and outward arterial remodeling in a flow cessation model

We used the carotid artery contralateral to the ligation as a model in which only outward remodeling occurs [22]. Twenty-eight days after ligation, the BALB/c mice showed in the contralateral arteries an increase in EEL area (23330 ± 5644 μm^2, P=0.001, Table) compared with nonligated control arteries, whereas in C.C3H-*Tlr4*$^{LPS\text{-}d}$ mice, no increase in EEL area was found (EEL area, 6952 ± 5509 μm^2, P=0.628 Table). The BALB/c mice also showed an increase in media area compared with the control arteries (6696 ± 1279 μm^2, P=0.001, Table). In contrast, media area in C.C3H-*Tlr4*$^{LPS\text{-}d}$ mice was not increased (2006 ± 1086 μm^2, P=0.288, Table). Increase in carotid media and EEL area showed a significant difference between wild-type and C.C3H-*Tlr4*$^{LPS\text{-}d}$ mice (Figure 3, A and B).

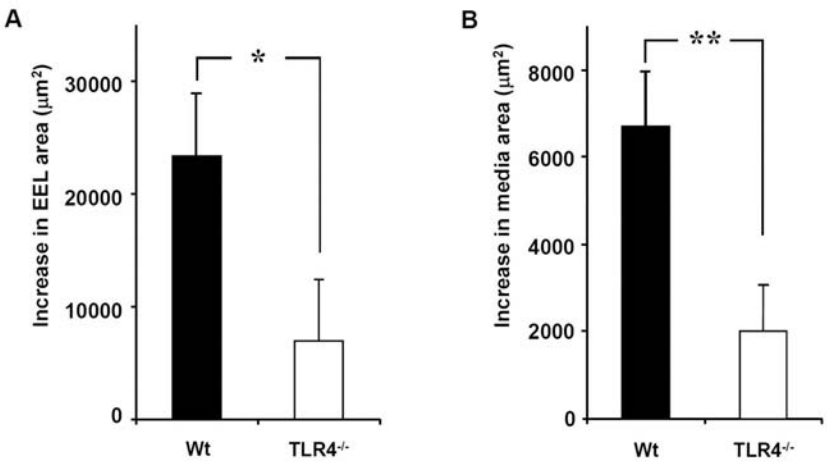

Figure 3.
Increase in EEL area (A) and media area of the left carotid artery after ligation of the right carotid artery in wild-type (Wt) BALB/c and TLR4-defective (TLR4-/-) C.C3H-Tlr4$^{LPS\text{-}d}$ mice 28 days after right carotid artery ligation (B). n=14 to 17 mice per group. *P=0.047, **P=0.014.

Expression of TLR4 and endogenous TLR4 ligands in a flow cessation model

Next, we studied whether outward remodeling resulted in an increase of TLR4 or endogenous TLR4 ligand levels in the contralateral unligated carotid artery. We observed an upregulation of TLR4 mRNA (~25 times) and TLR4 protein levels (~3 times) over time that was significant at 28 days after ligation (P=0.046, P=0.029, respectively, Figure 4A). We also measured the EDA and Hsp60 mRNA levels at 0 and 28 days after ligation in

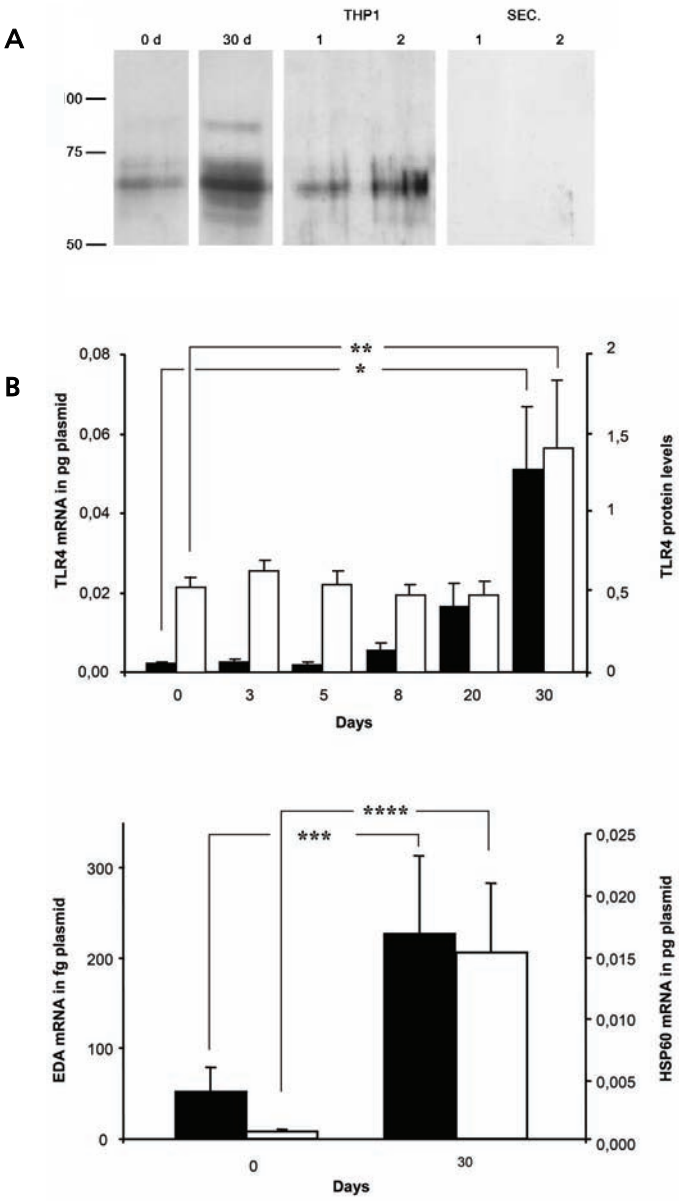
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Figure 4.
Left carotid artery TLR4 mRNA and protein levels over time after ligation of the right carotid artery in wild-type BALB/c mice (A). Upper panel, Example of TLR4 Western blotting at 0 and 30 days after ligation and TLR4 Western blotting on protein of the THP1 monocyte cell line (1=2 µg, 2=4 µg protein) and the same blot (SEC) incubated with the immune serum replaced with nonimmune rabbit IgG. Bottom panel, Black bars represent TLR4 mRNA levels, white bars represent TLR4 protein levels, expressed in arbitrary units. Left carotid artery EDA and Hsp60 mRNA levels 28 days after right carotid artery ligation in wild-type BALB/c mice (B). Black bars represent EDA mRNA levels, white bars represent Hsp60 mRNA levels. n=5 to 9 mice per group. TLR4, EDA, and Hsp60 mRNA is represented as the amount of plasmid containing the PCR product to which it correlates in the dilution series of this plasmid used in the quantitative real-time PCR. Localization of TLR4 protein expression of the left carotid artery of the BALB/c mice (C, left panel) and negative control (4 µg/mL nonimmune rabbit IgG; C, right panel). *P=0.046, **P=0.029, ***P=0.007, ****P=0.041. Bar=25 µm.

wild-type BALB/c mice. Both endogenous TLR4 ligands were significantly upregulated at 28 days (EDA, 4 times, P=0.007, and Hsp60, 24 times, P=0.041, Figure 4B).
Staining for TLR4 protein expression demonstrated that the endothelial cell layer, media, and adventitia contained TLR4-positive cells (Figure 4C, left panel). Negative control with nonimmune rabbit IgG showed no positive staining (Figure 4C, right panel) or bands after Western blotting (Figure 4A, top panel).

Discussion

Inappropriate arterial remodeling is currently thought to be the main cause of prevalent vascular pathologies, including atherosclerosis and restenosis [25], but the components and regulatory mechanisms of this process are still unclear.
First, we demonstrated that TLR4 activation in an atherosclerotic (ApoE3 Leiden) mouse model can induce outward remodeling and plaque formation.
The increase in atherosclerotic plaque formation after LPS application is consistent with the formation of a smooth muscle cell–rich lesion after adventitial LPS application in rats [26] and with the role of TLR4 in neointima formation, as described before in mouse [6] and plaque formation in humans [7].
Next, we questioned if TLR4 is involved in outward arterial remodeling with neointima formation and used the femoral cuff model in BALB/c and C.C3H-*Tlr4*$^{LPS\text{-}d}$ mice. This showed that TLR4 is involved in outward remodeling. Although no relation was found between intima formation and outward remodeling in the C.C3H-*Tlr4*$^{LPS\text{-}d}$ mouse, it remains difficult to determine what the contribution of neointima formation is in this process. For this, we investigated if TLR4 is involved in outward arterial remodeling

without neointima formation by using the carotid artery contralateral to the ligated artery. This shows that TLR4 is involved in outward arterial remodeling without neointima formation or injury. Thus, outward remodeling by lumen area increase depends on TLR4 presence independent of neointima formation.

The finding that TLR4 is involved in outward remodeling with and without neointima formation suggest an important role for TLR4 in all outward arterial remodeling.

Previously, Kiechl *et al*. [7] demonstrated a relation between Asp299Gly TLR4 polymorphism and carotid intima media thickness. It remains to be determined if human Asp299Gly TLR4 polymorphism is also associated with less outward remodeling. Less outward remodeling might lead to more lumen loss, but, since outward remodeling is associated with an unstable plaque phenotype [18], this might lead to more stable plaques. The plaque phenotype is determined by collagen turnover [27] and inflammation [28]. Earlier findings show that TLR4 is associated with collagen turnover [3,29], initiation of an inflammatory response [6,30], and production of chemokines and cytokines such as monocyte chemoattractant protein-1 [6], suggesting a potential role for TLR4 in plaque stabilization. Recently, Boekholdt *et al*. [31] found that TLR4 polymorphism modify the efficacy of statin therapy and the risk of cardiovascular events. This points again to a role of TLR4 in cardiovascular disease, although the exact mechanistic role of TLR4 remains obscure.

Since in the carotid ligation model no neointima formation occurs and no exogenous TLR4 ligand was used, we studied whether outward remodeling resulted in an increase of TLR4 or endogenous TLR4 ligands levels. TLR4 was upregulated on mRNA and protein levels that localized to the endothelial, medial, and adventitial layers. Also, two endogenous TLR4 ligands, EDA and Hsp60, showed an increased mRNA expression at 28 days after ligation. This indicates that during outward arterial remodeling, endogenous ligands are the main TLR4 activators, and no exogenous ligands are necessary, as was found earlier for murine atherosclerosis [32]. Nevertheless, a causal role for endogenous TLR4 ligands in remodeling still has to be established.

The involvement of TLR4 in arterial remodeling might point to a role for TLR4 in arteriogenesis, in which outward remodeling is an essential process. Furthermore, TLR4 activation results in increased production of monocyte chemoattractant protein-1 and other cytokines [6,30], which stimulates arteriogenesis [33]. Similarly, a bolus of LPS improves peripheral and collateral conductance after femoral artery occlusion in the rabbit [34].

Limitations

Outward remodeling in the TLR4-deficient mouse is not prevented by the femoral cuff, since in both ApoE3 Leiden (Figure 1C) and wild-type BALB/c (Figure 2a), outward remodeling occurs. However, we cannot exclude that the cuff prevents even more outward remodeling in ApoE3 Leiden and BALB/c. Arteries were pressure-fixed with constant intraventricular pressure (50mmHg). Although we did not determine intraluminal arterial pressure, procedure and intraventricular pressure was the same in the different mice groups. In summary, we showed that TLR4 is involved in the outward arterial remodeling process, probably through upregulation of TLR4 and its endogenous ligands, EDA and Hsp60, which might also apply to arteriogenesis.

Acknowledgments

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Table

EEL, media, intima/plaque, and lumen area of ApoE3 Leiden, wild-type, and TLR4-deficient mice after femoral cuff placement and carotid ligation

	EEL	Media	Intima/Plaque	Lumen
Femoral cuff				
ApoE3 (gel)	28226 ± 2217	11660 ± 700	8448 ± 1902	8118 ± 1149
ApoE3 (LPS)	38704 ± 2415	14131 ± 897	15803 ± 1565	8770 ± 1377
Wild-type (gel)	22789 ± 3076	8985 ± 845	2353 ± 1076	11449 ± 2650
Wild-type (LPS)	30956 ± 2485	9624 ± 387	9133 ± 1714	12198 ± 1595
TLR4-deficient (gel)	16241 ± 1262	7976 ± 334	1279 ± 183	6986 ± 1197
TLR4-deficient (LPS)	17213 ± 2275	8001 ± 420	3859 ± 912	5354 ± 1554
Carotid ligation				
Wild-type, unligated	117839 ± 19577	18039 ± 2377	0	99799 ± 17634
Wild-type, ligated	141169 ± 5644	24735 ± 1279	0	116433 ± 4883
TLR4-deficient, unligated	133173 ± 13300	21764 ± 1881	0	111409 ± 11638
TLR4-deficient, ligated	140125 ± 5509	23770 ± 1086	0	116355 ± 4915

Data are mean ± SEM (in μm^2), n=7 to 17. Outward remodeling of the carotid artery is determined in the carotid artery contralateral to the ligated artery.

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Chapter 3

The nuclear factor-kappa B p50 subunit is involved in flow-induced outward arterial remodeling

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Abstract

Aims

Outward arterial remodeling is a structural enlargement of the artery that is associated with unstable inflammatory atherosclerotic lesions. Toll-like receptor (TLR) activation is known as a key pathway in outward arterial remodeling. TLR activation results in nuclear translocation of the transcription factor Nuclear Factor-kappa B (NF-κB) that controls the transcription of many inflammatory genes. The NF-kB subunit p50 is generally considered to be an inhibitory subunit of the NF-kB complex. We therefore hypothesize that NF-kB p50 inhibits outward arterial remodeling.

Methods and results

Carotid artery ligation in mice, induced outward remodeling in contralateral arteries of NF-kB p50-/- (p50-/-) and wild type (WT) arteries. p50-/- arteries showed more outward arterial remodeling than WT arteries (19894.0 ± 3136.7 μm^2 vs. 6120.7 ± 2741.2 μm^2, respectively, P=0.006). *In vitro*, lipopolysaccharide induced higher cytokine expression levels in p50-/- cells compared to WT cells. *In vivo*, more outward remodeling in p50-/- arteries was associated with a decrease in collagen density and an increased influx of macrophages.

Conclusions

The NF-kB p50 subunit is involved in outward arterial remodeling. This is probably due to modulation of macrophage influx and adventitial collagen, leading to enhanced flow-induced outward arterial remodeling after targeted deletion of NF-kB subunit p50.

Introduction

Arterial lumen loss is determined by atherosclerotic plaque formation, as well as structural changes in arterial diameter; arterial remodeling. Arterial remodeling is defined as a change in arterial size compared with a control or reference artery. An increase in size is defined as outward remodeling, whereas a decrease in size is defined as inward remodeling. Depending on the direction of remodeling (inward or outward), the change in arterial diameter accelerates luminal narrowing or prevents the lumen from being occluded. Structural geometrical enlargement of the arterial wall, or outward arterial remodeling, seems a favorable process since it compensates for lumen loss due to intima or plaque formation. However, this compensatory enlargement of the arterial wall is associated with an unstable plaque phenotype [1], more prone to rupture and may lead to an acute arterial occlusion. Little is known about the mechanisms that drive outward remodeling, but matrix turnover and inflammation are important features [2], as well as Toll-like receptor 4 (TLR4) activation [3]. TLR4 activation results in nuclear translocation of various transcription factors, including Nuclear Factor-kappa B (NF-kB) that controls transcription of inflammatory genes. NF-kB is composed of homo- and heterodimers of Rel family proteins (p65 (RelA), RelB, c-Rel, p50 (NF-kB1) and p52 (NF-kB2)) [4]. NF-kB activation via Toll-like receptors or cytokines results in translocation of predominantly p50/p65 heterodimers to the nucleus. The NF-kB p50 subunit lacks a transactivation domain [5] and forms heterodimers that are transcriptionally active. Homodimers of p50 bind to DNA but inhibit transcription activity by other NF-kB dimers, thereby acting as a brake on NF-kB signaling [6,7]. Indeed, several studies report that p50 has an inhibitory effect on TLR4 signaling. Upregulation of p50 homodimers attenuates lipopolysaccharide (LPS) response and is essential for LPS tolerance [8], and NF-kB p50 inhibits a LPS-induced shock [9].

In arteries, however, neointima formation and ICAM-1 mRNA expression is reduced after carotid artery ligation in NF-kB p50 null mice [10,11], suggesting a controversial, stimulatory role for NF-kB p50. For outward remodeling, TLR4 activation is essential [3]. The role of the NF-kB p50 subunit in outward arterial remodeling however, is unknown. Since NF-kB p50 is generally considered to be an inhibitory subunit of the NF-kB complex, we hypothesized that deletion of NF-kB p50 enhances outward remodeling. For this purpose, we studied NF-kB p50 null mice in a carotid artery ligation model for outward remodeling and determine arterial macrophage influx and collagen density during outward remodeling.

Materials and methods

Cell culture and cytokine antibody array

Mouse embryonic fibroblasts (MEFs) were isolated from 12.5 days WT and p50-/- embryos. MEFs were cultured in Dulbecco's modified Eagle's medium (DMEM, InVitrogen) with MEM nonessential amino acids 1x (InVitrogen), 50 µM 2-mercaptoethanol (Sigma), 100 U/ml penicillin, 100 µg/ml streptomycin (Gibco), and 10% FBS (Gibco). P3-4 cells, starved O/N in 0.1% FCS medium, were stimulated with PBS and 100 ng/mL LPS.

Medium was collected 24 h after stimulation. Medium of four different wells was used and assayed for cytokines using a cytokine antibody array. This assay was performed according to the manufacturer's protocol (RayBiotech, Norcross, GA, USA).

Animals

To investigate the role of the NF-kB p50 subunit in outward arterial remodeling we used B6;129P2-Nfkb1^{tm1Bal}/J mice, where the p50 gene is disrupted by insertion of a neomycin gene; p50-/-, and B6/129PF2 mice as controls (WT).

Prior to intervention, the animals were anesthetized with an intramuscular injection of 0.1 mL/10 g body weight of a cocktail (ketamine (6 mg/mL) and medetomidine (50 µg/ml) in 0.9% NaCl). We used 0.1 mL/10 g body weight Antisedan (0.5 mg/mL in 0.9% NaCl, Orion) as antagonist after the intervention.

The right common carotid arteries of 8 weeks old mice were completely ligated near the carotid bifurcation as previously described [12]. In this model, we studied the artery, contralateral to the ligated artery, in which flow was increased and no injury or damage was induced. Food consumption and weight gain were the same in all groups. At 0, 3, 9 and 28 days after the ligation, the contralateral artery was harvested for morphometry. First the arteries were perfused via the left heart ventricle with 10^{-4} M sodium nitroprusside in PBS (3 min) followed by perfusion with 10^{-4} M sodium nitroprusside in 4% paraformaldehyde at 99 mL/h to get maximal dilation of the arteries and normal intraventricular pressure for nitroprusside-treated mice (50–60 mmHg). Arteries were fixed in 4% paraformaldehyde in PBS before being embedded in paraffin.

For RNA and protein isolation, the carotid arteries were perfused with PBS and harvested at 0 and 3 days after the ligation. TriPure reagent was used according to the manufacturer's protocol to isolate RNA and proteins from the samples (Roche). During the studies we used 150 animals in total: 75 animals for morphology measurements

and 66 animals for RNA and protein isolation. Nine animals were lost shortly after the ligation procedure.
The ethics committee on animal welfare of the Utrecht University approved the animal experiments and the investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Measuring arterial remodeling
Arterial remodeling is defined as a change in arterial size compared with a control or reference artery. As a standard of arterial size, the External Elastic Lamina (EEL) area was used, calculated from the EEL length (formula: (EEL area = $\pi((\text{EEL length}/2\pi)^2)$).

Morphometry
Eight to 12 serial cross-sections (5 µm), spanning the whole vessel segment, were cut and stained with Elastin–von Gieson staining. The EEL length and Internal Elastic Lamina (IEL) length were quantified with image analysis software (AnalySIS version 3.2), and the IEL and EEL areas were calculated. Total cross-sectional medial area was calculated by subtraction of the IEL area from the EEL area.

Collagen density
Sections of left carotid arteries, harvested 28 days after ligation, were stained with picro Sirius red. Quantification of collagen density was performed with circularly polarized light and digital image microscopy after conversion into grey value images.

Macrophage quantification
To determine the number of inflammatory cells (macrophages) in the adventitia of the remodeled arteries, a MAC-3 staining was performed on sections from left carotid arteries harvested 0, 3, and 9 days after ligation. Sections were boiled in 10 mM citrate buffer for optimal antigen retrieval. MAC-3 staining was performed using a rat-anti-mouse MAC-3 antibody (o/n incubation at 4°C, 1:30, Becton Dickinson, Franklin Lakes, USA), goat-anti-rat biotin labeled antibody (1 h incubation at room temperature, 1:200, Southern Biotech, Birmingham, Alabama, USA) and streptavidin-HRPO (1 h incubation at room temperature, 1:1000, Southern Biotech, Birmingham, Alabama, USA). Subsequently the sections were incubated for 30 min in 3-amino-9-ethylcarbazole (AEC) and stained with hematoxilin before being embedded. MAC-3 positive cells in the vascular wall were counted manually and expressed as the amount

of macrophages/mm^2 adventitia. The surface area of the adventitia was determined using image analysis software (AnalySIS version 3.2). Per artery, macrophage numbers were determined in 3 non-consecutive cross-sections (at least 50 µm between cross-sections). In total, macrophage numbers were determined in 3–7 arteries per group.

MMP-9 activity assay

The MMP-9 activity assay was performed according to the manufacturer's instructions (Amersham, Munich, Germany) and the results were corrected for sample protein concentration.

Quantitative PCR

Primers from SuperArray Bioscience Corporation and sybergreen supermix for qPCR from Biorad were used following manufacturer's protocol to measure Col1a1 and Col1a2 mRNA levels with qPCR method in I-cycler iQ™ RT-PCR (Biorad, n=16 per group.

Statistics

All statistics were performed in SPSS 15.0. The Mann–Whitney test was performed to determine a difference between B6;129P2-Nfkb1tm$^{1Bal/J}$ mice (p50-/-) and B6/129PF2 mice as controls (WT). Values are presented as mean ± SEM. A value of $P<0.05$ was regarded significant.

Abbreviations used

GCSF (Granulocyte Colony-Stimulating Factor), MCP-5 (Monocyte Chemotactic Protein-5), SCF (Stem Cell Factor), GM-CSF (Granulocyte/Macrophage Colony-Stimulating Factor), EEL (External Elastic Lamina).

Results

Cytokine expression after NF-kB activation in WT and p50-/- cells

Stimulation of wild type (WT) and p50-/- mouse embryonic fibroblasts (MEFs) with 100 ng/mL LPS resulted in an increase of cytokine levels in the medium, n=4 (Figure 1). IL-6 and IL-12p40p70 medium levels after LPS stimulation were higher in both WT and p50-/- cells compared to unstimulated cells and higher in stimulated p50-/- cells compared to WT ($P=0.021$ for both; Figure 1). Levels of GCSF, IL-5, IL-9, MCP-5, and SCF were low and an increase after stimulation was only detectable in p50-/- cells.

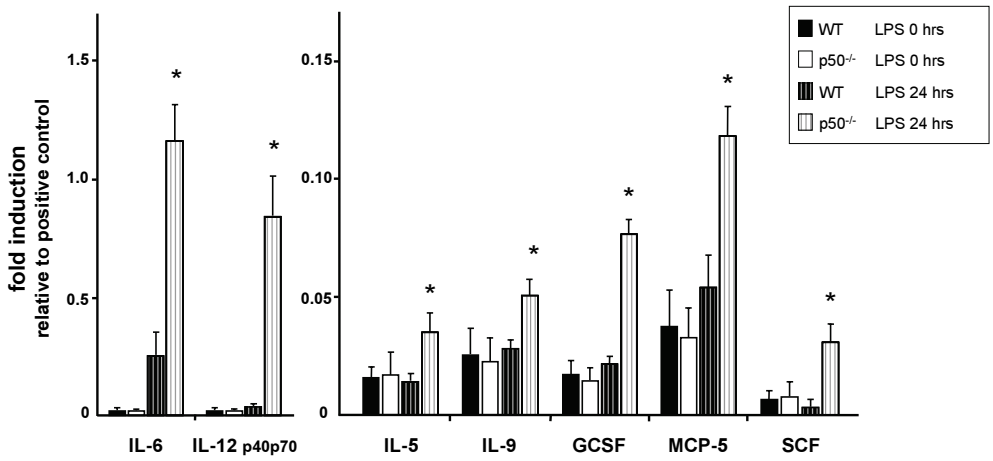

Figure 1. Cytokine expression in vitro
MEFs were stimulated for 24 h with LPS (100 ng/mL) and PBS as a control. Medium of stimulated NF-kB p50-/- cells contains higher levels of most cytokines compared to WT cells (n=4). Differently expressed cytokines are presented in this figure, *P<0.05. Error bars represent SEM.

NF-kB p50 subunit is involved in outward remodeling

To investigate the role of the p50 subunit in the process of outward arterial remodeling, we performed carotid artery ligation of the right carotid artery in WT and NF-kB p50-/- animals. Morphological measurements (Figure 2, Table 1) were performed on the contralateral arteries harvested at 0 and 28 days after ligation, n=10-12 animals per group. Outward remodeling, measured as the increase in EEL area (EEL area at 28 days - EEL area at 0 days), was more pronounced in arteries from p50-/- animals compared with WT animals; 19894.0 ± 3136.7 μm^2 in the p50-/- vs. 6120.7 ± 2741.2 μm^2 in WT animals, P=0.006. Also the media area of the p50-/- arteries increased more than WT arteries; 5054.7 ± 1274.2 μm^2 in p50-/- vs. - 55.8 ± 700.6 μm^2 in WT arteries, P=0.016.

Collagen density in arteries during outward remodeling

Collagen density was measured in the adventitia of the contralateral arteries at 0 and 28 days after ligation, n=11–12 animals per group (Figure 3a and b). At day 0, collagen density did not differ between WT and p50-/- arteries. At 28 days, the collagen density was lower in p50-/- arteries compared to WT (73.3 ± 4.8 greyvalue/μm^2 in p50-/- vs. 88.7 ± 2.8 greyvalue/μm^2 in WT arteries, P=0.014). In WT arteries, collagen density did not differ between 0 and 28 days after ligation. In p50-/- arteries collagen density was

lower at 28 days after ligation compared to 0 days. Collagen density in the media remained unchanged in WT and p50-/- arteries before and after ligation (data not shown).

Influx of macrophages during outward remodeling

At baseline and at 3 days after ligation, there were no changes in the number of macrophages in the adventitia of WT and p50-/- contralateral arteries (214.7 ± 30.7 and 238.9 ± 50.9 macrophages/mm^2 at baseline and 140.1 ± 55.1 and 231.1 ± 44.8 macrophages/mm^2 at 3 days; Figure 3c). At 9 days after ligation, however, more macrophages were observed in the contralateral p50-/- arteries compared to WT arteries; 388.1 ± 55.5 and 232.3 ± 28.6 macrophages/mm^2 adventitia, respectively (P=0.046).

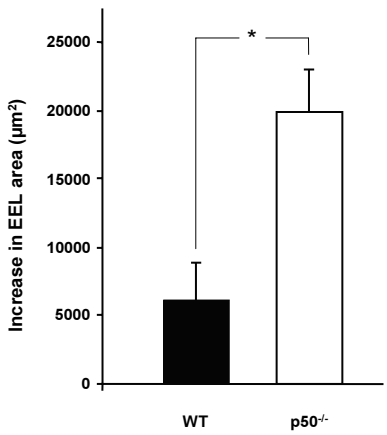

Figure 2. Outward remodeling in vivo
Increase in EEL area of the contralateral artery of WT arteries (black) and p50-/- (white) mice at 28 days after ligation, n=10–12 animals per group. *P=0.006, error bars represent SEM.

Table 1. EEL, media and lumen area of WT and p50-/- carotid arteries after ligation

	EEL	media	lumen
WT, 0 days	125592 ± 4763	21396 ± 886	104196 ± 4093
WT, 28 days	131713 ± 2741	21340 ± 819	110373 ± 2459
p50$^{-/-}$, 0 days	105697 ± 3021	17366 ± 735	88331 ± 2412
p50$^{-/-}$, 28 days	125591 ± 3276	22421 ± 1331	103171 ± 2914

Data are mean ± SEM in µm^2, n=10–12. Outward remodeling is determined in the carotid artery contralateral to the ligated artery.

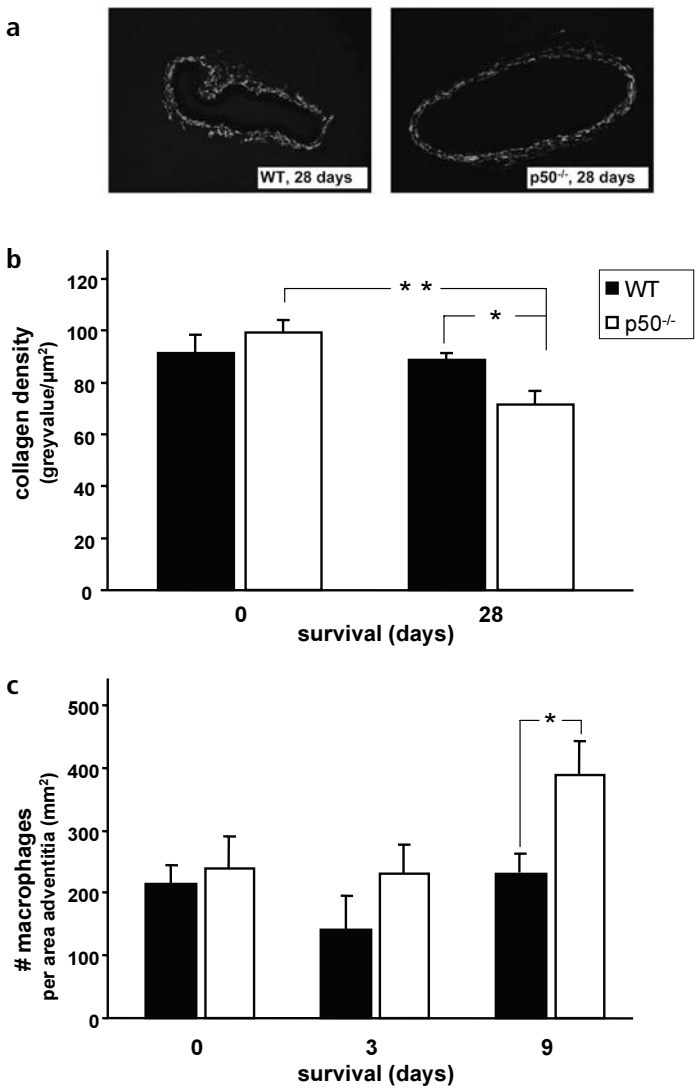

Figure 3. Collagen density and macrophages

(a) Picro Sirius staining of contralateral WT (left) and p50-/- arteries (right) at 28 days after ligation, magnification is 200x, (b) Collagen density is lower in p50-/- compared to WT arteries at 28 days, *P=0.014. **P=0.001 comparing the collagen density at 0 and 28 days in p50-/- animals. (c) Number of macrophages in adventitia of contralateral arteries of p50-/- (white bars) and WT mice (black bars) at 0, 3 and 9 days after ligation. *P=0.046 comparing WT and p50-/- arteries at 9 days. Error bars represent SEM.

Cytokine expression, collagen mRNA and MMP activity in the remodeling artery

In the arteries of p50-/- animals, collagen mRNA levels did not change during outward remodeling (Table 2). At baseline MMP-9 levels in p50-/- arteries were higher compared to WT arteries (Table 2). At 3 days after ligation, IL-1alpha, IL-2, IL-4, IL-5, and IL-10 levels were higher in p50-/- arteries compared to expression levels in WT. Levels of IL-6, IL-17, IFN-gamma, and TNF-alpha were not detectable.

Table 2. Cytokine and collagen mRNA expression levels and MMP-9 activity levels in left artery

	WT 0 days	$p50^{-/-}$ 0 days	WT 3 days	$p50^{-/-}$ 3 days
IL-1alpha (pg/mL)	537.15 ± 23.63	206.44 ± 35.42 *	366.06 ± 52.83	491.02 ± 36.77 †
IL-2 (pg/mL)	1647.20 ± 96.47	480.28 ± 47.65 *	768.62 ± 151.01	1365.81 ± 125.10 ‡
IL-5 (pg/mL)	154.29 ± 7.07	67.12 ± 5.96 *	85.99 ± 10.98	133.96 ± 11.09 ‡
IL-10 (pg/mL)	1570.00 ± 110.25	595.10 ± 111.32 *	829.65 ± 161.69	1381.81 ± 126.92 †
GM-CSF (pg/mL)	401.05 ± 29.69	142.58 ± 23.46 *	220.21 ± 42.42	306.38 ± 30.71
IL-4 (pg/mL)	694.91 ± 59.33	277.63 ± 55.26 *	356.07 ± 105.62	648.88 ± 75.51 ‡
Col1a1 (arbitrary units)	60.38 ± 15.95	33.51 ± 6.16	31.84 ± 10.86	28.12 ± 5.78
Col1a2 (arbitrary units)	135.10 ± 28.03	100.82 ± 12.63	49.32 ± 8.03	72.69 ± 8.25
MMP-9 (ng/mL)	1.07 ± 0.34	2.78 ± 0.55 §	3.08 ± 0.36	3.12 ± 0.37

Expression levels of cytokines, collagen mRNA, and MMP-9 activity levels at 0 and 3 days after ligation in contralateral arteries of WT and p50-/- mice are presented ± SEM. *P ≤ 0.001 compared to WT at baseline, †P<0.05 and ‡P<0.01 compared to WT 3 days, §P<0.01 compared to WT at baseline.

Discussion

The regulatory role of the NF-kB p50 subunit is unclear. The described inhibitory role of the NF-kB p50 subunit in immunological processes seems to be aberrant during intima formation in the artery. The regulatory role of NF-kB p50 in outward arterial remodeling is still unknown. In this study, we investigated the role of the NF-kB p50 subunit in outward remodeling. Using the carotid artery ligation model in WT and NF-kB p50-/- mouse, we show that the regulatory NF-kB p50 subunit is involved outward arterial remodeling.

Cytokine expression after NF-kB activation in WT and p50-/- cells

NF-kB activation in response to LPS stimulation was investigated in vitro, in cells lacking the NF-kB p50 subunit. These p50-/- cells were activated by LPS and responded with even higher cytokine expression levels than WT cells suggesting an inhibitory role of the p50 subunit. This is in line with the findings of Kastenbauer and Ziegler-Heitbrock [8], and Gadjeva *et al*. [9], showing an inhibitory role of p50 on the response to LPS stimulation. Frantz et al. also found an increase of cytokine levels in p50-/- tissue after myocardial infarction [13]. In contrast, they and others [14] suggest that, using shift-assays, NF-kB cannot be translocated in cells lacking the p50 subunit. In general, the NF-kB p50 subunit is considered as an inhibitory subunit of NF-kB signaling, because p50 is able to bind to DNA, but lacks a transactivation domain [5]. This would explain the higher levels of cytokines, down stream of NF-kB, after stimulation with LPS.

NF-kB p50 subunit is involved in outward remodeling

Having established that cells lacking the NF-kB p50 subunit can be activated, we increased blood-flow in the carotid artery to induce outward remodeling. Outward remodeling occurred in both WT and p50-/- mice. However, the enlargement of the p50-/- arteries at 28 days was higher compared to WT animals suggesting an inhibitory role of the p50 subunit on outward arterial remodeling. Other studies on the role of p50 in artery related processes showed a stimulatory effect of p50; less neointima hyperplasia was formed in the occluded carotid artery of p50-/- animals compared to WT animals [10,11]. An important difference between the two animal models is the increase in flow and probably subsequent high shear stress that develops in the contralateral artery after ligation of the carotid artery resulting in outward remodeling. In this model we studied the artery, contralateral to the ligated artery, in which flow was increased and no injury or damage was induced. To study intima formation,

Squadrito *et a*l. [10] and Ruusalepp *et al*. [11] used the ligated arteries in which the blood flow is completely stopped, so flow and shear stress are low. High shear stress is an activator of outward arterial remodeling [15]. In the context of atherosclerosis, most research focuses on low shear stress, since low shear stress is related with sites in the artery prone for atherosclerotic lesion development [16]. It could be speculated, whether the role of p50 in the artery might be shear stress dependent, resulting in a stimulatory role during low shear stress and an inhibitory role during high shear stress. This differential role of NF-kB during shear stress has been described previously. High shear stress activates NF-kB [17-19], while prolonged laminar shear stress primes the endothelial cells and suppresses pro-inflammatory activation via NF-kB [18,19]. Low shear stress and disturbed flow-induced NF-kB in a constitutive way [20,21] and makes the vascular wall vulnerable for atherosclerotic lesion development [22,23].

Collagen is the major extracellular matrix component in arteries and an important determinant of their structure and size. In WT arteries, the collagen density of the adventitia did not change after ligation as has been shown previously [2] and is in line with Sluijter et al. showing that collagen turnover increased during arterial remodeling, but collagen content did not change [24]. In p50-/- arteries, however, collagen density was decreased pointing to an altered collagen turn-over in these arteries possibly facilitating more outward remodeling. Altered arterial collagen density was also found in TLR4-/- mice, that were previously shown to manifest less outward arterial remodeling, and showed higher collagen density levels [2] that probably inhibited the enlargement of the arteries.

Like collagen, also macrophages are essential for outward remodeling [25-27]. Mice lacking macrophages and blood monocytes showed less collateral formation and less macrophages around the remodeling arteries [27]. Furthermore, activation of monocytes and macrophages during arteriogenesis or local MCP-1 application, led to a higher number of macrophages around the remodeling arteries, and an increased perfusion [25]. In atherosclerotic rabbit model, higher numbers of macrophages in the adventitia and the media were related with more outward arterial remodeling [28]. So in general; more macrophages lead to more outward remodeling. This is in accordance with the in vitro experiments that showed a higher level of MCP-5, a human MCP-1 homologue [29], mRNA in the p50-/- cells after activation. Consistently, we found more macrophage infiltration in the adventitia of p50-/- arteries compared to WT arteries.

In another mouse model, to investigate cerebral aneurysms, p50-/- animals showed a decreased incidence of cerebral aneurysm formation, with less macrophage infiltration into the arterial wall [30]. This is, however, a different disease model and a much later

time point then the rapid influx of macrophages after flow increase.
Cytokine, MMP-9 activity and collagen mRNA levels are in line with these findings, as we found higher cytokine levels at 3 days and higher MMP-9 activity at baseline in p50-/- arteries compared to WT while collagen mRNA levels do not differ.
In conclusion, using an artery with increased blood flow as a model, targeted deletion of the regulatory NF-kB p50 subunit enhanced outward arterial remodeling, probably via a decrease in adventitial collagen density and an increase in macrophage infiltration. This identifies the NF-kB p50 subunit as an important regulator of shear stress-induced outward remodeling and clarifies the role of this subunit in the artery during remodeling.

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Chapter 4

Extra domain A of fibronectin is involved in outward arterial remodeling

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Submitted

Abstract

Background

Outward arterial remodeling is a structural enlargement of the artery that is associated with unstable inflammatory atherosclerotic lesions and involves collagen turn-over and cell migration. During arterial remodeling, fibronectin containing extra domain A (EDA) is highly expressed. EDA enhances binding of fibronectin to integrins and thereby facilitates cell migration. The exact role of EDA in geometrical arterial remodeling, however, is unknown. Since fibronectin containing EDA is highly expressed in flow-induced remodeling arteries, we hypothesized that EDA stimulates outward arterial remodeling.

Methods

Carotid artery ligation was performed in EDA-/- and WT mice. Outward remodeling was measured in the artery contralateral to the ligated artery by determining the vessel area at 28 days after ligation. Collagen turnover was assessed in the contralateral arteries by measuring collagen density, collagen mRNA levels and matrix metalloproteinase 9 (MMP-9) activity. Migration capacity of EDA-/- and WT cells was determined using a Boyden chamber migration assay and fetal bovine serum (FBS) as attractant.

Results

In EDA-/- arteries, no outward remodeling occurred and even inward remodeling was measured (-47.667 ± 8.931 μm^2 in EDA-/-, 19.616 ± 28.783 μm^2 in WT). No difference was found in collagen turnover or MMP-9 activity between EDA-/- and WT arteries. In the Boyden chamber assay, FBS increased the number of migrated WT cells (56.06 ± 3.98 vs. 13.5 ± 2.58), while there was no increase in migration of EDA-/- cells (31.95 ± 6.54 vs. 27.12 ± 5.76).

Conclusions

Extra domain A of fibronectin is involved in flow-induced outward arterial remodeling, probably not due to changes in collagen turnover, but as the result of a diminished migration capacity of EDA-/- cells.

Introduction

Atherosclerosis is the underlying cause of major cardiovascular events such as myocardial infarction and stroke. Although the amount of atherosclerotic plaque is increasing in time, arterial lumen loss due to plaque accumulation does not occur frequently. The lumen is preserved as the artery increases its diameter structurally in a process called outward remodeling. This structural arterial enlargement can be induced by a sustained increase in arterial bloodflow with processes like inflammation, matrix turnover and cell migration playing an important role. This remodeling seems a favorable process; but is associated with an atherosclerotic plaque that is characterized by a lipid-rich core, a thin fibrous cap, and contains inflammatory cells. These unstable plaques are thought to be rupture-prone, leading after rupture to thrombus formation with subsequent arterial occlusion and possible myocardial infarction and stroke [1].
Arterial structure and form is determined by the extracellular matrix (ECM) containing mainly collagen and fibronectin. To change arterial size, this matrix structure has to be degraded and rebuilt and the cells embedded in this structure subsequently migrate within the tissue. Collagen and fibronectins give structure and strength to arteries [2] and form a substrate for cell adhesion, spreading and thereby promoting cell migration [3,4].
During development and in pathological conditions specific forms of fibronectin are produced. Fibronectin containing the extra domain A (EDA) is an alternative spliced form of fibronectin produced during embryogenesis, wound healing and vascular injury [5-8]. EDA expression is increased during outward arterial remodeling [9], atherogenesis [10], and remodeling of the heart after myocardial infarction [11]. EDA binds to α9ß1 integrins [12], and incorporation of this domain enhances binding of fibronectin to α5ß1 integrins [13]. Integrin signaling is important by cell-matrix and cell-cell interactions and can activate the adhesion, migration and proliferation of cells [14]. EDA is also described as an endogenous ligand of Toll-like receptor 4 (TLR4) that is involved in intima formation, outward arterial remodeling and atherogenesis [9,15,16]. A direct role of EDA in atherogenesis has been established using EDA-/- ApoE-/- mice that showed smaller lesions with less foam cells and lipids compared to atherosclerotic ApoE-/- mice [10]. Involvement of EDA in outward remodeling, however, is unknown. Since arterial EDA expression is increased during arterial remodeling and EDA drives integrin signaling that stimulates cell migration, we hypothesize that EDA enhances outward arterial remodeling.

In this study, we investigate the causal role of fibronectin containing EDA in the process of outward remodeling, using the carotid artery ligation model in EDA-/- mice. No outward remodeling was observed in EDA-/- mice and even inward remodeling was measured. Following flow-increase, no changes were found in collagen turn-over, but EDA-/- cells showed reduced migration capacities. This identifies a causal role for fibronectin containing EDA in outward arterial remodeling probably due to reduced cell migration.

Materials and methods

Animal experiments

Complete ligation of the right common carotid arteries was performed in 8 weeks old EDA-/- (UC Davis School of Medicine, California) and BALB/c (WT) mice. In the carotid ligation model, we studied the artery contralateral to the ligated artery, in which flow was increased and no injury or damage was induced. Mice were anesthetized with a cocktail (ketamine (6 mg/mL) and medetomidine (50 µg/mL) in 0.9% NaCl) via intramuscular injection of 0.1 mL/10 g body weight. Antisedan (0.1 mL/10 g body weight, 0.5 mg/mL in 0.9% NaCl, Orion) was used as antagonist. At 0 and 28 days after the ligation, contralateral arteries were harvested for morphometry (n=8-10 per group). First the arteries were perfused via the left heart ventricle with 10^{-4} M sodium nitroprusside in PBS (3 min) followed by perfusion with 10^{-4} M sodium nitroprusside in 4% paraformaldehyde at 99 mL/h to get maximal dilation of the arteries and normal intraventricular pressure for nitroprusside-treated mice (50–60 mmHg). Arteries were fixed in 4% paraformaldehyde in PBS before being embedded in paraffin.

To study neo intima formation, the femoral cuff model was used. Under sterile conditions, a non-constrictive poly-ethylene cuff was placed around the femoral arteries. After 0 and 28 days the arteries were harvested as described above for the carotid arteries, and fixed in 4% paraformaldehyde before paraffin embedding.

For RNA and protein isolation, the carotid arteries were perfused with PBS and harvested at 0, 3, 6, 9, 20 and 28 days after the ligation (n=8-10 per group). TriPure reagent was used according to the manufacturer's protocol to isolate RNA and proteins from the samples (Roche).

The ethics committees on animal welfare of the Utrecht University and UC Davis approved the animal experiments.

Measuring arterial remodeling

Arterial remodeling is defined as a change in arterial size compared with a control or reference artery. As a measure for arterial size, the External Elastic Lamina (EEL) area was used, calculated from the EEL length (formula: (EEL area=πEEL length/2π)^2)). As reference artery size we used the average EEL area of the non-ligated left artery at 0 days after ligation.

Morphometry

Five serial cross-sections (5 µm), spanning the whole vessel segment, were stained with Elastin–van Gieson staining. The EEL length, Internal Elastic Lamina (IEL), length, and the lumen circumference were quantified with image analysis software (AnalySIS version 3.2, Soft Imaging Systems, Germany), and the IEL, EEL, and luminal area (LA) were calculated. The intimal area was determined as IEL area-LA.

Collagen density

Sections of left carotid arteries, harvested 0 and 28 days after ligation, were stained with picro Sirius red. Quantification of collagen density was performed with circularly polarized light and digital image microscopy after conversion into grey value images.

Quantitative PCR

Primers from SuperArray Bioscience Corporation and sybergreen supermix for qPCR from Biorad were used following manufacturer's protocol to measure Col1a1 and Col1a2 mRNA levels with qPCR method in I-cycler iQ™ RT-PCR (Biorad, n=8-10 per group).

MMP-9 activity assay

The MMP-9 activity assay was performed according to the manufacturer's instructions (Amersham, Munich, Germany) and the results were corrected for sample protein concentration.

Boyden chamber assay

Mouse embryonic fibroblasts (MEFs) were isolated from 12.5 days WT and EDA-/- embryos. MEFs were cultured in Dulbecco's modified Eagle medium (DMEM, InVitrogen) with MEM nonessential amino acids 1x (InVitrogen), 50 µM 2-mercaptoethanol (Sigma), 100 U/ml penicillin, 100 µg/ml streptomycin (Gibco), and 10% FBS (Gibco). Migration of EDA-/- and WT cells were performed in 8µm pore size

transwell dishes (#3422, Corning). Membranes were coated with collagen O/N at 37°C and the cells were starved in serum-free medium for 3 hrs. 500.000 cells in 100 µl serum-free DMEM were added to the inserts. The lower chambers were filled with 600 µl serum-free or 10% FCS in DMEM. After 3 hrs incubation at 37 °C, cells that did not migrate through the filter were removed by cotton swab. The migrated cells were fixated in 3.7% paraformaldehyde, and stained with Hoechst (33342, Invitrogen Molecular Probes, 1:50.000). Membranes were removed from the transwells and mounted onto glass slides using fluoromount-G (SouthernBiotech). Numbers of migrated cells (4 wells per condition) were counted with AnalySIS (AnalySIS version 3.2, Soft Imaging Systems, Germany).

Statistics

All statistics were performed in SPSS 15.0. A Mann–Whitney test was used to compare differences between groups. Values are presented as mean ± SEM. A value of $P<0.05$ was regarded significant.

Results

EDA is involved in outward remodeling

To investigate the role of EDA in outward arterial remodeling, carotid artery ligation was performed in EDA-/- and WT mice. EEL and luminal areas were determined of the arteries contralateral to the ligated artery at 0 and 28 days after ligation (n=8-10 animals/group/timepoint). The difference in EEL area (area at 28 days -area at 0 days) was used as a measurement for remodeling (Figure 1a). This showed an increase in arterial size in WT mice at 28 days compared to day 0 (19.616 ± 28.783 μm^2) in EDA-/- animals arterial size was decreased at 28 days compared to day 0 (-47.667 ± 8.931 μm^2, P=0,007). Similarly, shown in Figure 1b, the increase in lumen area at 28 days in WT animals was 23.893 ± 25.443 μm^2 while a decrease was found in EDA-/- mice (-42.885 ± 8.359 μm^2 in EDA-/- animals, P=0,005).

Collagen density and turnover

The role of collagen turnover during remodeling in EDA-/- and WT arteries was investigated by determining collagen density at 0 and 28 days after ligation (Figure 2). These collagen density levels did not differ between WT and EDA-/- mice at baseline and at 28 days after ligation. In both WT and in EDA-/- arteries, no changes in collagen

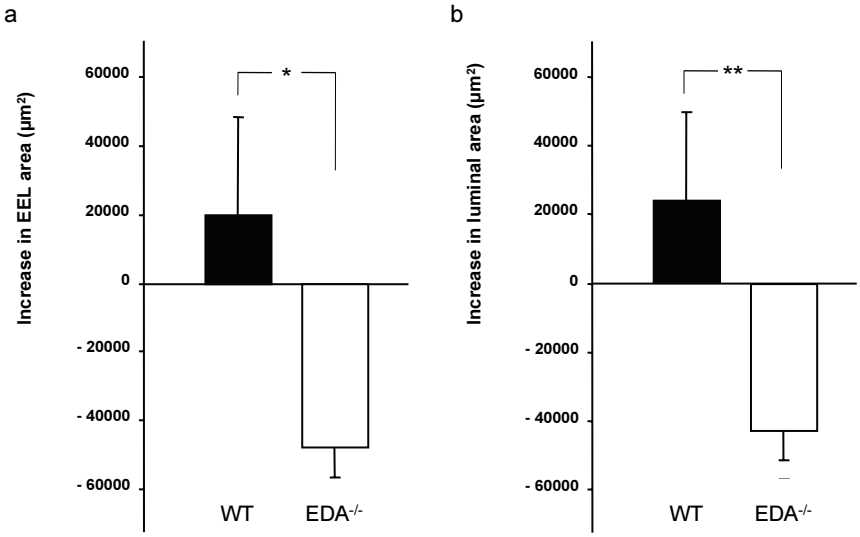

Figure 1. Outward remodeling
Increase in EEL and luminal areas of the contralateral arteries of WT (black bar) and EDA-/- (white bar) mice at 28 days after ligation, n=8-10 animals per group and * P=0.005, ** P=0.007, error bars represent SEM.

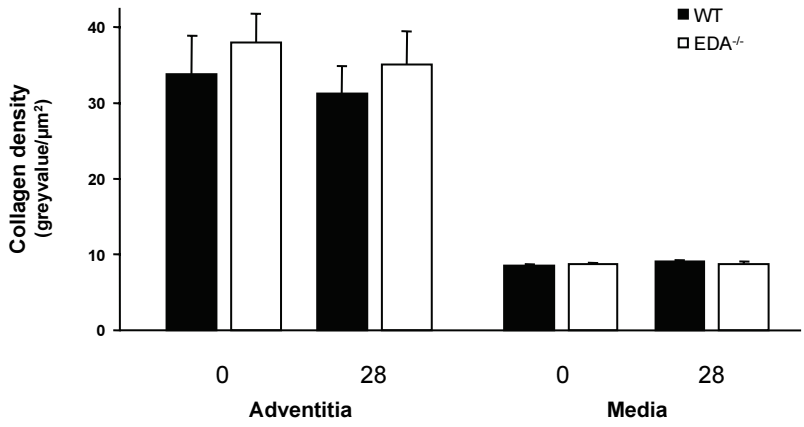

Figure 2. Collagen density
Collagen densities in adventitia and media from WT (black) and EDA-/- (white) arteries are not different at baseline, and at 28 days after ligation. At 0 days, P=0.343 and 0.607 for adventitia and media respectively, and at 28 days P=0.635 and P=0.465.

density were found between baseline and 28 days after ligation.
To determine if collagen synthesis was different between in EDA-/- and WT mice, Col1a1 and Col1a2 mRNA levels were determined at 0, 3, 6, 9, 20, 28 days after ligation. This showed no significant differences between EDA-/- and WT at all timepoints (Table 1). For collagen degradation MMP-9 activity was determined at 0, 3, 6 and 9 days. No differences were found in MMP-9 levels between EDA-/- and WT at all timepoints (Table 1).

Migration of cells in vitro

The migration capacity of mouse embryonic fibroblasts from EDA-/- and WT mice were studied using a Boyden chamber assay (Figure 3). Without chemoattractant, no difference was found between the number of WT and EDA-/- cells migrating through the filter. After adding fetal bovine serum (FBS) to the lower well of the Boyden chamber to attract the cells, we found that the number of migrating WT cells was

Table 1. Normalized Col1a1 and Col1a2 mRNA expression levels and MMP-9 activity levels (ng/mL) in the contra lateral carotid arteries

	Survival (days)	WT	KO	P-values
col1a1	0	16.45 ± 7.17	8.30 ± 1.56	0.69
	3	23.44 ± 9.41	25.66 ± 11.40	0.88
	6	7.76 ± 2.69	10.14 ± 2.94	0.36
	9	7.56 ± 1.53	10.63 ± 2.16	0.27
	20	7.51 ± 2.67	7.41 ± 1.11	0.39
	28	7.01 ± 1.77	8.71 ± 1.22	0.34
col1a2	0	4.63 ± 2.04	4.82 ± 1.33	0.40
	3	5.66 ± 1.00	9.79 ± 2.93	0.36
	6	7.59 ± 3.42	5.84 ± 1.10	0.67
	9	2.60 ± 0.49	4.39 ± 0.93	0.12
	20	3.68 ± 1.12	3.06 ± 0.64	0.66
	28	4.65 ± 1.04	4.91 ± 0.43	0.49
MMP-activity	0	0.97 ± 0.63	0.00 ± 0.00	0.07
	3	5.28 ± 1.79	3.10 ± 0.89	0.57
	6	5.22 ± 1.67	7.81 ± 2.57	0.56
	9	3.77 ± 0.81	2.48 ± 1.04	0.28

P-values were not corrected for repeated measurements. No differences were measured between WT and EDA-/- mice.

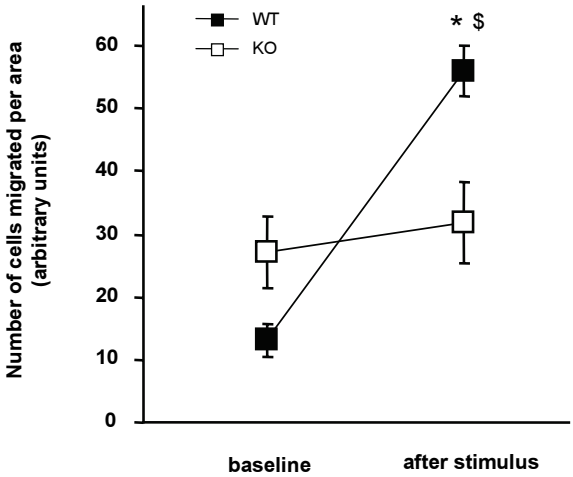

Figure 3. Migration in vitro
Migration of WT (black) and EDA-/- (white) MEFs without and with FBS as stimulus.
The number of migrated WT cells increased after stimulation (*P=0.034), while no migration of EDA-/- cells was induced (P=0.724). More WT cells migrated towards FBS than EDA-/- cells ($P=0.050).

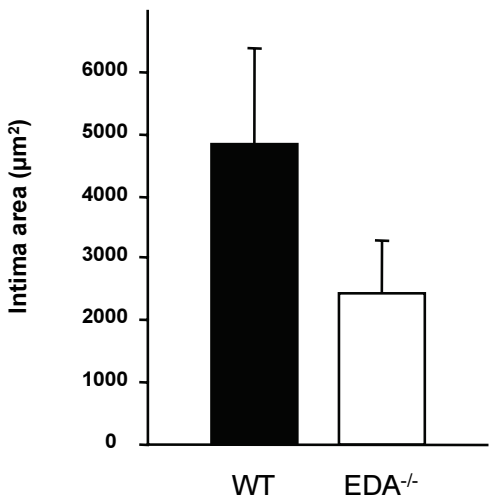

Figure 4. Intima formation
Intima formation after 28 days induced by cuff placement in WT and EDA-/- mice. A trend towards less intima formation in EDA-/- arteries was measured.

increased (56.06 ± 3.98 vs. 13.15 ± 2.58, P=0.034) as compared to without FBS. No difference was found in the number of migrating EDA-/- cells with and without FBS, resulting in a significant difference between EDA-/- and WT cells (EDA-/- 31.95 ± 6.54 vs. WT 56.06 ± 3.98 cells/membrane, P=0.050).

Migration of cells in vivo

Having established that Mouse Embryonic Fibroblasts EDA-/- cells show less migration compared to WT cells, we used a mouse femoral cuff model for intima formation as an in vivo model to monitor cell migration. Neointima was measured at 28 days after cuff placement in WT and EDA-/-mice (Figure 4). In the EDA-/- arteries, neointima formation was lower at 28 days compared to WT animals.

Discussion

Outward arterial remodeling is one of the hallmarks of a rupture-prone plaque that is considered the underlying cause of most myocardial infarctions or stroke. Until now, however, it is unclear which molecules are essential for outward remodeling to occur.
In this study, we investigated the role of the EDA domain of fibronectin in a mouse model for outward remodeling and found that without the EDA domain outward remodeling does not occur. The EDA domain in fibronectin is highly expressed during tissue remodeling like in wound healing, embryogenesis, and pathological processes such as tumor development, osteoarthritis and rheumatoid arthritis and ventricle remodeling of the heart after infarction [5-6,11,17-19].
EDA is expressed in arteries in pathological processes like balloon injury and atherosclerosis, and without EDA expression atherosclerotic plaque formation is diminished [8,10,20].
In this study we established a causal role for EDA in outward arterial remodeling. However, since EDA is described to bind to Toll-like receptor 4 and integrins, it remains unclear, how EDA achieves its role in remodeling.
Toll-like receptor 4 is described to respond to EDA and plays a causal role in outward arterial remodeling and ventricle remodeling after myocardial infarction [9,11]. In both arterial and ventricle remodeling collagen accumulation and decreased degradation has been described as an important feature of TLR4 deficiency that limited the remodeling response [21]. In our study, we do not find a difference in either collagen accumulation or collagen turn-over, pointing to another mechanism.

EDA is also important for binding integrins. EDA itself serves as a binding domain [12], and the incorporation of EDA into fibronectin also modifies binding capacities of other domains in fibronectin [13]. Integration of EDA in fibronectin up-regulates integrin-binding affinity to alpha5beta1 and enhances migration of cells [13].
We also investigated the migratory role of EDA in EDA-/- cells and found that migration of these deficient cells towards a chemo-attractant was strongly reduced. Since proliferation is induced by fibronectin containing EDA [22] and neointima formation is also determined by cell migration, the reduction in neointima formation in EDA-/- mice strongly points to an in vivo role of EDA in cell migration. We cannot exclude, however, that EDA similar to LPS [23], via TLR4 enhances cell migration without affecting collagen turn-over.
In contrast to TLR4 deficient mice, EDA-/- mice do not only show no outward remodeling but also shrinkage. This might be due to the fact that fibronectin promotes collagen gel contraction [24,25] that may result in arterial shrinkage and points to specific role for the EDA domain in collagen contraction. This, however, remains to be established.
In human atherosclerotic lesions, high EDA levels are associated with a stable plaque phenotype [26] that is associated with a low rate of outward remodeling. There are several reasons that can explain the discrepancy between EDA levels in stable human plaques and the result in our mouse model. First, the human plaque is the endstage of the disease in which EDA has a different role then in the onset of remodeling in the mouse. Secondly, EDA in human plaques can only be measured at one point in time and it is not known if high levels of EDA are found in plaques that are becoming unstable and start remodeling. Thirdly, EDA levels are measured locally in the plaque while outward remodeling of the artery occurs in the whole artery, and especially the adventitia containing most collagen is an important layer for arterial size and structure.
In conclusion, we showed that fibronectin containing EDA is an essential molecule for outward arterial remodeling probably not via collagen turn-over but by modulating cell migration.

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Chapter 5

Levels of extra domain A containing fibronectin in human atherosclerotic plaques are associated with a stable plaque phenotype

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Abstract

Background

Extra Domain A (EDA), splice-variant of fibronectin, is a Toll-like receptor 4 (TLR4) ligand. Recently, EDA has been demonstrated to enhance atherogenesis in mice but human data on the role of EDA in atherosclerotic disease are lacking. We hypothesized that EDA is associated with unstable plaque phenotypes and that plasma EDA could serve as biomarker for atherosclerosis.

Methods

EDA levels were assessed in carotid endarterectomy specimen (206 patients) and related with plaque phenotype. In a second patient cohort, systemic EDA levels in atherosclerotic patients (73 patients) were compared to risk-factor matched controls (68 patients).

Results

EDA plaque levels were associated with characteristics of stable plaques; more smooth muscle cells (P=0.003), more collagen (P=0.071), less fat (P=0.023). Concomitantly, asymptomatic patients showed higher EDA values in the plaque compared to symptomatic patients (P=0.004). EDA plasma levels did not differ between atherosclerotic patients versus controls (P=0.134).

Conclusion

EDA plaque levels are higher in asymptomatic patients and are associated with a stable plaque phenotype. EDA is not a plasma marker for atherosclerotic disease. These results suggest that local presence of endogenous TLR4 ligand EDA is not associated with an unstable plaque phenotype in humans.

Keywords: atherosclerosis • plaque • fibronectin • extra domain A

Introduction

Fibronectin is a major component of the extracellular matrix (ECM). Fibronectins are high molecular weight glycoproteins, that play key roles in cell adhesion, migration, growth and differentiation [1]. Alternative splicing of the primary transcript of the fibronectin gene results in the insertion or exclusion of certain domains within the fibronectin protein [2]. Extra domain A (EDA or EIIIA) is a fibronectin domain that is included by alternative splicing during embryogenesis, wound healing, and cellular damage [2,3].

Expression of fibronectin containing EDA is increased in pathophysiological conditions, for example in graft versus host disease leading to fibrosis of the skin, fibrosis in lung, liver and kidney, diabetes, rheumatoid arthritis, cardiac transplantation, vascular intimal proliferation, and vascular hypertension [4-13].

EDA is known to activate Toll-like receptor 4 (TLR4) [14]. TLRs are the first line of defense in the innate immune response following recognition of pathogen-associated molecular patterns. To date, ten human TLRs have been identified. TLR4 recognizes exogenous ligands, like bacterial lipopolysaccharide, but also endogenous ligands, like EDA and heat shock protein 60 [14-16].

The presence of TLR4 has been documented in murine and human atherosclerosis [17]. Recently, we and others showed that TLR4 activation accelerates arterial occlusion by neointima formation, plaque formation, geometrical remodeling and plaque destabilization [18-21]. It remains unclear which ligands activate TLR4 and thereby promote atherosclerotic luminal narrowing. Exogenous ligands are not obligatory for development of murine atherosclerosis [22] and are not involved in TLR4 dependent flow induced expansive remodeling [18].

The endogenous TLR4 ligand EDA is expressed in human and murine atherosclerotic lesions [23]. Evidence that EDA may act as a trigger for atherogenesis is accumulating. EDA levels are elevated in plasma of ApoE knockout mice [23] and EDA mRNA levels are increased during TLR4-dependent outward remodeling [18]. Furthermore, in atherosclerotic mice lacking EDA, atherosclerotic lesion areas were found to be reduced in number and size.

Human studies in which EDA levels are studied in atherosclerotic plaques and plasma in relation with clinical presentation are lacking. We hypothesized that local EDA levels in the atherosclerotic plaque are associated with a more inflammatory and advanced stage of atherosclerotic disease. We therefore determined EDA levels in human atherosclerotic plaques and plasma samples obtained in two patient cohorts to assess

associations with plaque characteristics and the presence of clinically manifest atherosclerotic disease.
We report that EDA levels in the plaque are associated with a stable plaque phenotype. No association was observed between plasma EDA and the presence of clinically manifest atherosclerotic disease. Our results suggest that EDA is not a serological biomarker for atherosclerotic disease but that plaque EDA could serve as a potential local marker associated with a stable plaque phenotype.

Materials and methods

Clinical studies

Athero-Express

Athero-Express is an ongoing longitudinal cohort study, initiated in 2002 by two Dutch hospitals [24]. The study has been approved by the ethics committees of both hospitals and written informed consents were obtained from all participants. The study is designed to investigate the expression of atherosclerotic tissue derived biological markers in relation to plaque phenotype and the long term outcome of patients undergoing carotid endarterectomy (CEA). Patients who undergo CEA fill in an extensive questionnaire and diagnostic examinations are performed. The Athero-Express biobank furthermore contains blood samples drawn before surgery and freshly frozen atherosclerotic tissue to study protein and RNA expression. We used plaque samples from the Athero-Express study to answer the research question whether EDA plaque levels are associated with stable or unstable plaques.

SMART

The SMART (Second Manifestations of ARTerial disease) study is an ongoing, single-center, prospective cohort study of approximately 3500 patients. The patients are referred to the University Medical Center Utrecht for the first time because of atherosclerotic vascular disease or treatment of atherosclerotic risk factors [25]. The study was approved by the ethics committee of the University Medical Center Utrecht, and written informed consent was obtained from all participants.
For the present study, patients were selected that fulfilled the following three criteria: (1) a history of clinically manifest atherosclerotic disease (referral for percutaneous peripheral or coronary intervention, surgery to treat aneurysm formation, CABG, surgery to treat peripheral arterial disease, stroke); (2) atherosclerotic disease at the

time of hospital-entry and (3) one cardiovascular risk factor (hypertension, diabetes mellitus or hyperlipidemia). All patients had been screened for the presence of clinically silent atherosclerotic lesions in the carotid and femoral arteries and aorta using three approaches. First, atherosclerotic luminal narrowing in the femoral artery was evaluated by measuring the ratio of the systolic blood pressure measured at the ankle to the systolic blood pressure measured in both arms (ankle-brachial pressure index, ABPI). When patients without symptoms had a lowered ABPI (≤0.9) they were classified as having silent atherosclerosis. Second, stenosis of the common and internal carotid arteries was measured bilaterally with Doppler-assisted duplex scanning. When carotid stenosis exceeded 70%, patients were diagnosed having clinically silent atherosclerosis. Third, ultrasonography of the abdomen was performed to measure the anteroposterior juxtarenal diameter and the distal anteroposterior diameter of the aorta. When distal anteroposterior diameter ≥3 cm or ≥1.5 times anteroposterior juxtarenal diameter, patients were diagnosed having clinically silent atherosclerosis.
Control patients from the SMART study were frequency-matched for sex, age and one of three major risk factors (hypertension, diabetes mellitus and hyperlipidemia). These patients revealed neither clinically evident nor silent atherosclerosis.
The SMART cohort was used to answer the research question whether EDA could serve as a plasma biomarker for the presence of atherosclerotic disease.

Handling of atherosclerotic specimens

Atherosclerotic specimens obtained by carotid endarterectomy were dissected in segments of 0.5 cm in length. The segment with the largest plaque size (numbered 0) was fixated in formaldehyde 4% and paraffin embedded. The adjacent segments (numbered + or – 1,2,3 etc.) were immediately frozen in liquid nitrogen and stored at –80˚C. Paraffin sections of the atherosclerotic plaques were immunohistochemically stained and scored for the presence of macrophages (CD68), smooth muscle cells (alpha actin), collagen (picro Sirius red) and fat. Collagen staining was determined using polarized light microscopy on picro Sirius red stainings and scored as 1) no or minor staining = staining along part of the luminal border; 2) moderate or heavy staining= staining along the entire luminal border. The percentage atheroma of the total area of the plaque is visually estimated using hematoxylin stains. Three groups were considered based on the percentage of atheroma in the plaque: fibrous plaques containing <10% fat; fibro-atheromatous, 10-40%; or atheromatous, > 40% fat. The CD68 (macrophage) and smooth muscle alpha actin staining were analyzed quantitatively by computerized analyses using Analysis® software. Color thresholds

were set and adjusted until the computerized detection met the visual interpretation. The stained areas were scored quantitatively as a percentage of the plaque area. All semiquantitative scores were performed independently by two observers.
Proteins were isolated from the adjacent (+1) segments using 1ml Tripure™ Isolation Reagent (Boehringer, Mannheim) according to the manufacturer's protocol.

Mammary arteries

Mammary arteries were used as non atherosclerotic control arteries. Segments of mammary arteries (n=6) were obtained during bypass surgery. Levels of EDA protein in the mammary arteries were compared to 9 Athero-Express protein samples, measured all together on one ELISA plate.

Immunohistochemistry

Sections of atherosclerotic lesions were immunohistochemically stained for EDA (IST-9, Abcam, Cambridge), IgG1 as isotype control, macrophages (CD68), SMCs (alpha-actin), and endothelial cells (CD31).
EDA and IgG1 stainings were developed with fast blue, macrophages with fast red, the endothelial cells and SMCs with AEC. Haematoxylin was used for counterstaining.

EDA enzyme-linked immuno sorbent assay

Fibronectin containing EDA was measured with an enzyme-linked immunosorbent assay (ELISA) developed at the University Medical Center Utrecht [[8]]. Microtiter plates were coated overnight with IgG1 monoclonal antibody IST-9 (1.06 µg/mL) directed against Extra Domain A of fibronectin (Abcam, Cambridge, UK) in carbonate buffer (Na_2CO_3 15 mM, $NaHCO_3$ 35 mM, pH 9,6) at 4°C. Wells were washed with PBS/0.1% Tween and then blocked with phosphate-buffered saline (PBS)/3% bovine serum albumin (BSA)/0.1% Tween. Samples were diluted to an appropriate concentration with PBS/0.1% Tween/3% BSA (1:20 for plasma and 1:100 for protein extracts in 1% SDS). Ligand capture was detected by addition of peroxidase-conjugated rabbit-anti-human fibronectin antibodies (1.3 µg/mL) (Dako, Glostrup, Denmark). Binding of the antibody was detected by adding 1 µg/mL O-phenylene-diamine in 0.05 M citrate-phosphate buffer (pH 5) with 2.5 mM H_2O_2. The reaction was stopped by adding 1 M sulfuric acid. Absorbance was read at 490nm on microplate reader model 550, Biorad, 655nm filter was used as reference-filter. As a standard, cellular fibronectin was purified from cultured human fetal lung fibroblasts [26]. EDA levels measured by ELISA were corrected for protein concentrations of the samples.

MMP, IL and Emmprin measurements in plaques

MMP-2 and -9 are most intensively studied MMPs in relation to atherosclerosis and have been associated with matrix degradation in unstable lesions. MMP-8 has been related with thrombus formation. Recently we demonstrated an increase of MMP-8 and MMP-9 activity levels in unstable plaques, whereas MMP-2 activity levels are higher in stable lesions [27].

In 119 protein samples from a randomly selected subgroup of the Athero-Express study, matrix metalloproteinase (MMP)-2, MMP-8 and MMP-9 activities were measured using the Biotrak activity assays RPN 2631, RPN 2635, and RPN 2634 (Amersham Biosciences), respectively.

Extracellular matrix metalloproteinase inducer (Emmprin) expression levels were determined by Western blotting [27] (sc-9753, Santa Cruz Biotechnology).

Interleukin (IL)-6 and IL-8 are pro-inflammatory cytokines that have been associated with enhanced inflammatory response in atherosclerotic lesions. We measured IL-6 and IL-8 levels by a multiplex suspension array system according to the manufacturer's protocol (Bio-Rad Laboratories).

Statistics

The Mann-Whitney U or Kruskal-Wallis test was performed to compare different variables. Linear regression analyses were performed to correlate plasma and plaque EDA. A P-value <0.05 was regarded significant.

Results

Baseline characteristics of Athero-Express and SMART study population

The baseline characteristics of the Athero-Express and SMART study population are presented as supplementary data. Besides the difference in intima media thickness as measure of extent of atherosclerotic disease, which is higher in atherosclerotic patients, the cholesterol levels in atherosclerotic patients are lower than in the controls. This is probably the result of statin treatments the atherosclerotic patients received. No differences were observed in the other baseline characteristics (age, gender, body mass index, hypertension, hyperlipidemia, diabetes mellitus, smoking, cholesterol, triglycerides, HDL-cholesterol, glucose) between atherosclerotic patients and controls in the SMART study population.

EDA levels in the atherosclerotic plaque

EDA expression levels in plaques compared to non-atherosclerotic arteries

Atherosclerotic plaques revealed higher EDA levels compared to non-atherosclerotic mammary arteries. (n=9 and n=6 respectively, 22.9 ± 5.5 versus 6.8 ± 1.7 µg/mL, P=0.003, supplementary figure 1a) Mammary arteries didn't show EDA staining, confirming the difference in EDA expression measured by ELISA (supplemental figure 1b-e). To determine which cells do express EDA in the plaque, sections were immunohistochemically stained for EDA. EDA expression was predominantly observed in the extracellular matrix around macrophages, but also in SMC-rich areas and around endothelial cells (Figure 1).

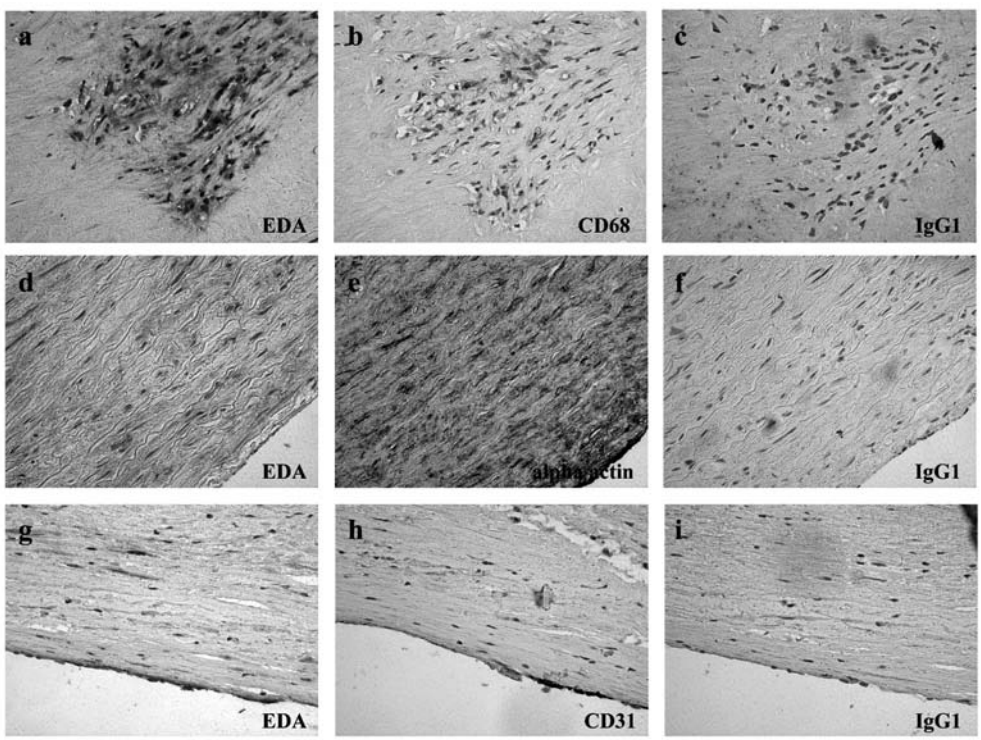

Figure 1. EDA expression in atherosclerotic lesions

EDA staining (1a, d, g) is predominantly co-localized with staining for macrophages (CD68, 2b), but also observed in smooth muscle cell rich areas (alpha actin, 1e) and around endothelial cells (CD31, 1h). IgG controls are negative (1c, f, i). All pictures are taken with 400x magnification.

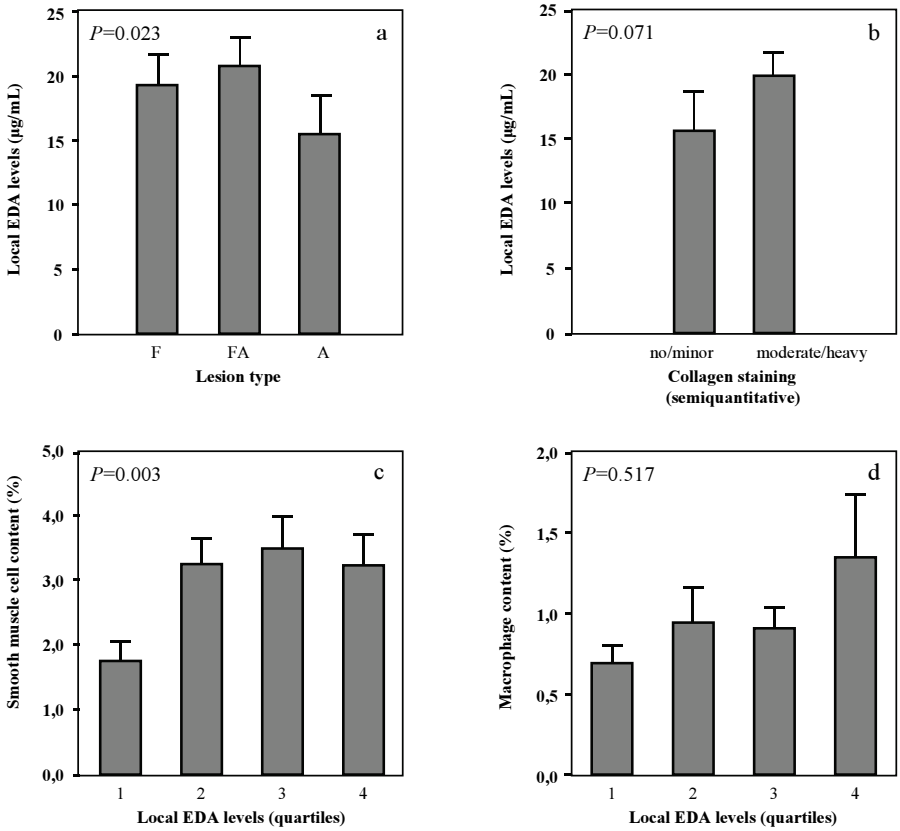

Figure 2. Relation of local EDA levels with amount of collagen, fat, macrophages and smooth muscle cells in the plaque

The plaques were divided into three groups; fibrous (F, n=67), fibroatheromatous (FA, n=72) and atheromatous (A, n=63), based on fat content (<10%, <40% or >40% fat, respectively). Higher local EDA levels were detected in the fibrous plaques (a, P=0.023). EDA levels in plaques with no/minor collagen staining (n=52) are compared to plaques with moderate/ heavy collagen staining (n=147). Local EDA levels are associated with more collagen content (b, P=0.071) and a higher number of smooth muscle cells in the plaque (c, P=0.003). No relation is found between the number of macrophages in the plaque and local EDA levels (d, P=0.517). Error bars in figure 2a-d represent the standard error of the mean (SEM).

EDA levels and plaque phenotype

Atheromatous plaques (n=63) showed significantly lower EDA values compared to fibrous (n=67) and fibroatheromatous plaques (n=72), (15.5 ± 3.0, 19.4 ± 2.3, and 20.8 ± 2.2 µg/mL, respectively, P=0.023, Figure 2a).

Fibrous plaques are commonly associated with strong collagen and smooth muscle cell staining, while the atheromatous plaque is associated with an inflammatory unstable

plaque phenotype. Figures 2b-c show that plaques with higher EDA expression, contain more collagen (although not significantly different, there is a strong trend towards more collagen in plaques with more EDA in the plaque) and more smooth muscle cells (b and c, P=0.071 and P=0.003, respectively). The lower percentage of SMC of plaques in quartile 1, expressing the lowest EDA levels, differs from the SMC percentages of the plaques in the other quartiles. No statistical relation is observed between EDA levels and the inflammatory macrophage cell content (d, P=0.517). Smooth muscle cells mostly outnumber the macrophages. This might obscure a possible relation between macrophage presence and EDA. Therefore we assessed the relation of macrophages and EDA expression in the plaques that contained low numbers of smooth muscle cells. Also in this group no relation between EDA levels and macrophage content was observed in the plaques (data not shown).

Table 1 . Plaque EDA levels in µg/mL (± SEM) in quartiles of inflammatory components

	Quartiles				
	1	2	3	4	P-value
MMP-2	9.6 (± 1.8)	17.3 (± 2.6)	19.3 (± 3.1)	29.3 (± 4.6)	0.001
MMP-9	20.4 (± 2.3)	18.2 (± 2.7)	24.7 (± 5.2)	12.4 (± 2.2)	0.121
MMP-8	18.0 (± 2.9)	16.2 (± 2.4)	20.7 (± 3.6)	20.8 (± 4.5)	0.929
Emmprin 45 kD	10.5 (± 3.6)	17.3 (± 2.9)	22.2 (± 3.2)	24.8 (± 3.2)	< 0.001
Emmprin 58 kD	14.1 (± 3.5)	21.8 (± 2.9)	17.6 (± 2.2)	21.4 (± 4.3)	0.072
Ratio emmprin 45kD/58kD	13.6 (± 3.6)	18.7 (± 3.9)	17.9 (± 2.3)	26.5 (± 3.6)	0.010
IL-6	25.0 (± 4.0)	14.1 (± 2.0)	16.3 (± 2.9)	19.0 (± 3.1)	0.138
IL-8	24.0 (± 3.1)	19.9 (± 3.5)	19.3 (± 3.2)	11.2 (± 2.3)	0.002

We also determined the correlation (Spearman test) between EDA expression levels and the levels of inflammatory components. MMP2, rho=0.377, P<0.001; MMP9, rho=-0.222, P=0.015, MMP8, rho=-0.031, P=0.740; Emmprin 45kD, rho=0.424, P<0.001; Emmprin 58kD, rho=0.137, P=0.131; Ratio emmprin 45/58kD, rho=0.329, P<0.001; IL-6, rho=-0.082, P=0.253; IL-8, rho=-0.257, P<0.001.

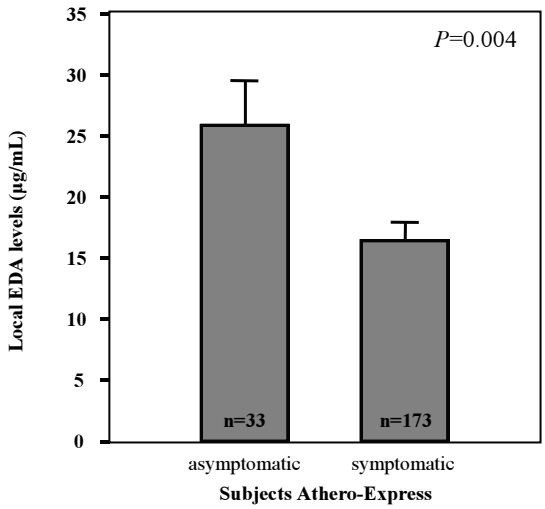

Figure 3. EDA levels in plaques from asymptomatic versus symptomatic subjects from the Athero-Express study

Asymptomatic patients show higher EDA plaque values compared to symptomatic patients (27.0 ± 3.8 versus 17.1 ± 1.6 µg/mL, P=0.004). Error bars represent SEM.

In the atherosclerotic plaque samples the expression levels of MMPs, MMP inducer Emmprin, IL-6 and IL-8 were determined (Table 1). Emmprin is expressed in two glycosylated forms in plaques; 45kD and 58kD. Previously, smooth muscle cell rich, stable lesions were associated with high Emmprin 45kD and MMP-2 levels. In contrast, unstable inflammatory lesions were associated with Emmprin 58kD and MMP-9 levels [27]. The expression of 45kD variant of MMP inducer Emmprin was positively correlated with EDA in the plaque, while the 58kD variant showed a trend to correlation with EDA (45kD, P<0.001; 58kD, P=0.072). In table 1 the EDA levels in µg/mL (± SEM) are shown for the different components divided into quartiles. EDA expression increased when more MMP-2 was expressed in the plaque (P=0.001). No association between EDA expression levels and MMP-8 and -9 was observed in the plaques (P=0.929 and P=0.121). A low ratio of Emmprin 45kD/58kD is an indicator of the activity status of Emmprin. This ratio was positively associated with higher EDA expression levels (P=0.010). We observed no relation between IL-6 and EDA levels (P=0.138). IL-8 levels showed an inverse relation with EDA expression in the plaques (P=0.002).

EDA levels in symptomatic versus asymptomatic subjects

Verhoeven *et al.* [28] reported that patients with vulnerable plaque phenotypes more

often suffered from symptoms related with cerebral ischemia compared to patients with stable plaques. We investigated whether plaques obtained from symptomatic versus asymptomatic showed different EDA expression levels. Patients undergoing carotid endarterectomy who had suffered from any clinical symptom related to carotid atherosclerosis, like TIA, stroke or amaurosis fugax, were considered symptomatic. Concomitantly, patients who did not suffer from clinical symptoms were asymptomatic: their indication for surgical therapy depended on co-morbidity and vertebral-basilar (in)sufficiency.
Higher EDA plaque values were found in asymptomatic subjects compared to symptomatic subjects (27.0 ± 3.8 µg/mL versus 17.1 ± 1.6, P=0.004, Figure 3).

Systemic EDA levels

EDA levels in plasma from atherosclerotic patients and controls (SMART)

Having established that EDA levels are increased in atherosclerotic specimen compared with non atherosclerotic arteries, we investigated whether systemic EDA levels in plasma were related with clinical manifestation of atherosclerotic disease.
EDA levels were measured with ELISA in plasma of 73 atherosclerotic patients and 68 risk-factor matched controls. All control patients scored negative when screened for the presence of clinically silent peripheral atherosclerotic disease (see methods). Plasma EDA levels did not differ between patients and controls (6.4 ± 0.5 versus 7.6 ± 0.6 µg/mL, respectively, P=0.134, Figure 4a). All patients in the SMART cohort underwent duplex ultrasound measurements to assess carotid intima media thickness. SMART subjects were divided in quartiles based on their measured carotid intima-media thickness which is representative for the severity of atherosclerotic disease. Although a tendency was suspected towards an inverse relation between intima media thickness and EDA levels, no significant difference was found in EDA plasma levels between intima media thickness quartiles (P=0.182, figure 4b).

Discussion

Extra domain A is incorporated in fibronectin during embryogenesis and repair processes after tissue-injury. In this study we investigated whether human EDA levels local in the plaque and systemic in plasma could be associated with plaque characteristics and used as marker for atherosclerotic disease.

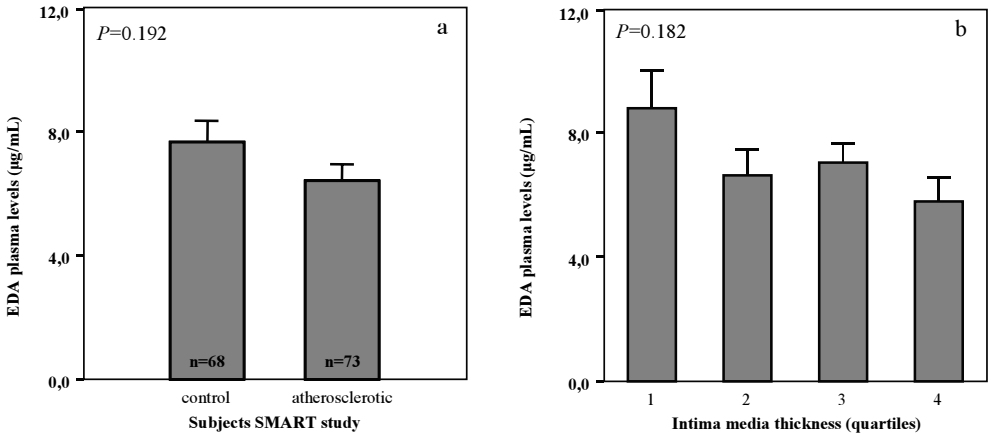

Figure 4. EDA levels in plasma in µg/mL of atherosclerotic patients (n=73) and risk-factor matched controls (n=68) from the SMART study

No difference is found between the patient and control-group (7.1 ±0.7 versus 6.9 ± 0.5 µg/mL, respectively, P=0.192, a). Error bars represent SEM. No difference in EDA levels was found in the SMART population divided into quartiles based on intima-media thickness (8.8 ± 1.2 µg/mL; 6.6 ± 0.8 µg/mL; 7.0 ± 0.6 µg/mL; 5.8 ± 0.8 µg/mL, P=0.182, b). Error bars represent SEM.

Local EDA levels in the plaque

In atherosclerotic lesions, EDA expression levels were higher compared to non atherosclerotic mammary arteries. Different cell types in the atherosclerotic lesions have been co-localized with EDA expression, like endothelial cells, macrophages and smooth muscle cells [2-12,23-29]. Local EDA levels were associated with a more stable plaque phenotype. Fibrous and fibroatheromatous lesions express higher EDA levels compared to atheromatous plaques. Furthermore, EDA expression is associated with more collagen and more smooth muscle cells, characteristic for a stable plaque phenotype. Concomitantly, higher EDA expression levels were observed in asymptomatic patients compared to patients with clinical symptoms related to atherosclerosis. This is in accordance with Verhoeven *et al.* [28], who showed that patients with vulnerable plaque phenotypes exhibit more symptoms related to atherosclerosis than patients with stable plaque phenotypes.

The finding that local EDA levels are higher in atherosclerotic specimen compared to a healthy vessel wall and considering the association of EDA with plaque phenotypes, suggests a role for EDA in atherosclerosis. The dual role of EDA in fibronectin as major component of the ECM and as activator of the immune system via TLR4 [14] make EDA

a good candidate to be involved in the initiation and progression of atherosclerosis, in which both inflammation and matrix turn-over are important processes.
As a ligand of TLR4 [14] and with atherosclerotic EDA null mice having less atherosclerosis and a more stable plaque phenotype [23], we expected a correlation between high EDA plaque levels and a more inflammatory and unstable plaque phenotype. However, in plaques with atheromatous phenotypes the EDA levels were found to be lower and EDA levels were not associated with the presence of macrophages. These surprising observations might be explained by the possible differences in the mechanisms for atherosclerotic lesion development between mice and humans. In the present study atherosclerotic lesions have been investigated that resulted in a clinical syndrome, which is not observed in genetically modified atherosclerotic mice. In these lesions high EDA levels were related with stable, non-inflammatory plaques. This observation does not exclude an inflammatory role for EDA during the initiation and early progression of the atherosclerotic plaque as shown in EDA KO mice [23], which points to EDA as a key molecule in the initiation phase of atherosclerosis. Damage to arterial wall cells, for example to the endothelium, is one of the first triggers to atherosclerosis. The response of the arterial wall to damage is scarring, which includes ECM formation, SMC proliferation and also EDA production as shown by Dubin *et al*. [11]. EDA in the plaque may activate TLR4 and initiate an inflammatory response, like a fraction of EDA is able to activate TLR4 and initiate an inflammatory cascade [14]. But TLR4 activation is also important for collagen turnover [20] and VSMC proliferation [30]. EDA might trigger SMC proliferation via TLR4 activation, leading to stabilization and reorganization of the plaque, which results in conversion of an unstable inflammatory plaque into a stable, smooth muscle cell rich plaque. TLR4 expression has been described in macrophages in murine and human atherosclerotic lesions [17,31]. Immunohistochemistry showed EDA-staining around macrophages in the lesions, however, no association was observed between the number of plaque macrophages and plaque EDA levels. In contrast to smooth muscle cells, the more SMC, the more EDA is measured in the plaque, suggesting that not macrophages but smooth muscle cells are the dominant EDA producing cells in the human atherosclerotic plaque. Glukhova *et al*. [12] demonstrated that EDA is expressed in the intima of human and experimental animal arteries by SMC that underwent a phenotype switch; from contractile to synthetic/ fibroblast-like SMCs. Synthetic SMCs do have a high proliferation and migration rate and produce extra cellular matrix components. These capacities are important during the initiation of atherosclerosis when SMCs migrate from the medial layer into the intima, where they will proliferate

and produce extracellular matrix components. In more advanced atherosclerotic lesions these synthetic SMCs may have a different role. These smooth muscle cells produce next to fibronectin containing EDA [2,3] also collagen [32] and other matrix molecules for repair of the arterial wall. This is in agreement with the previously observed correlations between EDA, collagen content, MMP2 and Emmprin 45kD levels [27] in the plaque. SMC proliferation and their production of ECM components may stabilize or reorganize the plaques from unstable into more stable atherosclerotic lesions.
The absence or even inverse relation between EDA and IL-8, IL-6, MMP-8, and MMP-9 supports the idea that local inflammation is not associated with local EDA levels. Tendencies towards inverse associations between the presence of macrophages, IL-8 and EDA were observed. Therefore, this study is not likely underpowered to support the hypothesis of a positive association between local inflammation and EDA.

EDA levels in plasma

Although plaque EDA levels are associated with plaque phenotype, EDA plasma levels did not correlate with the severity of atherosclerosis, measured as the intima-media thickness in the SMART population. In addition, EDA levels did not differ between atherosclerotic patients and risk factor-matched controls. EDA is a high molecular weight protein associated with the extracellular matrix. This may explain why EDA is not released from the arterial wall during atherosclerosis. However, in diabetic patients EDA is released from the vessel wall into the circulation and EDA levels are associated with diabetes [8]. EDA is involved in many other processes and disorders related to pathophysiological circumstances in the vessel wall [4-13]. EDA levels in plasma may also represent other pathological or physiological changes and might mask the changes due to atherosclerosis. Furthermore, also processing of EDA into smaller fragments might be necessary to get an immune reaction. These are two possible explanations why we did not find a difference in EDA containing fibronectin levels between atherosclerotic subjects and risk factor matched controls. In athero-express plasma samples EDA levels were also assessed. The systemic levels did not correlate with EDA plaque levels, or with plaque phenotype (data not shown), which indicates that plasma EDA levels are not useful as marker for atherosclerosis.

Limitations of the study

Internal mammary arteries have been used as control arteries, since they are spared from atherosclerosis possibly due to biochemical or flow mediated reasons. The mammary arteries are not the optimal control compared to non-diseased arteries prone

to develop atherosclerosis. However, availability of non diseased carotid artery is a drawback. We did not execute a specific fat stain. However the fat content of the plaques was clearly visible in the used HE stainings.

In summary, we show that levels of plaque EDA in human atherosclerotic lesions are associated with stable plaque phenotypes probably due to the higher number of smooth muscle cells that are the potential producers of human plaque EDA. This observation implies that the detection of endogenous TLR ligand not necessarily represent an inflammatory environment destabilizing the vulnerable plaque, but also may trigger the stabilization of atherosclerotic lesions. This is an observational study and therefore inferences regarding causality cannot me made. However, considering previous results from our own laboratory (unpublished data) and from Tan *et al*. [23] we feel that our data support the concept that EDA is actively involved in atherogenesis and should not be considered an innocent bystander. No differences were found in plasma levels of EDA between groups investigated, suggesting that EDA in plasma can not be used as diagnostic or prognostic plasma marker for atherosclerosis. The difference in EDA levels between stable and vulnerable plaque phenotypes identify EDA as a potential local plaque marker to image atherosclerotic plaques.

Acknowledgements

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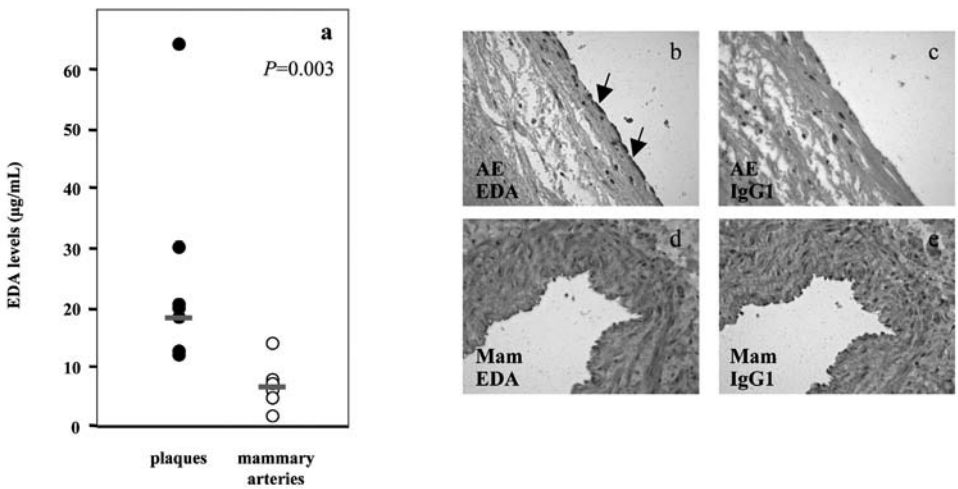
Supplements

Supplemental Table 1. Baseline characteristics of Athero-Express study population

Athero-express	
Total number of patients	206
Male gender (%)	67
Hypertension (%)	69
Hyperlipidemia (%)	60
Diabetes Mellitus (%)	19
Statin use (%)	61
Smoking (%)	88

Supplemental Table 2. Baseline characteristics SMART study population

SMART	Controls	Atherosclerotic patients	P-value
Total number of patients	68	73	
Age (years)	56 ± 9	60 ± 11	0.14
Male gender (%)	50	49	0.99
Body mass index (%)	26 ± 4	26 ± 3	0.84
Hypertension (%)	8	7	0.96
Hyperlipidemia (%)	82	85	0.80
Diabetes Mellitus (%)	10	8	0.71
Smoking (%)	47	59	0.11
Intima/media thickness (mm)	0.79 ± 0.20	0.95 ± 0.24	< 0.001
Cholesterol (mmol/L)	6.75 ± 0.24	5.77 ± 0.14	0.001
Triglyceriden (mmol/L)	2.89 ± 0.29	2.09 ± 0.10	0.248
HDL-cholesterol (mmol/L)	1.13 ± 0.04	1.15 ± 0.04	0.499
Glucose (mmol/L)	6.30 ± 0.38	6.23 ± 0.25	0.257



Supplemental Figure 1. Expression of EDA is elevated in atherosclerotic lesions
Expression of EDA is higher in protein samples from atherosclerotic arteries compared to mammary arteries (a, n=9 and n=6 respectively, 22.9 ± 5.5 versus 6.8 ± 1.7 µg/mL, P=0.003). Immunohistochemistry showed EDA expression in the atherosclerotic plaque (b, AE-EDA, black arrows indicate EDA staining), while no staining was observed in mammary arteries (d, mam-EDA. Figure c and e are IgG1 control stainings for plaque and mammary artery respectively.)

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Chapter 6

Human atherosclerotic plaque levels of extra domain A of fibronectin are associated with TGF-beta signaling molecules including Endoglin

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Submitted

Abstract

Background

Atherosclerosis develops over decades to complex lesions that may lead to cardiovascular events like myocardial infarction and stroke. These complex lesions can be divided in fibrous stable lesions and inflammatory unstable lesions from which the latter is thought to be associated with the occurrence of cardiovascular events. We recently established that the extra domain A (EDA) containing fibronectin, an embryonic form of fibronectin regulated via alternative splicing, is associated with stable, fibrous atherosclerotic lesions. Although it is known that EDA can be regulated by Transforming Growth Factor ß (TGFß) it is, however, unclear how EDA expression is regulated in atherosclerotic plaques and if Endoglin (CD105), an accessory component of the TGFß receptor complex, is involved in this. To study this, we investigated the localization and association between the levels of EDA and TGFß signaling molecules including Endoglin in human atherosclerotic lesions and investigated the regulatory role of Endoglin on TGF-beta stimulation in vitro.

Methods

Human atherosclerotic lesions were collected after endarterectomy and EDA and Endoglin localization was determined using immunohistochemistry. EDA, Endoglin, TGFß, p-SMAD expression levels in the plaque were measured using ELISA and Western blotting. The regulatory role of Endoglin on EDA expression was studied in embryonic endothelial cells from Endoglin wildtype and knockout (Eng-/-) mice.

Results

In human atherosclerotic lesions, EDA and Endoglin expression co-localized. EDA positively correlated with Endoglin levels as well as with TGFß and p-SMADs. In vitro TGFß stimulation of endothelial cells obtained from Eng-/- mice, showed higher EDA mRNA levels compared to wildtype Eng+/+ cells.

Conclusion

Atherosclerotic plaque EDA levels are associated with TGFß signaling including Endoglin that is likely an inhibitory protein for TGFß induced EDA expression.

Introduction

Atherosclerosis is the underlying disease for major cardiovascular events like myocardial infarction and stroke and is the major cause of death in the Western world. Atherosclerosis is an inflammatory disease [1] in which early lesions may progress into advanced atherosclerotic plaques. Dependent on the composition of the advanced atherosclerotic lesion, stable and unstable lesions can be discriminated. Unstable lesions or rupture-prone lesions are characterized by a thin fibrous cap, a large atheroma, infiltrated and activated inflammatory cells that are predominantly macrophages, and low smooth muscle cell content. When the plaque ruptures, thrombus formation is initiated and this often results in acute occlusion of the artery.
Fibronectin is a major component of the extracellular matrix and plays a key role in cell adhesion, migration, growth and differentiation [2]. Extra domain A (EDA) is a fibronectin domain included by alternative splicing during embryogenesis, therefore also referred to as fetal fibronectin [3]. However, EDA is also prominent during the injury response of adult tissues. In healthy arteries EDA is almost absent, but EDA is expressed in atherosclerotic lesions [4,5]. Higher EDA protein levels are associated with stable plaque characteristics; that includes more collagen, more smooth muscle cells, less macrophages and less fat [5].
In mouse models the role of EDA in the initiation of atherosclerotic lesions showed that atherosclerotic ApoE-/- mice lacking the EDA exon showed less atherosclerotic lesion formation [4].
The splicing of EDA in fibronectin is tightly regulated. One of the stimuli that induce EDA containing fibronectin is Transforming Growth Factor ß (TGFß) [6].
The complex TGFß-signaling pathway is involved in a variety of processes like differentiation, proliferation, migration, extracellular matrix formation, modulation of the immune and inflammatory response. After activation, TGFß binds to the TGFß type II receptor (TßRII) which in turn recruits TßRI, e.g. activin-receptor like kinases (ALKs) 1 or 5. ALK1 expression is restricted to endothelial cells, while ALK5 is broadly expressed. Ultimately SMADs are phosphorylated and translocated to the nucleus where they act as transcriptional regulators of target genes. Endoglin (CD105) is an accessory component of the TGFß receptor complex, predominantly expressed on proliferating endothelial cells [7]. Endoglin is crucial for efficient TGFß/ALK1 signaling; however, the precise role of Endoglin in TGFß signaling is not completely understood. Endoglin KO mice die at day 9.5-11.5 of gestation, due to defective angiogenesis. Mice heterozygous for Endoglin have very fragile arteries, due to low SMC content and

irregular supporting layers of collagen and elastin, resulting in abnormal vessel walls. Both EDA and Endoglin are expressed in atherosclerotic lesions and both are induced by TGFß. In atherosclerotic lesions, TGFß expression is found in areas of extracellular matrix production and intimal thickening. Cell culture experiments showed that Endoglin expression decreases TGFß induced cellular responses, like fibronectin synthesis [8]. To date, it is unclear how Endoglin regulates TGFß induced EDA expression and how Endoglin and EDA are associated in the human atherosclerotic plaque. In this study we determined the association between EDA and Endoglin levels in human atherosclerotic lesions. Furthermore, we used in vitro experiments to determine the regulatory role of Endoglin in TGFß induced EDA expression.

Materials and methods

Atherosclerotic tissue

Atherosclerotic tissue and protein samples from atherosclerotic specimen were collected for the Athero-Express biobank. The Athero-Express is a multicenter study combining a biobank of carotid endarterectomy material and patient follow up data [9]. The study was approved by the local medical ethics committees of each participating center and performed in accordance with institutional guidelines. Informed written consent was obtained from all patients.

Immunohistochemistry

Atherosclerotic lesions were embedded in paraffin. Five-micrometer sections were cut, deparaffinized and hydrated.

Slides for Endoglin and SMAD staining were incubated in methanol containing 1.5% H_2O_2 for 30 minutes to block endogenous peroxidase. Sections were boiled for 15 minutes in 10 mmol/L citrate buffer, pH 6.0, and blocked in 3% BSA in PBS-0.1% tween (PBSA) for 30 minutes. Slides were incubated with the first antibody overnight at 4°C. Endoglin: α-human CD105, BD Biosciences, 1:500. SMAD 1/5/8: Phospho-SMAD-1/5, (Ser 463/465) Cell signaling, 1:500. SMAD 2: Phospho-SMAD-2, (Ser 465/467) Cell signaling, 1:500. Sections were then incubated for 1 hour at room temperature with powervision polyHRP anti-mouse IgG (Immunologic). Stainings were developed with DAB for 10 minutes. Haematoxylin was used as counterstaining.

EDA slides were boiled for 15 minutes in 10 mmol/L citrate buffer, pH 6.0, and blocked in 10% normal horse serum in TBS for 30 minutes. The slides were incubated with

mouse monoclonal [IST-9] to fibronectin IgG1 (Abcam, 1:750) overnight at 4ºC. Mouse IgG1 was used as a negative control. Slides were then incubated with biotinylated anti-mouse IgG in TBSA for 1 hour. Alkaline phosphatase streptavidin, 1:750, was added for 1 hour and the sections were developed in Fast Blue for 30 minutes. Haematoxylin was used as counterstaining.

Cell culture

Human Microvascular Endothelial Cells (HMECs) were cultured in 0.2µg/ml fibronectin coated tissue culture flasks with MCDB-131 medium containing 10% fetal bovine serum (FBS), 100µg/ml pen/strep, 10mM l-glutamine, 0.05µM hydrocortisone and 10ng/ml hEGF. Mouse Embryonic Endothelial Cells (MEECs) were obtained from WT and Endoglin knockout (Eng-/-) mouse embryos at gestational day E9.0. Cells were cultured in 0,1% gelatin coated tissue culture flasks with DMEM medium containing 10% FBS, 100µg/ml pen/strep, 10mM l-glutamine and VEGF.
Prior to stimulation, HMEC cells were grown in gelatin coated wells, and HMEC and MEEC were starved in medium containing 0.1% FBS. Cells were stimulated with 1 ng/mL TGFß1 (peproTech), and the ALK5 inhibitor (Tocris Bioscience) was added 2 hours prior to stimulation.

RNA isolation, cDNA synthesis and quantitative PCR

RNA was isolated using TriPure reagent according to the manufacturer's protocol (Roche). After DNase treatment, 500 ng RNA was used for cDNA synthesis using the iScript cDNA synthesis kit (Bio-Rad). Primers for mouse 18s, Endoglin and EDA and human ß-actin, Endoglin and EDA were designed using Beacon Desinger 4.0 (Premier Biosoft). Mm18s forward 5'tcaacacgggaaacctcac3' reverse 5'accagacaaatcgctccac3'. MmEDA forward 5'acgtggttagtgtttatgctc3' reverse 5'tggaatcgacatccacatcag3'. huß-actin forward 5'gatcggcggctccatcctg3' reverse 5'gactcgtcatactcctgcttgc3'. HuEDA forward 5'tccatgagctattccctgcac3' reverse 5'tgcaaggcaaccacactgac3'. HuEndoglin forward 5'aaacattcgtggcttcaag3' reverse 5' ggaggagtgtgctgggatc3'. Amplification was performed using 10µl IQ™ SYBR Green supermix (Bio-Rad) and 10µl cDNA. Values for mouse EDA and Endoglin were corrected for the amount of 18s. Values for human EDA and Endoglin were corrected for the amount of ß-actin.

Western blotting

Protein was isolated using TriPure isolation according to the manufactures protocol (Roche). 8 µg protein was boiled in loadingbuffer with 0,1M DTT for 5 minutes at 95˚C

and loaded onto an 8% acryl-bisacrylamide gel. Protein was transferred onto a Hybond-P transfer membrane (Millipore) overnight and then blocked with 3% blocking milk. Incubation with the first antibody anti-Endoglin (α-human CD105, BD Biosciences, 1:1000) for 1 hour at room temperature was followed by incubation with the second antibody (goat-α–mouse polyclonal HRP, Dako, 1:2000) for 1 hour at room temperature. After using a chemiluminescent peroxidase substrate (Sigma), the blots were quantified in real time, using the ChemiDoc XRS system (Bio-Rad) and analysis software Quantity One (Bio-Rad). After analysis, anti-ß-tubulin was added (Rabbit polyclonal to ß-tubulin, BioConnect, 1:5000) to the membrane followed by the second antibody (goat-α–rabbit polyclonal HRP, Dako, 1:2000). Expression was quantified after chemiluminescent peroxidase substrate (Sigma) addition, and the Endoglin values were corrected for loading by the ß-tubulin values.

EDA enzyme-linked immuno sorbent assay

Fibronectin containing EDA was measured with an enzyme-linked immunosorbent assay (ELISA) developed at the University Medical Center Utrecht. Microtiter plates were coated overnight with IgG1 monoclonal antibody IST-9 (1.06 µg/mL) directed against Extra domain A of fibronectin (Abcam, Cambridge, UK) in carbonate buffer (Na_2CO_3 15 mM, $NaHCO_3$ 35 mM, pH 9.6) at 4 ˚C. Wells were washed with PBS/0.1% Tween and then blocked with phosphate-buffered saline (PBS)/3% bovine serum albumin (BSA)/0.1% Tween. Samples were diluted to an appropriate concentration with PBS/0.1% Tween/3% BSA (1:100 for protein extracts in 1% SDS). Ligand capture was detected by addition of peroxidase-conjugated rabbit-anti-human fibronectin antibodies (1.3 µg/mL) (Dako, Glostrup, Denmark). Binding of the antibody was detected by adding 1 µg/mL O-phenylene-diamine in 0.05M citrate-phosphate buffer (pH 5) with 2.5mM H_2O_2. The reaction was stopped by adding 1M sulfuric acid. Absorbance was read at 490 nm on microplate reader model 550, Biorad, 655 nm filter was used as reference-filter. As a standard, cellular fibronectin was purified from cultured human fetal lung fibroblasts. EDA levels measured by ELISA were corrected for protein concentrations of the samples.

Statistics

The Mann-Whitney U or Kruskal-Wallis test was performed to compare different variables. Linear regression analyses (Spearman's rho) were performed to correlate expression levels in the plaque. A P-value <0.05 was regarded significant.

Results

Localization and levels of EDA and TGF-beta signaling molecules in human atherosclerotic plaques

Alternate sections of atherosclerotic lesions were stained for EDA, Endoglin, and p-SMAD 2 using immunohistochemistry.

Figure 1 shows co-localization of EDA and Endoglin staining. SMAD 2 also co-localized with EDA and Endoglin.

Expression levels of EDA, Endoglin, TGFß, p-SMAD 1/5/8 and p-SMAD 2 were determined in protein extractions of human atherosclerotic specimen using ELISA and western blotting. Figure 2 shows positive correlations for EDA expression levels with Endoglin (n=47, rho=0.417, P<0.01), TGFß (n=197, rho=0.309, P<0.001), p-SMAD 1/5/8 (n= 43, rho=0.508, P<0.01) and p-SMAD 2 (n=47, rho=0.587, P<0.01) levels.

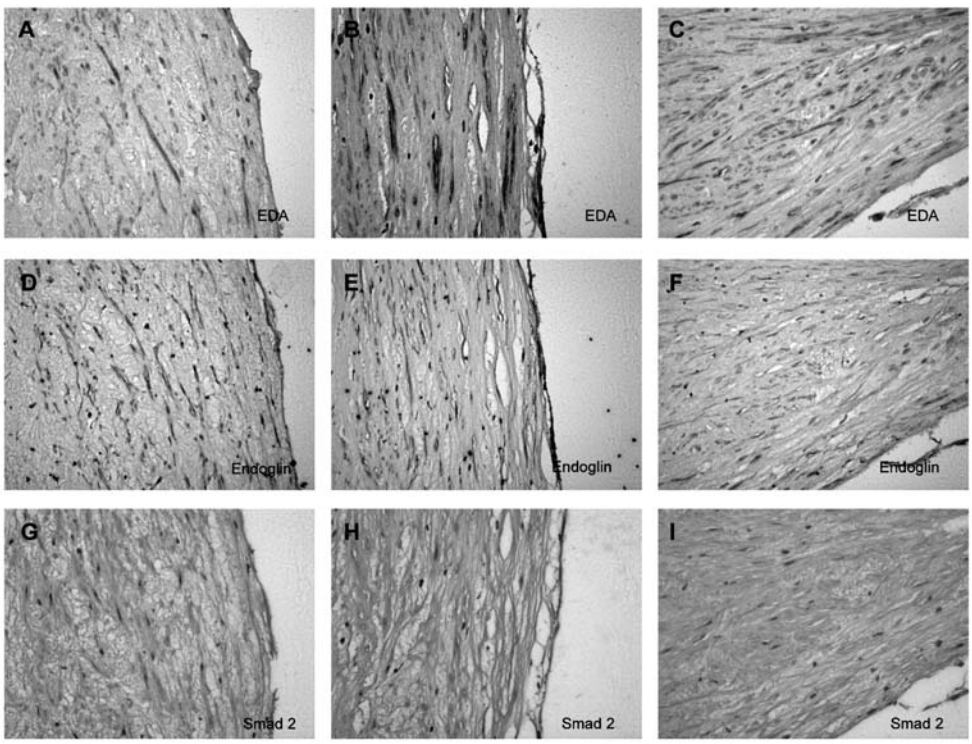

Figure 1. Immunohistochemistry of EDA, Endoglin, and SMAD 2

EDA and Endoglin staining show co-localization of expression of these two proteins in the atherosclerotic plaque. p-SMAD 2 staining was shown in the nuclei of cells, in the regions positive for EDA and Endoglin staining.

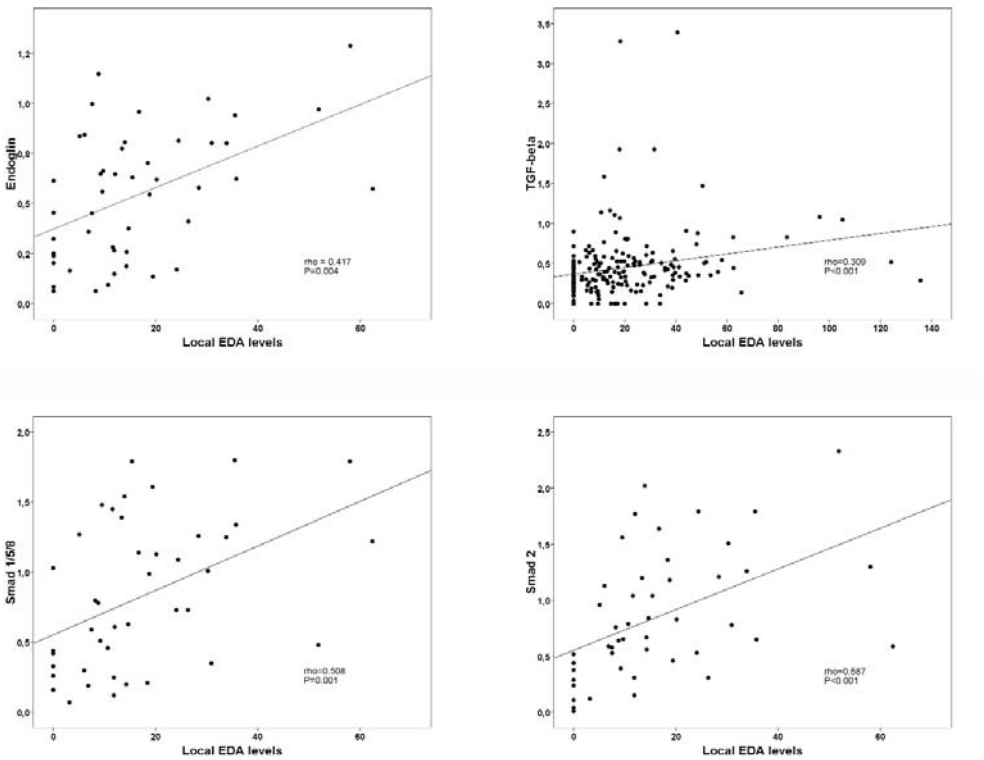

Figure 2. Correlations of EDA, Endoglin, TGF-beta and p-SMADs in atherosclerotic plaques
Expression levels of EDA, Endoglin, TGF-beta, SMAD 1/5/8 and SMAD 2 were determined in protein samples of atherosclerotic lesions. Positive correlations for EDA and Endoglin, rho=0.417, n=47, P<0.01 (A), EDA and TGFß, rho=0.309, n=197, P<0.01, EDA and SMAD 1/5/8, rho=0.508, n=43, P<0.01 (C), and EDA and SMAD 2, rho=0.587, n=47, P<0.01 (D).

EDA and Endoglin expression in endothelial cells

Human microvascular endothelial cells (HMECs) were stimulated with TGFß 1ng/ml for 6 and 24 hours (Figures 3a and b). Endoglin mRNA levels were significantly increased at 6 hrs (P=0.03) and EDA mRNA levels were increased at 24 hours after stimulation, P=0.05. Eng-/- en WT mouse embryonic endothelial cells (MEECs) were stimulated with TGFß for 24 hours and EDA mRNA expression levels determined. Both Eng-/- and WT cells showed a relative increase in EDA expression compared to PBS stimulation (Figure 3c, P<0.01 for Eng-/-, and P=0.01 for WT). The increase in EDA levels in Eng-/- cells is significantly higher compared to WT cells (P<0.01).

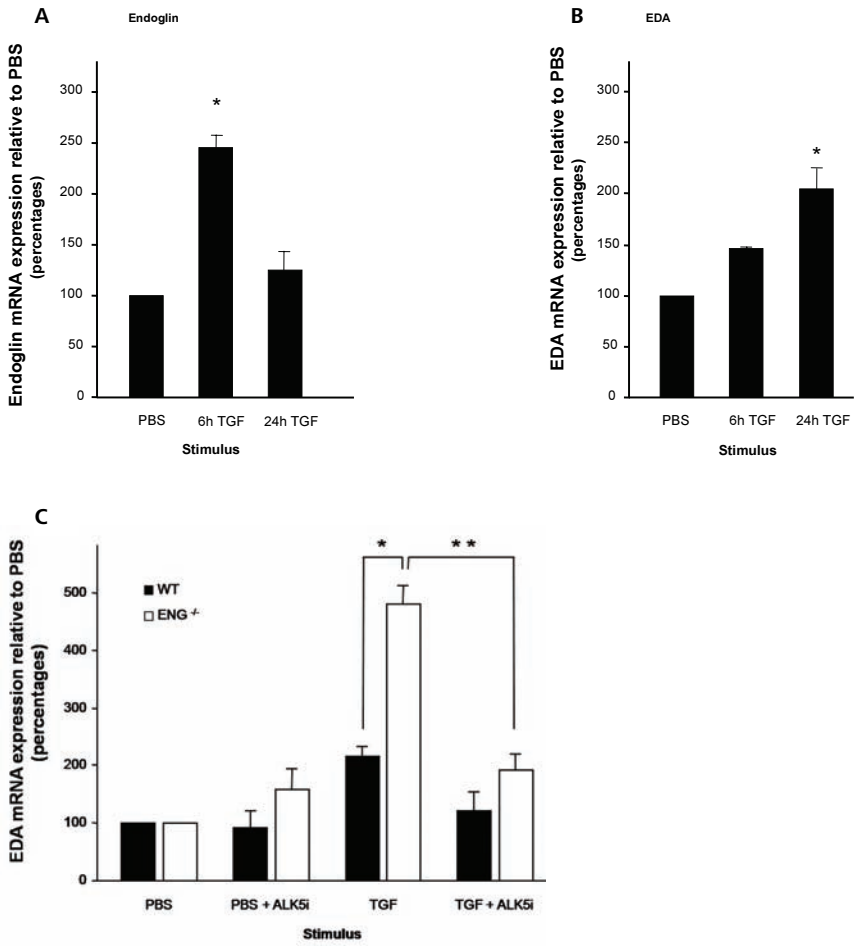

Figure 3. TGFß stimulation of HMECs and MEECs

HMEC stimulation with TGFß1 resulted in an increase in Endoglin mRNA expression at 6 hours after stimulation (A, P=0.03), and EDA mRNA expression was increased at 24 hours after stimulation (B, P=0.05, n=2). TGFß stimulation of Endoglin$^{-/-}$ MEECs for 24 hours resulted in an increased EDA expression compared to WT cells (C, *P<0.01, n=4). Addition of an ALK-5 inhibitor in MEECs stimulated with TGFß resulted in a reduction of EDA expression compared to cells without addition of the inhibitor (C, **P<0.01, n=4).

To investigate whether the increase in EDA expression was ALK5 dependent, WT and Eng-/- MEECs were treated with an ALK5 inhibitor before stimulation with TGFß EDA mRNA expression in Eng-/- cells was decreased compared to samples without the inhibitor (P<0.01, Figure 3c). The Eng-/- cells incubated with the ALK5 inhibitor did not show a higher relative increase in EDA expression compared to wildtype cells (P=0.22).

Discussion

In atherosclerotic arteries, expression levels EDA of fibronectin and Endoglin are increased compared to healthy arteries [4,5,10,11]. In human, EDA plaque levels are associated with a more stable plaque phenotype [5], and also Endoglin expression levels, that are elevated in both tissue samples and in the serum of atherosclerotic patients, are higher in atherosclerotic lesions with a stable plaque phenotype [11,12] and unpublished data Bot *et al.*, in press. In this study, we investigated localization and expression levels of EDA in human atherosclerotic plaques in association with other TGFß signaling molecules including Endoglin. Furthermore, we investigated the regulatory role of Endoglin in endothelial EDA expression.

EDA and Endoglin in atherosclerotic lesions

We showed co-localization of EDA and Endoglin expression in human atherosclerotic lesions, using immunohistochemistry. Furthermore, SMAD 1/5/8 and SMAD 2 staining was observed in the nuclei of the cells that stained positive for EDA en Endoglin, pointing to an activated TGFß pathway.

Next to co-localization of EDA and Endoglin in the atherosclerotic lesion, also the expression levels of EDA and Endoglin, and the other components of the TGFß signaling pathway (TGFß, phosphorylated SMADs) were positively correlated in atherosclerotic lesions. Expression of Endoglin in atherosclerotic lesions has been described previously [11,13]. An immunohistological study showed that Endoglin is expressed in endothelial cells of both advanced and early atherosclerotic lesions, and also in smooth muscle cells and macrophages in the plaques [11,13]. A correlation of Endoglin/TGFß/TGFßRII expression was observed in atherosclerotic lesions which was absent in non-atherosclerotic aortas [11]. Active plaque vascular endothelial cells were described to express Endoglin but also smooth muscle cells. We showed that EDA and Endoglin co-localize and that their expression levels are associate in the atherosclerotic plaque suggesting an interaction of the proteins.

The role of Endoglin in regulation of EDA expression

Since endothelial cells in human atherosclerotic plaques express both proteins, endothelial cells were chosen for in vitro experiments.

Stimulation of human endothelial cells with TGFß, first induced Endoglin expression followed by increased EDA expression when Endoglin expression was decreased.

Overexpression of Endoglin has been reported to result in less fibronectin and EDA

expression [14], suggesting an inhibitory role of Endoglin on TGFß induced EDA expression. To confirm this inhibitory role of Endoglin, mouse endothelial cells were used that do not have Endoglin and we confirmed that EDA levels were strongly increased in Endoglin null cells compared to wildtype cells. These data are in line with the results in Endoglin over-expressing cells, which produced less EDA and less fibronectin [14]. Stimulation with TGFß results in activation of TßRII and subsequently ALK5 and/or ALK1 is activated. Endoglin is required for efficient TGFß/ALK1 signaling [15] and indirectly inhibits TGF/ALK5 signaling. ALK5 specifically induces fibronectin expression [16,17]. Absence of Endoglin will therefore reduce inhibition of the ALK5 pathway and increase EDA expression that can be inhibited by ALK5 inhibition. Indeed, we showed that upregulation of EDA expression in Endoglin KO cells is mainly ALK5 dependent. The inhibitory role of Endoglin on EDA expression in the endothelial cells seems to be in contradiction with the positive correlation between EDA and Endoglin in the plaque. The sampling of atherosclerotic plaques, is a cross-sectional event and represents a snap-shot in time. The association of EDA with Endoglin and TGFß as wells as pSMADs in the plaque indicates that EDA is associated with TGFß signaling including TGFß induced inhibitors like Endoglin. Due to the cross-sectional way of plaque sampling, no conclusions can be deduced whether in the plaque, EDA expression increases first, followed by an increased Endoglin expression or how Endoglin and EDA are correlated in time like we observed in the in vitro experiments.

In summary, we showed that atherosclerotic plaque EDA levels are associated with TGFß signaling molecules including Endoglin that is likely an ALK5 dependent inhibitory protein for TGFß induced EDA expression.

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Chapter 7

Identification of gene expression regulated by both Toll-like receptor and transforming growth factor pathways in monocytes

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Submitted

Abstract

Background

Toll-like receptor 4 (TLR4) and Transforming Growth Factor beta (TGFß) are well known players in atherosclerotic disease but little is known about the interaction of these pathways in atherosclerotic plaques or in cells related to atherosclerosis and which mRNAs and proteins are regulated by both pathways. In this study, mRNAs regulated by TLR4 and TGFß pathway interaction were identified in human monocytes and validated in protein samples of human atherosclerotic lesions.

Methods

Microarray analyses were performed on monocytes stimulated with TGFß1 or the TLR4 ligand lipopolysaccharide (LPS), or both TGFß1 and LPS. Cluster analysis was performed using Spotfire (Tibco) and Omniviz (Biowisdom). Pathway analysis was done using Ingenuity and EASE. As both TLR4 and TGFß signaling are present in the atherosclerotic plaque, focus was on mRNAs that had higher expression levels with both TGF and LPS compared to TGF or LPS alone and with no expression at baseline. One of these mRNAs, Osteopontin, was validated measuring Osteopontin protein levels using ELISA on monocytes and in human atherosclerotic lesions with clinical data on the occurrence of secondary events.

Results

Cluster analysis identified several clusters of genes that showed increase or decrease after combined LPS and TGFß stimulation while other clusters contained genes that only responded to LPS or TGFß. For one cluster of 100 mRNAs, mRNA expression level was found to be higher when stimulated by both LPS and TGFß compared to TGFß or LPS alone with lower expression at baseline. In this group, deduced proteins were more involved in immune protein activity and lipoprotein binding and associated with the endocytic and phagocytic vesicle compared to LPS or TGFß alone. Validation of one of these mRNAs, Osteopontin, showed similar monocyte protein expression patterns as mRNA expression in the microarray, and revealed higher expression in atherosclerotic lesions from patients that suffered from an event during follow-up compared to patients that did not get a second event.

Conclusion

These studies show that several sets of mRNAs are regulated by both TLR4 and TGFß

stimulation and that these mRNAs may play an important role in the progression of atherosclerotic disease leading to events.

Introduction

Atherosclerosis is an inflammatory disease and the leading cause of mortality in the Western world. The onset of atherosclerosis starts already during childhood with the formation of fatty streaks. These lesions can progress over decades into advanced atherosclerotic plaques that may rupture and cause clinical symptoms such as a myocardial infarction or stroke. Post-mortem studies revealed that at the site of rupture the plaque mostly contains a large lipid core covered by a thin fibrous cap and infiltrated by a large amount of inflammatory cells underlining the concept that atherosclerosis is an inflammatory disease. Activation of monocytes going into the plaque as well as macrophages in the plaque can occur by a spectrum of growth factors, exogenous and endogenous ligands. It is often not taken into consideration, however, that when different pathways are activated they interact in the cell.
In this study, we investigated the potential cumulative effects and interaction between the Toll-like receptor (TLR) and Transforming Growth Factor beta (TGFß) pathways in monocytes. Both TLR and TGFß are recognized as important players in atherosclerotic disease.
Toll-like receptors are involved in the innate immune response, serving as the first line of defense against pathogens. TLR4 recognizes exogenous ligand lipopolysaccharide and endogenous ligands like EDA of fibronectin and HSP60. Stimulation of TLR4 by injections of LPS increased the rapid development of atherosclerotic disease in animal models [1,2]. Moreover, neointima formation and outward arterial remodeling were decreased in TLR4-/- mice [3,4] and ApoE-/-TLR4-/- mice show significantly reduced atherosclerotic lesion development [5]. In recent clinical studies, human TLR4 polymorphisms were associated with lower risks of carotid artery atherosclerosis [6,7], acute coronary syndrome [8] and myocardial infarction. These findings, however, could not be confirmed in all clinical studies [9-11].
TGFß mainly exerts an anti-inflammatory effect during atherosclerosis. In atherosclerotic lesion formation TGFß expression is found in areas of extracellular matrix production and intimal thickening. TGFß levels in serum of patients with advanced atherosclerosis are depressed [12] and low TGFß expression levels is found in human aortic regions vulnerable for lesion development [13]. Furthermore,

polymorphisms of the TGFß1 gene and mutations in TGF-receptors are associated with a higher risk of cardiovascular disease [14-16].
Evidence is emerging that the TGFß and TLR4 pathways can influence each other. In TGFß1 null mice, TLR4 mRNA expression was increased and associated with LPS hyper-responsiveness and lack of TGFß1 led to extensive inflammation and death within 3 weeks [17]. Both stimulatory and inhibitory effects of the TLR4 and TGFß pathways on each other have been described. The TGFß dependent fibrogenetic response after liver injury was enhanced after priming with TLR4 activation [18], while TGF-family member activin A reduced TLR4 expression induced by LPS, suggesting a suppressing role on TLR4 signaling [19]. Inhibiting TGFß in uterine natural killer cells increased cytokines production after TLR activation, also suggesting an inhibitory TGFß effect [20]. Recently, it was shown that Ecsit, an obligatory protein in the Toll-like signaling pathway, is also essential for signaling of the TGFß superfamily [21,22] establishing a novel molecular link between the TLR and TGFß family.
Although both TLR4 and TGFß are well known players in atherosclerotic disease, little is known about the interaction of these pathways in atherosclerotic plaques or in cells related to atherosclerosis and which mRNAs and proteins are regulated by both pathways.
In this study, we stimulated monocytes with LPS or TGFß or with both LPS and TGFß and performed microarrays to study differential expression on the transcriptome level. Several expression patterns were found with each a defined set of mRNAs. We focused on the genes that were expressed at low levels at baseline, higher after stimulating with TGFß1 or LPS, and even higher using both stimuli. Expression of one of the mRNAs was verified on protein level in monocytes and human plaques and related to secondary events after plaque removal.

Materials and methods

Cell culture

THP-1 cells were cultured in RPMI-1640 medium (InVitrogen) containing 50mM 2-mercaptoethanol (Sigma), 100 U/ml penicillin, 100 µg/ml streptomycin (Gibco), and 5% FBS (Gibco). Monocytes, in a density of 2*10^6 cells/4 mL were starved O/N prior to stimulation in medium containing 0.1% FCS. Cells were stimulated with LPS (100ng/mL) or TGFß1 (1ng/mL, Peprotech) or LPS and TGFß1 for 24 hrs (n=8/group). As a control PBS was added to the cells.

RNA isolation and cDNA and cRNA synthesis

RNA of the stimulated monocytes was isolated using Tripure reagent (Roche) according the manufacturer's protocol. High quality RNA was obtained with NucleoSpin RNA II kits (Machery-Nagel) inclusive DNase treatment. Illumina TotalPrep RNA Amplification Kits were used for cDNA and cRNA synthesis (Ambion) according the manufacturer's protocol. Labelled cRNA samples (n=8/group) were loaded on HumanRef-8 v3 Expression BeadChips (Illumina) and hybridized O/N following the manufacturer's protocol.

RNA expression profiling

Gene expression values of the chips were normalized in BeadStudio (version 3.2 Illumina) and used for further analysis. SAM analysis was performed in Omniviz (Biowisdom), with FDR set on 0.05 and 1.5-fold difference as a threshold. Spotfire was used for K-means clustering analysis.

Gene annotation enrichment analysis is performed with EASE. This tool is used to highlight the most relevant GO terms associated with a given gene list. Using EASE for enrichment analysis will give an EASE score, which is an one-tail Fisher Exact Probability Value adjusted for gene-enrichment analysis (more stringent).

Osteopontin enzyme-linked immuno sorbent assay

A commercial Osteopontin ELISA (R&D systems) assay was used according to the described procedures. From each patient, 1 µg of Tripure (Roche) isolated protein was used per well.

Athero-Express

Athero-Express is an ongoing vascular biobank project with the goal to investigate locally expressed plaque markers in relation to clinical presentation and clinical outcome [23]. The biobank project is running in two Dutch hospitals and was approved by the ethical committees of the Antonius hospital Nieuwegein, The Netherlands and the UMCU, Utrecht, the Netherlands. Written informed consent is obtained from all patients.

Follow-up protocol and clinical outcome events

After carotid surgery, patients were followed yearly up to 3 years. The primary outcome was a composite encompassing all cardiovascular events and interventions: vascular death, non-fatal myocardial infarction, non-fatal stroke, and vascular intervention that

was not planned at the time of inclusion.
Secondary endpoint was any major cardiovascular event: vascular death, non fatal myocardial infarction, non-fatal stroke and non-fatal aneurysm rupture.

Tissue sampling
Carotid endarterectomy was performed by an open, non-eversion technique with careful dissection of the atherosclerotic plaque. Following excision, the plaque was immediately transferred to the laboratory to undergo standardized processing. First, it was divided into 5mm cross-sectional segments. The culprit lesion, defined as the segment with greatest plaque burden, was fixated in 4% formalin for 7 days and then decalcified in EDTA and embedded in paraffin. The other segments were snap frozen in liquid nitrogen and stored at -80°C. Protein extraction was performed on the carotid segments adjacent to the culprit lesion by mechanical crushing followed by 1) protein isolation with TriPure reagent, according to the manufacturer's protocol (Boehringer Mannheim, Germany) and 2) by dissolving in 40 mM Tris-HCl (pH = 7.5) at 4°C.

Statistics
Gene expression data from the beadchip microarrays were normalized in BeadStudio (version 3.2 Illumina). The detection P-value of at least one of the eight sample was ≤0.05, which was chosen as cut off value for gene expressions to be included. Omniviz was used for the Significance Analysis of Microarrays (SAM). The False Discovery Rate (FDR) was set on 0.05 and 1.5-fold increase was chosen as cut off value for an increase in expression. Omniviz (Biowisdom) was used for the Significance Analysis of Microarrays (SAM). Clustering of the significant records was done in Spotfire (Tibco) using K-means clustering. With this method, genes with comparable expression patterns are clustered together. Gene annotation enrichment analysis is performed with EASE, a one-tail Fisher Exact Probability Value adjusted for gene-enrichment analysis.

Results

mRNA profile analysis of THP-1 cells
Whole transcriptome microarray analysis was performed on RNA from THP-1 cells stimulated for 24 hrs with PBS, LPS (100ng/mL), TGFß1 (1ng/mL), or LPS + TGFß1 (100 and 1ng/mL, respectively, n=8 per condition). Expression data were normalized in

Beadstudio. SAM analysis (OmniViz) comparing LPS+TGF vs. LPS and TGF resulted in 954 records (both up 247; both down 229; vs. TGF up and vs. LPS down 363; vs. TGF down and vs. LPS up 115), Several clusters of mRNAs with different expression patterns could be identified using Spotfire. In Figure 1 the Log2GM (geometric mean) levels are depicted on the y-axis, giving the fold increase in expression of the mRNAs for the different stimuli. Cluster 1-6 are clusters of mRNAs that show smaller differences in expression between the different stimuli, and clusters 7-12 show mRNAs with larger differences in expression. Clusters 1 and 5 show patterns of down-regulated mRNAs after stimulation with both TGFß and LPS. In clusters 2, 6, 8, and 9, stimulation with both TGFß and LPS led to higher expression than in the single stimulus groups and PBS group. Some mRNA levels seem affected predominantly by LPS stimulation (clusters 10, 11, and 12) while in other clusters LPS and TGFß separately had opposite effects resulting in average mRNA levels after giving both LPS and TGFß (clusters 3, 4, and 7). Focus was on the set of mRNAs which levels showed the following pattern a) (LPS + TGFß) > PBS, b) (LPS + TGFß) > LPS and (LPS + TGFß) > TGFß, and c) (LPS + TGFß) > LPS and TGFß > PBS. SAM analyses were performed in Omniviz and resulted in a) 2271, b) 247 and c) 100 differently expressed mRNAs, visualized in Figure 2. Hundred mRNAs show the expression pattern LPS + TGFß (+++) / TGFß (+) and LPS (+) / PBS (0) and were investigated and identified in detail.

Ingenuity and EASE analysis of one set of mRNAs

The 100 mRNAs with the expression pattern LPS + TGFß (+++) / TGFß (+) and LPS (+) / PBS (0) were classified based on biological function, using Ingenuity (Figure 3a). GO enrichment analyses for biological processes, molecular functions, and cellular compartments (Figure 3b-d) were performed using EASE. In total, 29 biological processes, 5 molecular functions, and 4 cellular components were significantly increased compared to what was expected based on analysis of all mRNAs on the chip. To determine which processes were typical for TGF plus LPS stimulation, GO enrichment analysis was also performed for mRNA which levels were increased after only LPS or only TGFß stimulation. Figure 3b shows the number of TGFß plus LPS induced biological processes after exclusion of overlapping GO-terms. For the same GO-annotations, the enrichment scores of LPS or TGFß induced classes are depicted in Figure 3b-d. For some biological processes (Figure 3b), the same enrichment scores are obtained after stimulation with LPS or TGFß or both (e.g. cellular processes, cell communication, immune response), but other processes like protein kinase cascade, inflammatory response, immune cell activation, regulation of metabolism are enhanced

in cells stimulated with both TGFß and LPS. For molecular function (Figure 3c) especially lipoprotein binding was overrepresented while for the cellular compartments (Figure 3d) these were the endocytotic and phagocytotic vesicle.

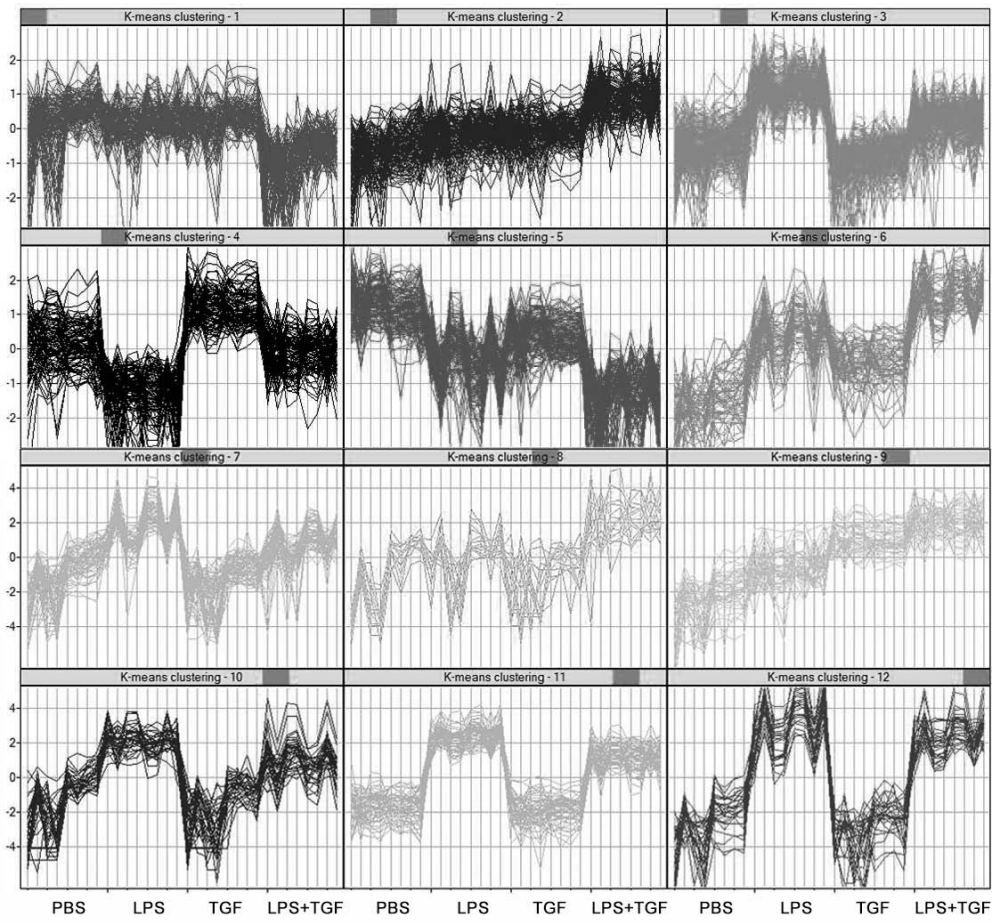


Figure 1. Gene expression patterns in THP-1 monocytes

Stimulation of THP-1 monocytes with PBS, LPS, TGF, or LPS + TGFß (x-axis) leads to different expression patterns. In this figure the genes are divided into 12 clusters, each cluster represents a group of genes with the same expression pattern. On the y-axis the log2GM (geometric mean) values are given. Cluster 1-6 are expression profiles of genes with smaller differences in expression between the different stimuli, and cluster 7-12 show genes with larger differences in expression. In gene clusters 2, 6, 8, and 9, stimulation with both TGFß and LPS led to higher expression than in the single stimulus groups and PBS group.

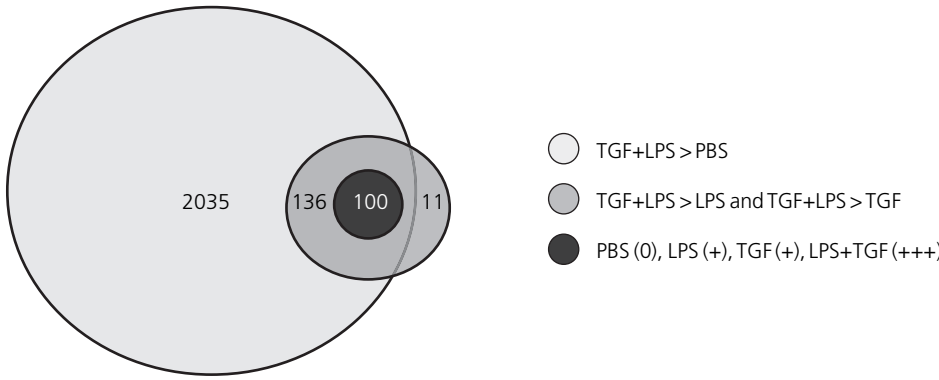


Figure 2. Visualization of SAM analyses
Visualization of genes analyzed with SAM that show the expression pattern of a) (LPS + TGFß) > PBS, 2035 genes (○), b) (LPS + TGFß) > LPS and (LPS + TGFß) > TGFß 147 genes (◍), and c) (LPS + TGFß) > LPS and TGFß > PBS, 100 genes (●).

A

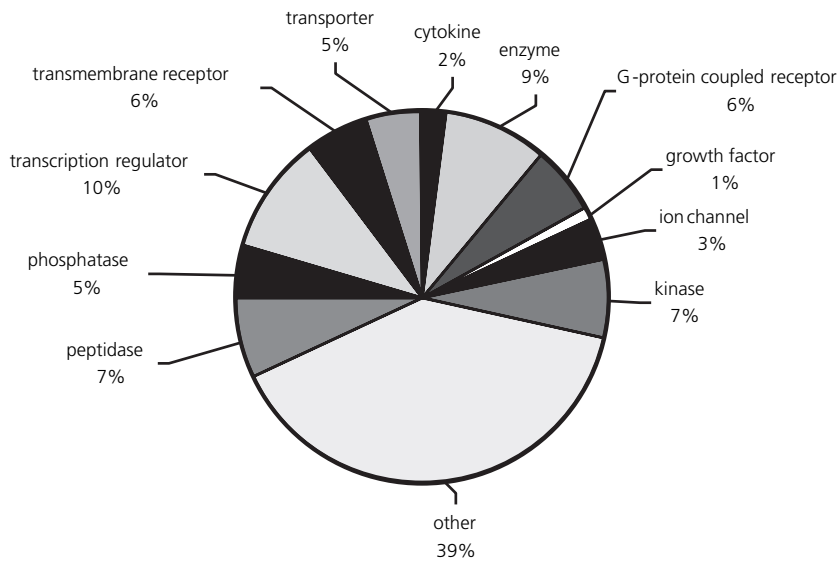
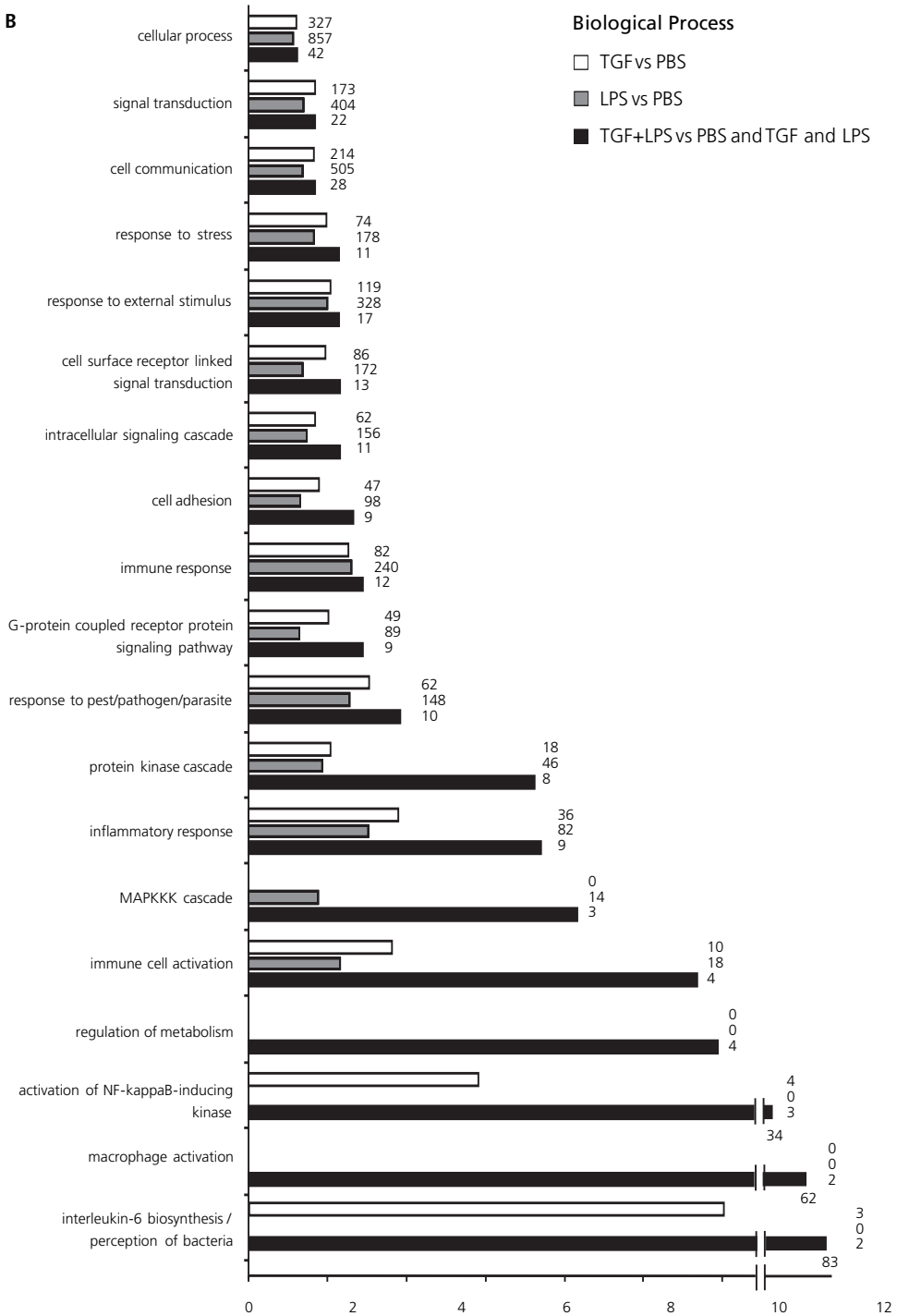
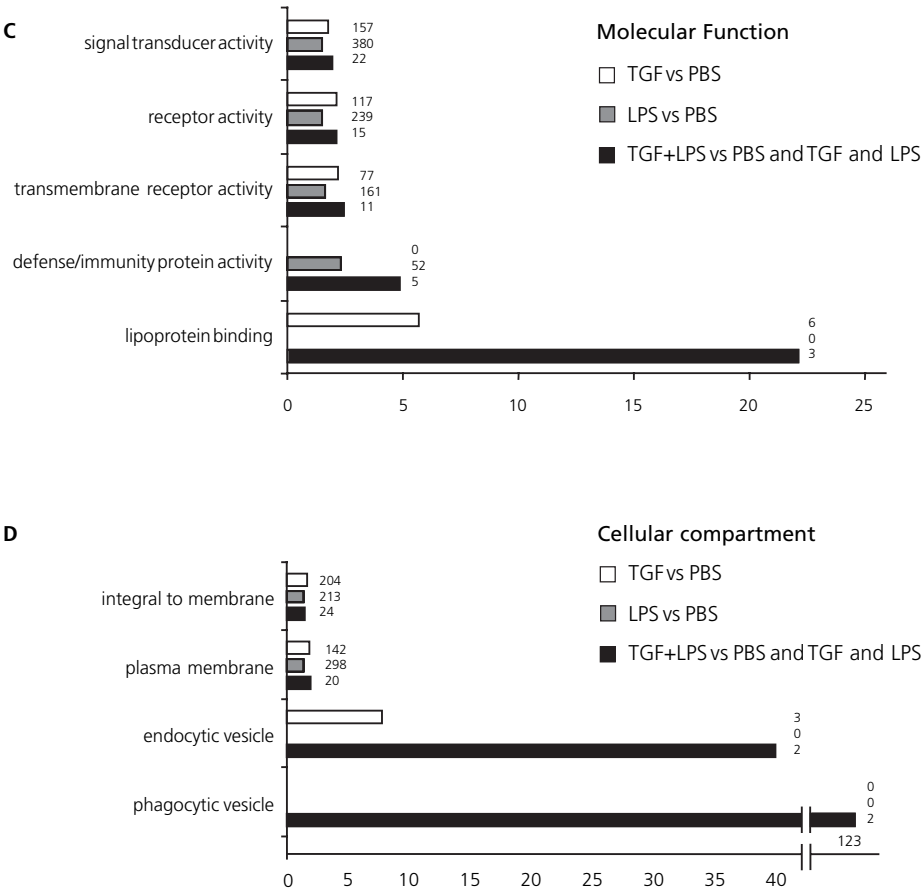


Figure 3. Visualization of Ingenuity and Ease analysis of mRNAs induced by both TLR4 and LPS signaling
Analysis of the 100 mRNAs with increased expression levels after both LPS and TGFß stimulation. The pie chart shows the different biological functions of the upregulated genes (a). Enrichment analysis based on GO annotations was performed for biological processes (b), molecular functions (c) and cellular compartments for genes higher in TGFß + LPS vs. TGFß, LPS, and PBS (■), higher in LPS vs. PBS (▣) and for genes. higher in TGFß vs. PBS (□)


B
Biological Process
TGF vs PBS
LPS vs PBS
TGF+LPS vs PBS and TGF and LPS
cellular process
327
857
42
signal transduction
173
404
22
cell communication
214
505
28
response to stress
74
178
11
response to external stimulus
119
328
17
cell surface receptor linked signal transduction
86
172
13
intracellular signaling cascade
62
156
11
cell adhesion
47
98
9
immune response
82
240
12
G-protein coupled receptor protein signaling pathway
49
89
9
response to pest/pathogen/parasite
62
148
10
protein kinase cascade
18
46
8
inflammatory response
36
82
9
MAPKKK cascade
0
14
3
immune cell activation
10
18
4
regulation of metabolism
0
0
4
activation of NF-kappaB-inducing kinase
4
0
3
34
macrophage activation
0
0
2
62
interleukin-6 biosynthesis / perception of bacteria
3
0
2
83
0
2
4
6
8
10
12



Validation of one of the mRNAs on protein level in monocytes

Osteopontin (OPN) was one of the mRNAs that had higher expression levels with both TGF and LPS compared to TGF or LPS alone with no expression at baseline. Based on its strong association with atherosclerotic disease, OPN was selected for validation experiments. The detection values for OPN in the microarray are shown in Figure 4a and OPN protein expression levels (n=4-8) in Figure 4b. As with the microarray results, OPN protein concentration was higher in cells stimulated with LPS + TGFß compared to LPS, TGFß and PBS stimulation (2.157 ± 2.027 ng/mL vs. 0.221 ± 0.146 ng/mL (LPS), 0.962 ± 0.307 ng/mL (TGF) and 0.073 ± 0.046 ng/mL (PBS), P=0.006, P=0.132, P=0.006, respectively).

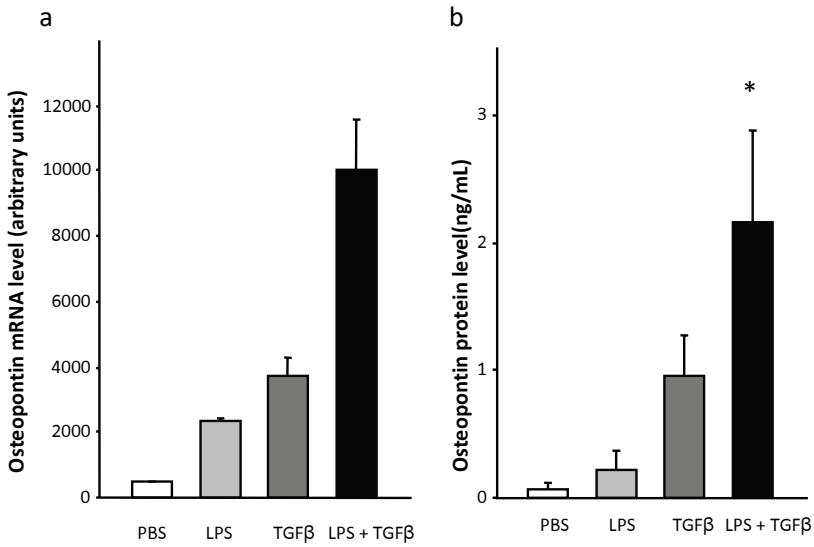


Figure 4. Validation of Osteopontin in monocytes
Osteopontin, one of the upregulated mRNAs when both TLR4 and TGFß pathways are activated (a), has been validated on protein level in monocytes (b).

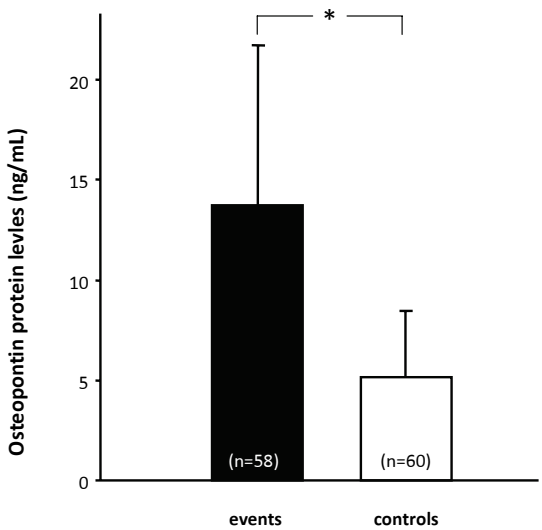


Figure 5. Validation of Osteopontin in atherosclerotic tissue
Validation of Osteopontin expression in atherosclerotic plaques shows that Osteopontin protein levels are higher in atherosclerotic plaques from patients that suffered from a secondary clinical event compared to plaques from patients without a second event. *P<0.001.

Verification of Osteopontin in vivo, Osteopontin in atherosclerotic lesions
Osteopontin protein levels were determined in protein samples of atherosclerotic plaques from patients that did or did not suffer from a secondary cardiovascular event during follow-up (Figure 5). OPN protein levels in plaques from patients that had a clinical event were higher compared to controls (13.716 ng/mL vs. 5.162 ng/mL, $P<0.001$).

Discussion

Evidence is accumulating that TLR and TGFß pathways communicate. Both stimulatory and inhibitory effects have been described; however, which genes are regulated by an interaction of both signals is not clear. In this study, mRNAs are identified which expressions are regulated by the combination of TLR4 and TGFß signals. The levels of mRNA after giving both stimuli were compared to the separate stimuli and PBS as baseline expression values. Combined LPS and TGFß stimulation induced specific sets of mRNA expression, for other sets mRNA expression was inhibited or sets only responded to LPS or TGFß alone. These wide range and even opposite effects might be an explanation for contradictive reports like in arterial remodeling showing that inward arterial remodeling can be inhibited using a TGFß signaling inhibitor [24] while adenoviral overexpression of TGFß [25] that would stimulate TGFß signaling, also inhibits inward arterial remodeling.
All different patterns that show an interaction between the two pathways are of interest. Since TLR4, TGFß and factors regulated by TLR4 are found in neointima formation, arterial remodeling and plaque formation [3,4,24,26], we focused on genes with the expression pattern that showed a synergistic effect of stimulation with both LPS and TGFß above separate stimulation and no increased expression at baseline (PBS). Enrichment analysis of the hundred genes with this expression pattern, showed that relatively more mRNAs are involved in the immune response, inflammatory response and protein kinase cascade, compared to the reference and compared to mRNAs up regulated by single stimulation with LPS or TGFß. Furthermore, relatively more mRNAs have a molecular function in defense/immune protein activity and especially lipoprotein binding. The lipoprotein binding function can be related to the found enrichment in endocytotic and phagocytotic vesicles that are known to be involved in lipoprotein uptake [27,28] and may point to an enhanced lipoprotein up-take by scavengers. Scavenger receptors are important in the inflammatory response in host defense [29],

cellular activation [30,31], adhesion [32], and cell-cell interaction. Scavenger receptors are also involved in foam cell formation and subsequent plaque lipid core formation in the progression of atherosclerosis [33]. Native and modified lipoproteins are recognized by scavengers [34,35], modified lipoproteins have also been shown to induce scavenger receptor expression in mouse macrophages [36].

Studies that investigated the effect of TGFß on TLR signaling mostly describe an inhibitory effect on the TLR response [19,37-39] But in the fibrotic response TLR4 seem to enhance TGFß signals [18]. We also found counterbalancing effects of stimulating both TLR and TGFß pathways, but focused on the synergistic stimulatory effect on gene expression. Although we have to take into account that some biological processes in the EASE analysis are represented by a small number of genes, the processes, functions and cell compartments are characteristic for atherosclerosis.
We cannot distinguish between a direct and indirect interaction between TLR4 and TGFß pathways since it is possible that factors secreted by activation of one pathway can influence the other pathway.
OPN was selected for validation on protein level in monocytes and plaques since mouse studies provided genetic evidence for a causal role of OPN in the development of atherosclerotic plaques. OPN plasma levels have shown to be related to coronary artery disease and to be an independent predictor of future cardiovascular events in patients with chronic stable angina [40].
Our microarray data show that OPN expression is increased by separate stimulation of TGFß, and LPS but is increased even more with both LPS and TGFß stimulation. TGF induced OPN expression is reported by Noda *et al.* [41], but inhibitory effects of TGFß on OPN are also described [42,43] as well as LPS induced OPN expression [44]. In monocytes OPN protein expression levels showed the same pattern as mRNA expression and confirming the microarray findings.
Determination of OPN levels in human atherosclerotic plaques showed that OPN was present in these plaques. Comparing plaques from patients that had an event during follow-up after carotid atherectomy to patients that did not have an event showed that OPN levels were much higher in patients with an event. This identifies OPN as a potential plaque biomarker for secondary events and suggests a role for OPN plaque progression towards rupture and subsequent events and is in line with OPN plasma studies. It also point to a similar role of the other genes following the same expression pattern.
We investigated the crosstalk between TLR4 and TGFß signals using transcriptome

analysis at one timepoint and we cannot investigate what happens over time or at protein level. Validation of Osteopontin on protein level, however, showed the value of this kind of mRNA analysis, although validation of other genes has to be performed. Next to this, we focused now on the genes that showed an increased expression when both TLR and TGFß pathways were activated. The genes regulated differently by the interaction of both stimuli are also of interest and will be investigated in more detail in the near future.

In summary, we identified different sets of mRNAs from which monocytes levels modified after both LPS and TGFß stimulation. One of these sets was associated with specific functions and cell compartments and Osteopontin was, as mRNA of this set, present in human plaques and Osteopontin protein levels associated with secondary events. This shows that several sets of mRNAs are regulated by both TLR4 and TGFß stimulation and that these mRNAs may play an important role in the progression of atherosclerotic disease leading to clinical events.

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Chapter 8

Summary and discussion

Atherosclerosis

Atherosclerosis is a chronic inflammatory disease of the vascular wall and the primary cause of cardiovascular events like myocardial infarction, heart failure and stroke. Accumulation of macrophage foamcells in the subendothelial layer of the artery leads to the formation of fatty streaks, the first stage in atherosclerotic disease. These early lesions may progress into advanced atherosclerotic lesions, characterized by recruitment of more macrophages and T-lymphocytes together with proliferation and migration of vascular smooth muscle cells. Based on plaque composition, stable and unstable lesions are discriminated. Stable lesions are characterized by a thick fibrous cap and are rich in collagen and smooth muscle cells, unstable lesions contain large lipid pools covered by a thin fibrous cap, dense infiltrates of inflammatory cells and intra-plaque hemorrhages. Unstable lesions are prone to rupture, and when the plaque contents are exposed to the blood, thrombus formation is induced that can result in acute occlusion of the artery.

Arterial remodeling

The size of the lumen of an atherosclerotic artery is determined by the size of the atherosclerotic lesion, together with the degree and direction of arterial remodeling. In the early stage of atherosclerosis arterial lumen loss due to plaque formation is compensated by outward arterial remodeling, a structural enlargement of the arterial diameter. However, in advanced atherosclerotic lesions, outward arterial remodeling is associated with the characteristics of unstable lesions and with plaques vulnerable for rupture. Also inward arterial remodeling is observed in atherosclerotic arteries, accelerating luminal narrowing. Important features of arterial remodeling are inflammation and immune cell activation, furthermore, collagen turnover and cell migration play important roles.

The immune response

Inflammation and the immune response are involved in the initiation and progression of atherosclerotic disease. The immune system responds effectively to harmful agents by close interplay between the innate and the adaptive immune systems. The innate immune response is the first line of defense against invading pathogens and can be initiated quickly. Monocytes, macrophages, dendritic cells and leukocytes are the effector cells during this rapid immune response. These cells express pattern recognition receptors, including scavenger receptors and Toll-like receptors that recognize whole classes of pathogens sharing the same pathogen associated molecular

pattern (PAMP). The adaptive immune response is highly specific and B-cells and T-cells are the most important cells during the adaptive immune response. B- and T-cell activation results in the production of antibodies and the development of a long-life memory for specific epitopes.

Toll-like receptors

Toll-like receptors are not only expressed by immune cells, but also by vascular cells [1-3]. In healthy arteries TLR4 is expressed in low levels, in atherosclerotic lesions, however, TLR4 expression is markedly increased [1]. TLR4 expression in adventitial fibroblasts is associated with intima formation [2] and in human atherosclerotic disease TLR4 polymorphisms are associated with intima media thickness.

Whether TLR4 is also involved in arterial remodeling, the other determinant of lumen loss next to intima and plaque formation is investigated in this thesis.

The TLR4 pathway in arterial remodeling is studied on different levels; 1) at the level of the receptor itself, in TLR4-/- mice and after adventitial LPS application in atherosclerotic ApoE3 Leiden mice; 2) downstream of the receptor, on the level of transcription factor NF-κB, studying the role of the p50 subunit in arterial remodeling, and 3) upstream of TLR4, on the level of endogenous TLR4 ligand EDA and its role in arterial remodeling. Furthermore, the expression levels of endogenous TLR4 ligand EDA are determined in human atherosclerotic lesions and plasma samples from atherosclerotic patients and linked to plaque phenotype and atherosclerosis-related clinical symptoms. EDA expression in human atherosclerotic plaque samples is also linked to Endoglin expression. Endoglin is an accessory TGFß receptor, and while evidence is emerging that TLR and TGFß signaling pathways interact, this has not been shown in atherosclerotic lesions before. Toll-TGF crosstalk is also investigated in a wider approach using microarray analyses on RNA of monocytes stimulated with LPS, TGFß1 or both, because monocytes play an important role during initiation and progression of atherosclerosis.

The Toll-like receptor 4 pathway in arterial remodeling

Toll-like receptor 4 in outward arterial remodeling

TLR4 is expressed in atherosclerotic lesions and associated to intima formation in mouse models [2] in intima media thickness in human [4,5]. In chapter 2, we demonstrated that TLR4 is also involved in outward arterial remodeling both with and without intima formation. In mice expressing a non-functional TLR4, intima formation was reduced, and subsequent outward arterial remodeling was absent. Outward remodeling without

intima formation, studied with the carotid artery ligation model, is also reduced in TLR4 deficient mice. Thus, TLR4 expression is upregulated during outward remodeling and is involved in two determinants of lumen size reduction during atherosclerosis; intima formation and arterial remodeling.

NF-kB p50 subunit in outward arterial remodeling

TLR activation results in translocation of NF-kB into the nucleus and transcription of inflammatory genes. The NF-kB family consists of 5 different subunits; p65, RelB, c-Rel, p50 and p52 [6]. In chapter 3 is described that the p50 subunit of NF-kB is involved in outward arterial remodeling. The NF-kB p50 subunit lacks a transcription domain and forms heterodimers with transcriptionally active subunits to induce gene expression. The p50 subunit is known as a regulatory subunit of NF-kB signaling. After activation of NF-kB, transcription of NF-kB p50 is also enhanced. This leads to the formation of NF-kB p50 homodimers that bind to the DNA and thereby inhibit binding of transcriptionally active NF-kB dimers, working as a negative feedback loop on NF-kB signaling.

In our study, using the carotid artery ligation model, targeted deletion of the regulatory NF-kB p50 subunit enhanced outward arterial remodeling. Without the brake on NF-kB signaling, as in p50-/- mice, the response can continue, so in this model p50 has an inhibitory effect on arterial enlargement. Controversially, carotid artery ligation in NF-kB p50 null mice resulted in a reduction of neointima formation and ICAM-1 mRNA expression [7,8], suggesting a stimulatory role for NF-kB p50. The different effects of the absence of p50 in outward remodeling and neointima formation might be due to a difference in shear stress. For outward remodeling, the flow and shear stress are increased in the artery, while for neointima formation the ligated artery is used in which the blood flow is completely blocked, so flow and shear stress are low [7,8]. The effect of the p50 subunit in the artery might therefore be shear stress dependent; stimulatory in a low shear stress environment and an inhibitory effect during high shear stress.

While arterial remodeling was increased in p50-/- arteries, the adventitial collagen density was decreased. During arterial remodeling, collagen turnover is increased, but collagen content does not change [9]. The decrease of collagen density in p50-/- arteries points to an altered collagen turnover, which possibly enhance arterial remodeling. Furthermore, more macrophages were observed in the adventitia of NF-kB p50 null mice. The attraction and presence of more monocytes is associated with an increase of arterial remodeling during atherosclerosis, and also during arteriogenesis [10-12]. Also during flow-induced arterial remodeling a higher number of monocytes

was associated with more outward remodeling. An increase in fluid shear stress also led to an increase in arteriogenesis [13], suggesting a comparable response during arteriogenesis and outward arterial remodeling.
The increased number of macrophages together with more outward remodeling might also be due to the absence of the brake on the NF-kB induced response, but whether this is the exact mechanism behind this increase needs to be investigated. Furthermore, it is unclear whether more macrophages enter the vascular wall from the bloodstream or from the adventitial site, nevertheless, the presence of more macrophages in the p50-/- arteries explains (at least part of) the mechanism behind the enhanced arterial remodeling in these arteries.

Fibronectin containing the extra domain A in arterial remodeling

During outward arterial remodeling, TLR4 mRNA and TLR4 protein expression levels are significantly upregulated as described in chapter 2. Since no exogenous TLR4 ligand was applied to trigger outward remodeling in the ligation model, we determined the mRNA expression levels of endogenous TLR4 ligands EDA and HSP60. The upregulation of both EDA and HSP60 suggest a role for these endogenous TLR4 ligands during outward arterial remodeling. To investigate whether EDA could be causally related to outward arterial remodeling we performed the ligation model in EDA-/- mice. Extra domain A is a domain of fibronectin, a major component of the extracellular matrix. Fibronectins are high molecular weight glycoproteins, that play key roles in cell adhesion, migration, growth and differentiation [14]. Via alternative splicing of the fibronectin transcript, different isoforms of the fibronectin protein are expressed [15]. Extra Domain A is incorporated in fibronectin during embryogenesis, wound healing, cellular damage and atherogenesis [15,16]. In chapter 4 the role of EDA of fibronectin is investigated in the process of outward remodeling. No outward arterial remodeling was induced in the contralateral arteries of EDA-/- mice, after carotid artery ligation, and even inward remodeling was measured. Collagen turn-over was not affected in EDA-/- animals, but EDA-/- embryonic fibroblasts showed reduced migration capacities. Cell adhesion and migration can be modulated by EDA via integrin binding [17]. EDA itself is an integrin binding domain and can also modulate integrin binding with other domains in fibronectin. The unchanged collagen turnover and the diminished migration capacity of EDA-/- cells indicates that the role of EDA in outward arterial remodeling is not restricted to TLR4 activation, but it may also affect other molecular pathways leading to integrin binding.

TLR4, NF-kB p50 and EDA affect different mechanisms during arterial remodeling

The deletion of TLR4 and p50 both have an effect on collagen turnover during outward remodeling, while the absence of EDA does not. The observation that EDA is involved in outward remodeling without an effect on collagen turnover suggests that EDA affects arterial remodeling via at least a slightly different mechanism than TLR4 and NF-kB p50. In absence of TLR4 an increase of collagen density was associated with less outward remodeling while in p50-/- arteries collagen densities were decreased and outward remodeling was enhanced. During remodeling in arteries from wildtype mice collagen densities before and after remodeling were not different, as is also described in other studies concerning arterial remodeling [9].

This suggests that during outward remodeling EDA does not only trigger TLR4 activation and subsequent NF-kB induced gene expression, but also leads to activation of other signals that influence adhesion and migration of cells. This is supported by the finding that EDA-/- cells had a reduced migration capacity. EDA is very important for the interaction with integrins, it serves as a binding domain for α9ß1 integrins and increases integrin-binding affinity of fibronectin to alpha5beta1 and thereby enhances migration of cells [17]. TLR4 activation can also induce cell migration in leukocytes [18] and adhesion of human T cells to fibronectin [19], and also NF-kB can promote cell migration [20]. Therefore we cannot exclude, that EDA similar to LPS [21], enhances cell migration without affecting collagen turn-over via TLR4.

Endogenous Toll-like receptor 4 ligand EDA in atherosclerosis

Extra domain A of fibronectin expression in stable human atherosclerotic lesions

EDA of fibronectin is an endogenous TLR4 ligand and induces matrix metalloproteinases and cytokines in a TLR4 dependent manner [22-24]. EDA containing fibronectin is expressed in human atherosclerotic lesions and is involved in atherogenesis in mice [25]. In ApoE-/- mice, that spontaneously develop atherosclerotic lesions, EDA levels in the arteries increase in time during atherogenesis [25,26]. Concomitantly, EDA-/- ApoE-/- mice showed reduced lesion formation and reduced lipid content in the plaques. In chapter 5, we studied EDA levels in human atherosclerotic plaques and plasma in relation with morphological presentation and clinical outcome. Consistent with previous reports [25,26], EDA expression could be detected in human atherosclerotic lesions, these expression levels in atherosclerotic plaques were higher than in mammary arteries used as controls. When EDA expression levels in the plaque were related to plaque phenotype, higher EDA levels are associated with a stable plaque phenotype, with a higher number of smooth muscle cells, less fat

and more collagen. This observation implies that the detection of an endogenous TLR4 ligand not necessarily represent an inflammatory environment destabilizing the vulnerable plaque, but also may trigger the stabilization of atherosclerotic lesions.
These data are obtained in an observational study and therefore inferences regarding a causal role of EDA in atherosclerosis cannot me made. However, considering the results from studies towards the role of EDA in atherogenesis from our own laboratory [26] and from Tan *et al*. [25] our data support the concept that EDA is actively involved in atherogenesis.

Endogenous TLR ligand Extra domain A of fibronectin in human atherosclerotic lesions and arterial remodeling

In human atherosclerotic lesions, high EDA levels are associated with a stable plaque phenotype [27] that is associated with a low rate of outward remodeling. During atherogenesis, EDA levels are increased and the absence of EDA results in less and smaller lesions and inward remodeling. These different associations of EDA with stable human plaques and EDA with intima formation and arterial remodeling may indicate that EDA expression in the atherosclerotic plaque may have a different function than in the arterial wall. Other possible explanations for the discrepancy between EDA levels in human plaques and the mouse models are the difference in timing during lesion development; the human plaque is the end stage of the disease in which EDA may have a different role then in the onset of remodeling and plaque formation what we study in the mouse model. In human studies like this, we can measure EDA in the atherosclerotic plaque at one point in time only. It might be that high levels of EDA are found in plaques that are becoming unstable and start remodeling. Another difference is that EDA levels in human atherosclerosis are measured in protein samples from locally in the plaque itself, while outward arterial remodeling occurs in the whole artery. The size and structure of an artery is determined by the layers of the arterial wall, in particular the adventitia that contains most collagen.
The possible role of EDA in plaque stabilization can be linked to the reduced migration capacity in the cells lacking EDA expression. EDA might stimulate migration of smooth muscle cells in the plaque, resulting in reorganization and stabilization of an atherosclerotic lesion, but this needs further investigation. This will be difficult to study since in mouse models atherosclerotic lesions develop sharing characteristics with early or even advanced human lesions, however, spontaneous rupture and arterial occlusion does not occur.

Interaction between Toll-like receptor 4 and Transforming Growth Factor ß signaling

Extra domain A of fibronectin and Endoglin expression in human atherosclerotic tissue

Both EDA, an endogenous TLR4 ligand, and endoglin, an accessory TGFß- receptor, are expressed in atherosclerotic lesions and expression of both proteins is induced by TGFß. In atherosclerotic lesion formation TGFß expression is found in areas of extracellular matrix production and intimal thickening [28-30]. Cell culture experiments showed that endoglin expression decreases TGFß induced cellular responses, like fibronectin synthesis [31]. Chapter 6 describes the association we found between EDA and TGFß signaling molecules TGFß, p-SMAD2, p-SMAD1/5/8, and Endoglin in human atherosclerotic lesions. The in vitro results show that Endoglin is an inhibitory protein for TGFß induced EDA expression in endothelial cells and this effect is ALK5 dependent. The association of EDA with Endoglin in the atherosclerotic lesion suggests an important role of TGFß signaling in the atherosclerotic plaque probably associated with plaques having stable plaque phenotypes. Furthermore the association between EDA and Endoglin is an indication for TLR4 and TGF communication in atherosclerotic lesions.

Cross-talk of TLR4 and TGF signaling in THP-1 monocytes and atherosclerotic lesions

An increasing number of studies report an effect of TGFß on the TLR response, or vice versa [32-36]. Accordingly, in TGFß1-/- mice TLR4 expression is increased, resulting in hyper responsiveness to LPS and an enormous inflammatory response, leading to death within 3 weeks [37]. The observed effect that in absence of TGFß TLR4 expression and activity are increased implies an interaction between these two signaling pathways.

Both TLR4 and TGFß are well known players in atherosclerotic disease. Little is known however, about the interaction of these pathways in atherosclerotic plaques or in cells related to atherosclerosis and what the effect is of both signals on mRNA transcription and which mRNAs are affected. In chapter 7, the cross-talk between TGFß and TLR4 is investigated using microarray analysis on monocytes stimulated with LPS or TGFß1 or with both LPS and TGFß1. The combination of TLR4 and TGFß activation had a diverse effect on different set of mRNAs; stimulatory, inhibitory or opposite effects could be observed. We focused on a group of hundred mRNAs that were expressed at low levels at baseline, at higher levels after stimulating with TGFß1 or LPS, and even higher using both stimuli. Identification of the functions of this group of mRNAs, showed relatively more genes involved in the immune response, inflammatory response and protein kinase cascade, compared to the reference and compared to mRNAs up regulated by

single stimulation with LPS or TGFß. Furthermore, relatively more mRNAs from the upregulated set have a molecular function in defense/immune protein activity and especially lipoprotein binding. The lipoprotein binding function can be related to the found enrichment in endocytotic and phagocytotic vesicles that are known to be involved in lipoprotein uptake and metabolism [38,39]. Verification of Osteopontin, one of the mRNAs with the up regulated expression pattern after activation of both TLR and TGFß pathway, showed the same expression pattern on protein level, confirming the data on mRNA level in the microarray. Osteopontin protein levels were than determined in atherosclerotic plaques and were associated with the occurrence of a secondary clinical event. This shows that several sets of mRNAs are regulated by both TLR4 and TGFß stimulation and that these mRNAs may play an important role in the progression of atherosclerotic disease leading to clinical events.

Concluding remarks

Atherosclerosis is a disease of the western world; lesions start to develop at a young age already and progress into lesions that give rise to clinical implications in presence of risk factors like smoking, hypertension, high fat diet, hypercholesterolemia, and stress. Rupture of atherosclerotic lesions leads to life threatening clinical implications like myocardial infarction and stroke. Better understanding and a more detailed knowledge of the mechanisms behind plaque destabilization, will lead to better prevention and treatment of cardiovascular risk factors and cardiovascular disorders. Next to plaque formation, also arterial remodeling is an important determinant of lumen loss and is also associated with plaque rupture leading to an acute arterial occlusion.

In this thesis the role of Toll-like receptor 4 signaling is investigated on different levels in outward arterial remodeling, in atherosclerosis and its interaction with the TGFß pathway. The role of the receptor itself, the role of the p50 subunit of the transcription factor NF-kB activated downstream of TLRs, and the role of endogenous TLR4 ligand EDA has been studied. A simplified overview of TLR4 signaling and other signals during arterial remodeling and atherosclerosis is shown in Figure 1.

Toll-like receptor 4 expression is essential for intima formation and outward arterial remodeling. And also downstream of TLR4, when the p50 subunit of NF-kB, the brake on NF-kB activation, is not expressed, outward remodeling is not limited and this results in enhanced arterial enlargement. Based on these findings, inhibition of the TLR4 pathway might be a valid target to prevent arteries from outward arterial remodeling or other tissue remodeling processes, like dilatation of the left heart ventricle after

myocardial infarction [40]. Possible ways to intervene in the TLR4 pathway are for example the use of antagonists or antibodies that block the ligand binding site of TLR4. Or more downstream of the receptor, overexpression of the p50 subunit will result in more inhibitory p50-homodimers, reducing the NF-kB dependent response. For other processes that depend on restructuring of arteries, like arteriogenesis, it might be beneficial to stimulate TLR4 signaling. However, TLR4 signaling is essential for the initiation of the immune response after PAMP recognition to defend the host from infections. Therefore any intervention affecting TLR4 signaling needs a restricted and local delivery of the drug and also the timing and duration of the modulating treatment will be of major importance.

The role of endogenous ligands of TLR4 in relation to atherosclerosis and arterial remodeling needs to be investigated in more detail. In mouse models the endogenous ligands EDA and HSP60 are upregulated during atherosclerosis [26] and outward remodeling (this thesis), and atherosclerotic mice without EDA expression show less atherosclerotic lesion development [25]. However, in human atherosclerosis EDA expression is associated with more stable atherosclerotic plaques and less clinical symptoms. Apparently, EDA expression has a different role during atherogenesis in mouse models than in human atherosclerotic lesions that already have developed. Is this difference due to the restriction of only one measurement at one timepoint in human lesions, and does EDA have different roles during initiation and progression of atherosclerotic plaques? Can EDA induce reorganization of the plaque towards a more stable phenotype by modulating cell migration and proliferation? These are all very interesting issues that need to be investigated before the role of EDA in atherosclerosis will be completely understood.

Both TLR4 and TGFß are expressed in atherosclerotic lesions. Evidence for interaction between TLR and TGFß signaling is accumulating, but was not investigated in atherosclerotic tissue or atherosclerosis related cells. We showed an interaction of Toll and TGF signaling in monocytes, important cells in atherosclerosis, on transcriptome level. Furthermore mRNAs triggered by both stimuli may have clinical importance as well, as Osteopontin expression is affected by both signals and is present atherosclerotic lesions and even more important related with clinical outcome. The results based on the microarray data, will help to identify potential markers for vulnerable plaques or targets for intervention or plaque imaging. These markers or imaging targets can help in risk stratification, which patient will benefit from treatment and which patient needs to be monitored intensively, because a next cardiovascular event is very likely to happen. To investigate the specific effects of TLR4 and TGFß

crosstalk on gene expression will be very informative. Do other upregulated mRNAs have the same relation to atherosclerosis as we showed for Osteopontin? And maybe these enhanced expressed mRNAs are also involved in other immune related diseases. Interestingly, the co-stimulation of TGFß and TLR4 resulted in diverse effects on different sets of mRNA. Also the mRNAs for which the interaction of TLR4 and TGFß signals had a different effect than enhanced stimulation might lead to new insights in the crosstalk of these two signaling pathways.

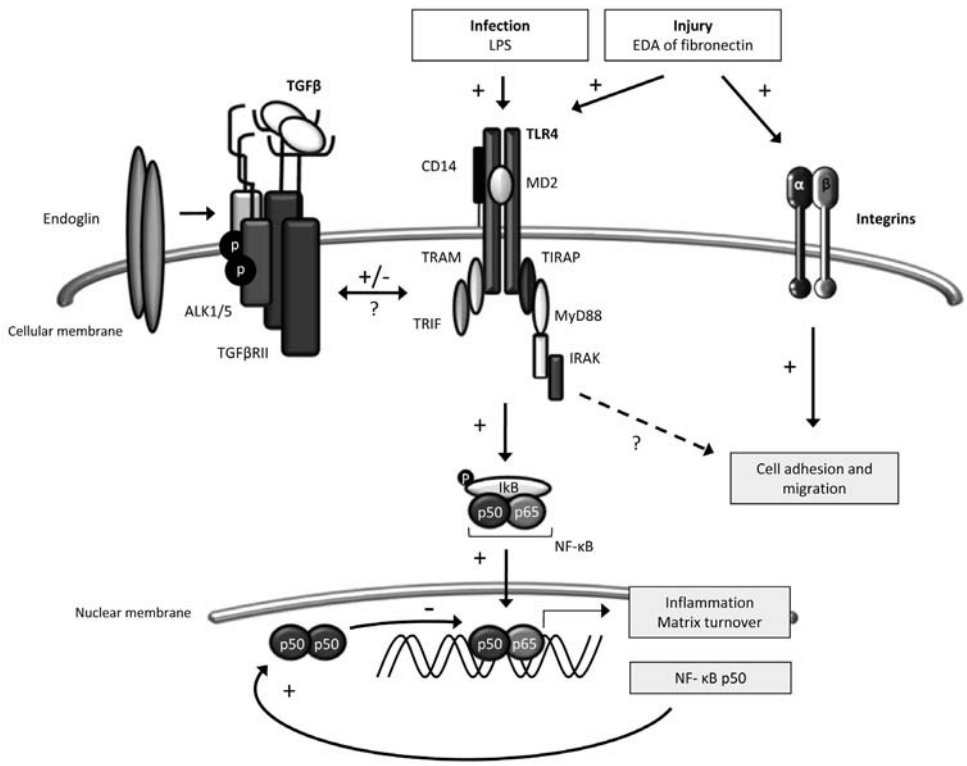

Figure 1. A simplified overview of TLR4 signaling during outward arterial remodeling, intima formation and atherosclerosis

This hypothetical scheme shows that EDA can trigger TLR4 signaling, but is also involved in integrin dependent processes like cell adhesion and migration. During outward remodeling p50 expression has a inhibitory effect on NF-kB activation, possibly via a negative feedback loop of p50 homodimer formation. Furthermore, TLR4 and TGFß signaling pathways interact and this cross-talk has diverse effects on different sets of mRNA (stimulatory or inhibitory).

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Nederlandse samenvatting

Atherosclerose

Atherosclerose, of aderverkalking, is de belangrijkste oorzaak van hart- en vaatziekten, zoals een hartinfarct, hartfalen en een herseninfarct. Risicofactoren voor het ontwikkelen van atherosclerose zijn hoge bloeddruk (hypertensie), suikerziekte (diabetes mellitus), roken, een vetrijk dieet, stress en hoge niveaus LDL-cholesterol in het bloed. Deze risicofactoren leiden tot beschadiging en functieverlies van het endotheel, de laag cellen die de binnenkant van een bloedvat bekleedt. Monocyten, een soort witte bloedcellen, blijven dan plakken aan het endotheel en migreren de bloedvatwand in.

Monocyten spelen een belangrijke rol bij een ontstekingsreactie. In het weefsel veranderen zij in macrofagen. Deze herkennen en verwijderen lichaamsvreemde cellen of deeltjes, maar ook beschadigde lichaamseigen cellen. Daarnaast produceren de macrofagen ontstekingsfactoren, zoals cytokines en chemokines, om nog meer ontstekingscellen te activeren en te laten weten waar de ontsteking is.

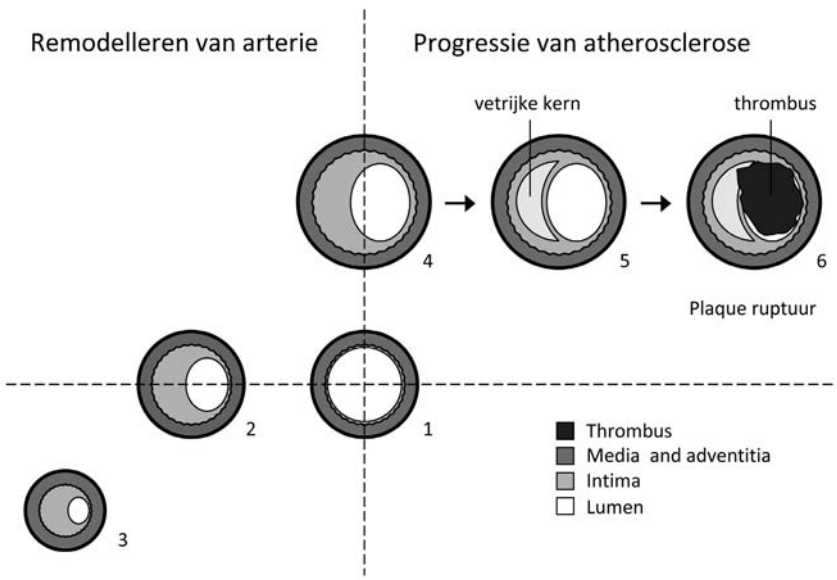
De initiatiefase van atherosclerose wordt gekenmerkt door schuimcellen; macrofagen die er door het opnemen van vet schuimachtig uitzien. Deze schuimcellen hopen zich op onder de endotheellaag en dit wordt een ‘fatty streak’ genoemd. Deze vroege beschadigingen, of laesies, kunnen zich ontwikkelen tot meer complexe atherosclerotische beschadigingen. Deze plaques ontstaan door het aantrekken van macrofagen en specifieke immuuncellen (T-lymfocyten), en het delen, vermeerderen en verplaatsen (proliferatie en migratie) van vasculaire gladde spiercellen. Afhankelijk van de samenstelling van een plaque, worden stabiele en onstabiele atherosclerotische laesies onderscheiden.

Stabiele plaques worden gekenmerkt door gladde spiercellen en een dikke fibreuze kap, waar bindweefsel bestaand uit collageen zorgt voor stevigheid van het weefsel. Onstabiele plaques hebben een dunne kap en bevatten veel vet, veel ontstekingscellen en kleine bloedingen. Onstabiele plaques kunnen makkelijk scheuren. Wanneer de vettige inhoud van zo’n plaque in contact komt met het bloed, stolt het onmiddellijk. Dit kan leiden tot een acute afsluiting van de slagader (Figuur 1). Op die manier kan een atherosclerotische plaque leiden tot bijvoorbeeld een hart- of herseninfarct.

Remodellering van de vaatwand

Een arterie is geen stugge pijp die alleen maar dient voor bloedtransport naar de organen. Een samenspel van hemodynamische, mechanische en biologische prikkels met het omliggende steunweefsel (de extracellulaire matrix) heeft invloed op de grootte en structuur van een arterie.

Bij plotselinge veranderingen in de bloedstroom zal een bloedvat tijdelijk verwijden of vernauwen. Als deze veranderingen in de bloedstroom chronisch worden, zal het bloedvat ook structurele aanpassingen ondergaan. Dit proces wordt arteriële remodellering genoemd. Ontstekingsreacties, immuuncelactivatie, celmigratie en collageenopbouw en –afbraak zijn belangrijke invloeden. Neemt de vaatholte (het lumen) in diameter toe dan wordt dit expansief remodelleren genoemd. Wordt de diameter kleiner dan is dat inwaartse remodellering. De grootte van het lumen van een atherosclerotische arterie wordt bepaald door de grootte van de aanwezige atherosclerotische plaque, samen met de mate en de richting van het remodelleren van de vaatwand (Figuur 1).



Figuur 1. Schematische weergave van arterieel remodelleren en intima/plaque vorming
In een gezond bloedvat is er geen intima en vormen endotheelcellen de bekleding van het lumen van het bloedvat (1). Intima vorming (2) leidt tot vernauwing van het lumen. De grootte van het lumen wordt bepaald door intima vorming en arterieel remodelleren samen. Inwaarts remodelleren (3) versnelt het kleiner worden van het lumen, terwijl expansief remodelleren (4) kan compenseren voor intima vorming. Expansief remodelleren lijkt dus gunstig, maar is geassocieerd met de ontwikkeling van onstabiele plaques (5). De dunne kap van dit type plaque vormt de barrière tussen de vetrijke kern van de plaque en het bloed en kan makkelijk scheuren. Wanneer de vetrijke inhoud van de plaque in contact komt met het bloed wordt onmiddellijk een trombus gevormd (6) die het bloedvat acuut afsluit.

In eerste instantie kan het verkleinen van het lumen door plaquevorming gecompenseerd worden door het naar buiten remodelleren van het bloedvat. Echter, bij meer ontwikkelde plaques wordt expansieve remodellering van het bloedvat geassocieerd met onstabiele plaques, die ruptuurgevoelig zijn. Wanneer een atherosclerotisch vat naar binnen toe remodelleert, versnelt dat het proces van vaatvernauwing.

De immuunreactie

Bij het ontstaan en de ontwikkeling van atherosclerose zijn ontstekings- en immuunreacties betrokken. Het immuunsysteem reageert heel effectief op schadelijke stoffen door een intensieve samenwerking tussen het aangeboren en verworven afweersysteem. Het aangeboren immuunsysteem vormt de eerste verdediging tegen binnendringende ziekteverwekkers, ook wel pathogenen genoemd, en wordt heel snel geactiveerd. Witte bloedcellen, zoals monocyten, macrofagen, dendritische cellen en leukocyten zijn betrokken bij deze vroege immuunrespons. De genoemde immuuncellen brengen specifieke eiwitten tot expressie op het celoppervlak. Scavenger- en Toll-like receptoren zijn voorbeelden van zulke eiwitten. Deze receptoren herkennen patronen die voorkomen op veel verschillende pathogenen. Het verworven afweersysteem is heel specifiek en B-cellen en T-cellen zijn de belangrijkste immuuncellen tijdens deze zogenoemde 'late immuunrespons'. De activatie van B- en T-cellen leidt tot de productie van antilichamen die heel specifiek bepaalde pathogenen herkennen. Dit is ook nodig voor de ontwikkeling van een levenslang geheugen voor het herkennen van deze structuren.

Toll-like receptoren

Toll-like receptoren komen niet alleen tot expressie op cellen van het immuunsysteem, maar ook op cellen in de vaatwand. In gezonde bloedvaten komt Toll-like receptor 4 (TLR4) heel weinig tot expressie. In atherosclerotische vaten is de expressie duidelijk verhoogd. Activatie van TLR4 op bindweefselcellen (fibroblasten) in de buitenste laag van de vaatwand (adventitia) gaat gepaard met meer intimavorming. Intimavorming is de eerste stap in atherosclerose vorming. In een humane studie is een minder actieve vorm van TLR4 geassocieerd met minder intimavorming in de halsslagader. In dit proefschrift is onderzocht of TLR4 ook betrokken is bij vaatwandremodellering, het proces dat naast plaquevorming de lumengrootte van een bloedvat bepaalt. Om dit te onderzoeken is er gekeken naar het signaal dat de Toll-like receptor 4 de cel instuurt (Figuur 2). Het TLR4 signaal in arteriële remodellering is bestudeerd op drie verschillende niveaus;

1) op het niveau van de TLR4 receptor zelf - waarbij gebruik gemaakt is van muizen die geen functionele TLR4 kunnen maken en muizen die bij een vetrijk dieet atherosclerose krijgen;
2) op het niveau van transcriptie factor NF-kB – dit eiwit verplaatst zich na activatie van TLR4 naar de kern van de cel, waar het de aanmaak van onstekingsfactoren reguleert;
3) op het niveau van het ligand EDA – dit eiwit komt vrij bij celschade en kan dan de TLR4 activeren.

Daarnaast zijn EDA niveaus gemeten in humane atherosclerotische plaques en in het bloed van atherosclerotische patiënten. De EDA niveaus zijn vervolgens gerelateerd aan plaquetype (stabiel of onstabiel) en klinische symptomen veroorzaakt door atherosclerose. De EDA niveaus in humane atherosclerotische plaques zijn ook bestudeerd in relatie tot eiwitniveaus van een ander signaal; het Transforming Growth Factor ß (TGFß) signaal. Steeds vaker wordt een wisselwerking tussen het TLR-signaal en het TGFß-signaal beschreven, maar in atherosclerotische plaques is dit is nog nooit onderzocht. De wisselwerking tussen deze twee signalen is verder bestudeerd in monocyten, omdat deze cellen een belangrijke rol spelen tijdens de beginfase en de ontwikkelingsfase van atherosclerose.

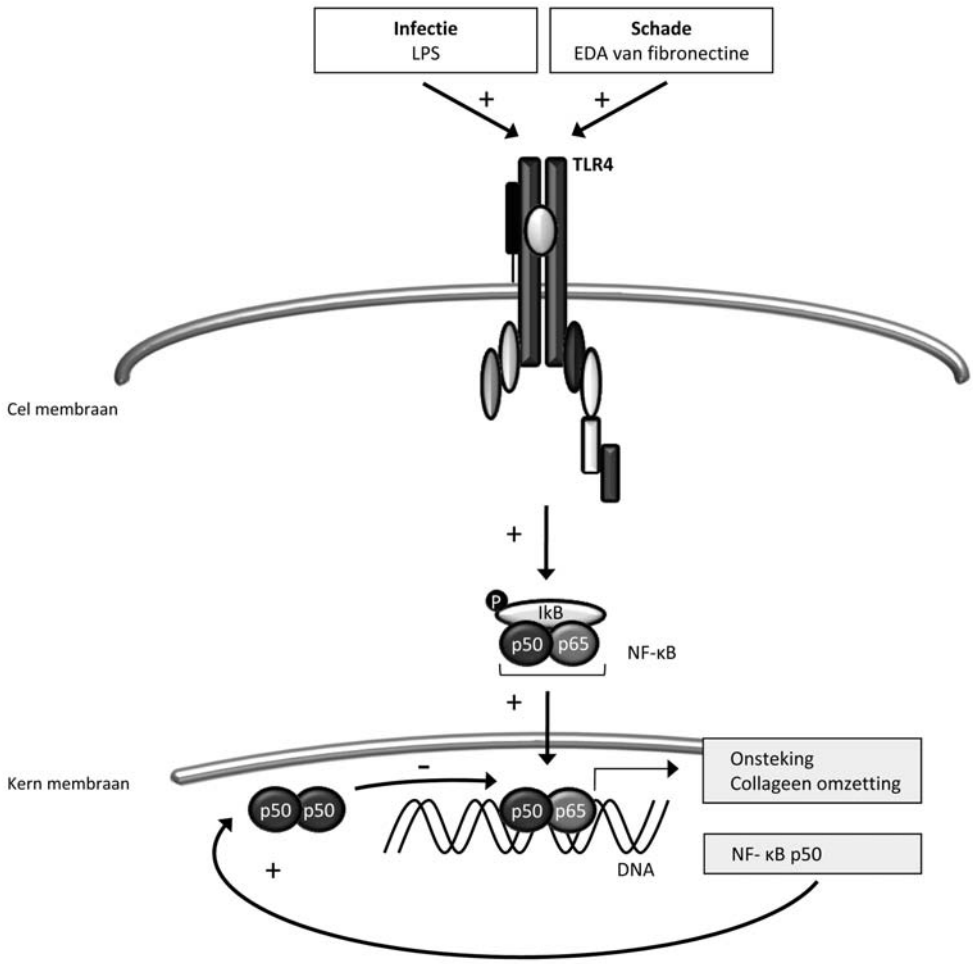
Het Toll-like receptor 4 signaal in vaatwand remodelleren

TLR4 in expansief vaatwand remodelleren

TLR4 komt tot expressie in atherosclerotische plaques en is geassocieerd met intimavorming in muismodellen en mensen. In **hoofdstuk 2** is beschreven dat TLR4 ook betrokken is bij het proces van expansief arterieel remodelleren. Muizen die geen functionele TLR4 tot expressie brengen vertonen minder intimavorming en het daarop volgende expansief remodelleren is ook afgenomen. Ook in een model zonder intimavorming is het expansief remodelleren ook afgenomen in TLR4-/- muizen. Alles bij elkaar genomen is tijdens vaatwand remodelleren de TLR4 expressie verhoogd en is TLR4 betrokken bij de twee determinanten van lumen afname tijdens atherosclerose, namelijk intimavorming en arteriële remodellering.

NF-kB p50 subunit in expansief vaatwand remodelleren

Activatie van TLR4 zorgt voor verplaatsing van transcriptiefactor NF-kB naar de kern van de cel, waar genen worden aangezet die belangrijk zijn voor de ontstekingsreactie. De transcriptie factor NF-kB bestaat altijd uit 2 subunits (dimeer) en er zijn totaal vijf verschillende subunits bekend; p65, RelB, c-Rel, p50 en p52. In **hoofdstuk 3** wordt de rol van de p50 subunit in expansief vaatwand remodelleren beschreven. De p50



Figuur 2. Toll-like receptor 4 signaal

Activatie van TLR4, door exogene of endogene liganden, zorgt voor verplaatsing van transcriptiefactor NF-kB naar de kern van de cel. Daar worden genen aangezet die belangrijk zijn voor de ontstekingsreactie en collageen omzetting. Na activatie van NF-kB worden meer p50 subunits gemaakt. p50 kan wel aan DNA binden, maar geen genen aanzetten. NF-kB dat uit twee p50 subunits bestaat, houdt de bindingsplaats op het DNA bezet en kan zo de NF-kB activiteit beïnvloeden.

subunit kan wel aan het DNA binden, maar geen genexpressie starten. Daarom vormt p50 altijd een combinatie met een van de andere subunits wanneer er genen aangezet moeten worden. Wanneer NF-kB uit twee p50 units bestaat en aan het DNA bindt, is de bindingsplek voor NF-kB bezet en kunnen geen genen worden aangezet. Zo kan de p50 subunit de activiteit van NF-kB beïnvloeden (Figuur 2). Wij hebben de p50 subunit bestudeerd. Bij afwezigheid van de regulatie-subunit p50 treedt meer arterieel remodelleren op. In het model dat wij hebben gebruikt, heeft p50 dus een remmende werking op expansief arterieel remodelleren. Het achterliggende mechanisme voor de verhoogde mate van remodelleren in muizen zonder p50, hebben we onderzocht door te kijken naar de collageendichtheid in de vaten en het aantal macrofagen in de bloedvatwand. De bloedvaten zonder p50, hebben na het remodellerings-proces een lagere collageendichtheid, dat iets zegt over het steunweefsel in het bloedvat en bevatten meer macrofagen tijdens het remodelleren. Deze bevinding sluit aan bij de kennis dat het aantrekken van macrofagen nodig is voor het remodelleren van vaten tijdens atherosclerose.

Extra Domein A bevattend fibronectine in vaatwand remodelleren

Tijdens het proces van expansief vaatwand remodelleren wordt meer TLR4 aangemaakt en is meer van het TLR4 eiwit aanwezig, zoals beschreven in hoofdstuk 2. In het gebruikte model wordt geen exogene (lichaamsvreemde) stof toegediend om de TLR4 te activeren. Daarom is gekeken naar endogene liganden van TLR4. Endogene liganden zijn bijvoorbeeld EDA en HSP60, structuren die door het lichaam zelf gemaakt worden en ook de TLR4 kunnen activeren. Zowel EDA als HSP60 niveaus zijn verhoogd tijdens expansief arteriële remodellering en dit wijst op een rol voor deze endogene liganden in het proces van arteriële remodellering. Extra Domein A (EDA) is een domein van fibronectine, een belangrijke component van de extracellulaire matrix. EDA wordt niet standaard ingebouwd in fibronectine, alleen tijdens processen als embryogenese, wondgenezing, bij cellulaire schade en bij atherosclerose. In hoofdstuk 4 is onderzocht of EDA ook een oorzakelijk verband heeft met arteriële remodellering. In muizen die geen EDA kunnen maken, wordt geen expansief remodelleren gemeten en is er zelfs een afname van de diameter van het vat. In de aanmaak en afbraak van collageen is geen verschil met controledieren, maar cellen afkomstig van muizenembryo's zonder EDA migreren minder. Deze afname in migratie van cellen is een mogelijke verklaring voor de vermindering in expansief remodelleren in muizen zonder EDA.

Expressie van Extra domein A van fibronectine in stabiele humane atherosclerotische plaques

Extra Domein A van fibronectine kan de TLR4 activeren en zo de productie van matrix metalloproteinases en cytokines beïnvloeden. In hoofdstuk 5 zijn EDA niveaus bepaald in humane atherosclerotische plaques en bekeken in relatie tot de samenstelling van de plaque (morfologie) en de klinische symptomen van de patiënten. Zoals ook bekend uit eerdere studies, zijn de EDA expressieniveaus in atherosclerotische plaques hoger dan in de mammaria arteriën. Mammaria dienen als controle, omdat in deze vaten bijna nooit atherosclerose voorkomt. In stabiele plaques worden hogere EDA niveaus gevonden en er was een relatie tussen hogere EDA levels en meer gladde spiercellen, minder vet en meer collageen. Dit laat zien dat de aanwezigheid van een endogeen TLR4 ligand duidend op inflammatie niet per se gerelateerd is met onstabiele plaques, maar ook juist de stabilisatie van plaques kan stimuleren.

Interactie tussen het Toll-like receptor 4 signaal en Transforming Growth Factor ß signaal

Extra Domein A van fibronectine en Endoglin expressie in humaan atherosclerotische plaques

EDA, een endogeen TLR4 ligand, en Endoglin, een co-receptor voor TGFß, komen allebei tot expressie in atherosclerotische laesies en worden beiden aangezet door TGFß. Tijdens de vorming van atherosclerotische plaques komt TGFß tot expressie in de gebieden waar extracellulaire matrix gemaakt wordt en waar de intima verdikt is. In celkweekexperimenten heeft Endoglin een remmende werking op processen die door TGFß in gang worden gezet, zoals onder andere de aanmaak van fibronectine. In hoofdstuk 6 wordt de relatie beschreven tussen EDA en moleculen van het TGFß signaal, waaronder Endoglin in humane atherosclerotische laesies. De celexperimenten laten zien dat Endoglin de aanmaak van EDA na TGFß stimulatie remt en dat dit effect afhankelijk is van een ander eiwit; de co-receptor ALK5.

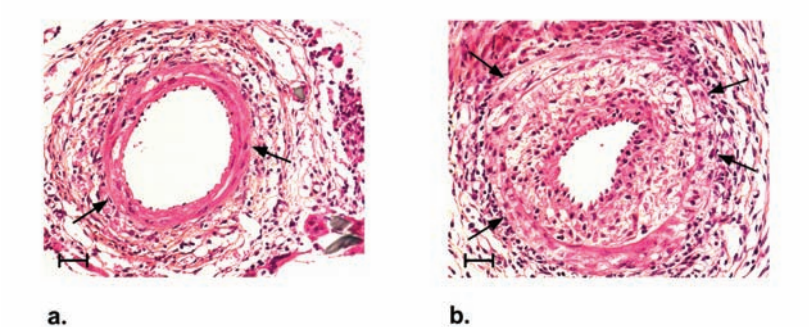
De associatie tussen EDA en Endoglin expressie in atherosclerotische plaques is een indicatie dat er ook een wisselwerking is tussen het TLR4 en TGFß signaal in atherosclerose.

Cross-talk van TLR4 en TGF signalen in THP-1 monocyten en atherosclerotische plaques

Steeds meer onderzoeken tonen een effect van TGFß op het TLR4 signaal aan, en andersom. Zo hebben bijvoorbeeld muizen zonder TGFß een verhoogde expressie en activiteit van TLR4. Dit duidt op een wisselwerking (cross-talk) tussen deze twee signalen. In atherosclerose zijn de afzonderlijke signalen via TLR4 en TGFß wel bekend. Maar weinig is bekend over de wisselwerking tussen deze signalen in de atherosclerotische plaque of in cellen die veel gezien worden in de plaque. Ook welk effect beide signalen op elkaar hebben en op welke genen ze van invloed zijn is niet bekend. In hoofdstuk 7 wordt de wisselwerking tussen het TGFß en TLR4 signaal onderzocht met een micro-array analyse op monocyten die met LPS (zet TLR4 signaal aan) of TGFß (zet TGFß signaal aan) of met TGFß plus LPS behandeld zijn. De activatie van de combinatie van beide signalen heeft een divers effect op verschillende sets van mRNAs; stimulerend, inhiberend of tegengestelde effecten van de twee signalen zijn gemeten. We hebben ons gericht op een groep van 100 mRNAs die laag tot expressie komen zonder stimulus toe te voegen en die bij het aanzetten van één van de twee signalen verhoogd tot expressie komen, maar die nog hoger tot expressie komen wanneer zowel het TGFß als het TLR4 signaal wordt aangezet. De meeste genen uit deze specifieke groep zijn betrokken bij immuun- en ontstekingsreacties en de eiwit kinase cascade. Kinases kunnen eiwitten een klein beetje veranderen waardoor ze actief of juist inactief worden.

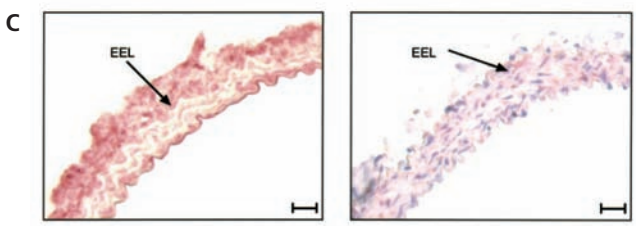
Osteopontine is één van de mRNAs die in de micro-array een patroon liet zien van: weinig aanwezig in de controle situatie, meer aanwezig als één signaal aanstaat en nog hoger wanneer beide signalen aanstaan. Ook op eiwitniveau vinden we dit expressiepatroon terug. Osteopontine niveaus zijn ook gemeten in atherosclerotische plaques en geassocieerd met een tweede klinisch incident, zoals een hart- of een herseninfarct. De resultaten van deze studie laten zien dat verschillende sets van mRNAs worden gereguleerd door zowel het TGFß als het TLR4 signaal en dat deze genen een belangrijke rol kunnen spelen in atherosclerose en de daaropvolgende klinische incidenten.

Color supplement



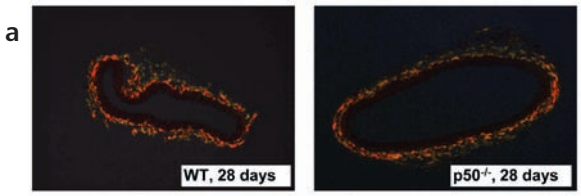
chapter 2

Figure 1.
HPS staining of femoral artery cross sections of APOE3 Leiden mouse treated with periadventitial cuff and gelatin (A) and with cuff and gelatin containing LPS (B). Arrows indicate EEL. EEL area (C) and plaque area (D) of the femoral artery of APOE3 Leiden mice after cuff placement with or without stimulation of LPS. n=10 mice per group. *P=0.009, **P=0.011. Bar =50µm.



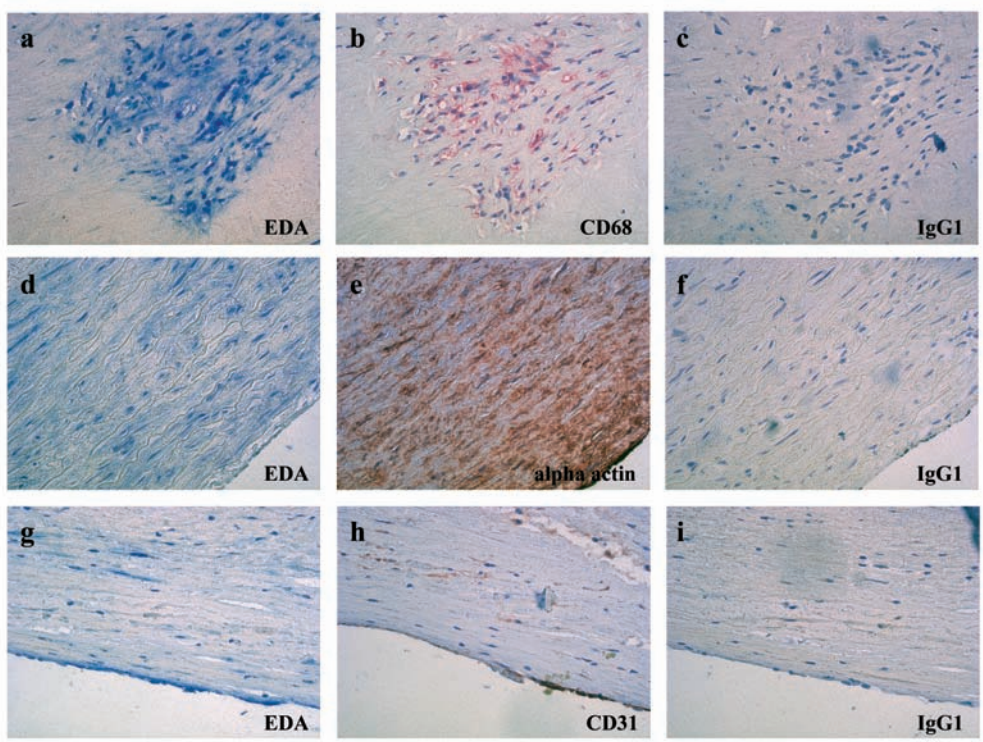
chapter 2

Figure 4.
Left carotid artery TLR4 mRNA and protein levels over time after ligation of the right carotid artery in wild-type BALB/c mice (A). Upper panel, Example of TLR4 Western blotting at 0 and 30 days after ligation and TLR4 Western blotting on protein of the THP1 monocyte cell line (1=2 µg, 2=4 µg protein) and the same blot (SEC) incubated with the immune serum replaced with nonimmune rabbit IgG. Bottom panel, Black bars represent TLR4 mRNA levels, white bars represent TLR4 protein levels, expressed in arbitrary units. Left carotid artery EDA and Hsp60 mRNA levels 28 days after right carotid artery ligation in wild-type BALB/c mice (B). Black bars represent EDA mRNA levels, white bars represent Hsp60 mRNA levels. n=5 to 9 mice per group. TLR4, EDA, and Hsp60 mRNA is represented as the amount of plasmid containing the PCR product to which it correlates in the dilution series of this plasmid used in the quantitative real-time PCR. Localization of TLR4 protein expression of the left carotid artery of the BALB/c mice (C, left panel) and negative control (4 µg/mL nonimmune rabbit IgG; C, right panel). *P=0.046, **P=0.029, ***P=0.007, ****P=0.041. Bar=25 µm.



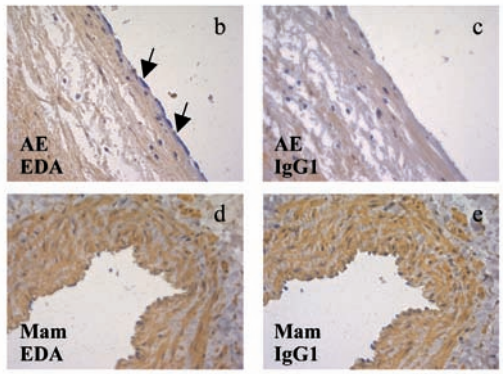
chapter 3

Figure 3. Collagen density and macrophages
(a) Picro Sirius staining of contralateral WT (left) and p50-/- arteries (right) at 28 days after ligation, magnification is 200x, (b) Collagen density is lower in p50-/- compared to WT arteries at 28 days, *P=0.014. **P=0.001 comparing the collagen density at 0 and 28 days in p50-/- animals. (c) Number of macrophages in adventitia of contralateral arteries of p50-/- (white bars) and WT mice (black bars) at 0, 3 and 9 days after ligation. *P=0.046 comparing WT and p50-/- arteries at 9 days. Error bars represent SEM.



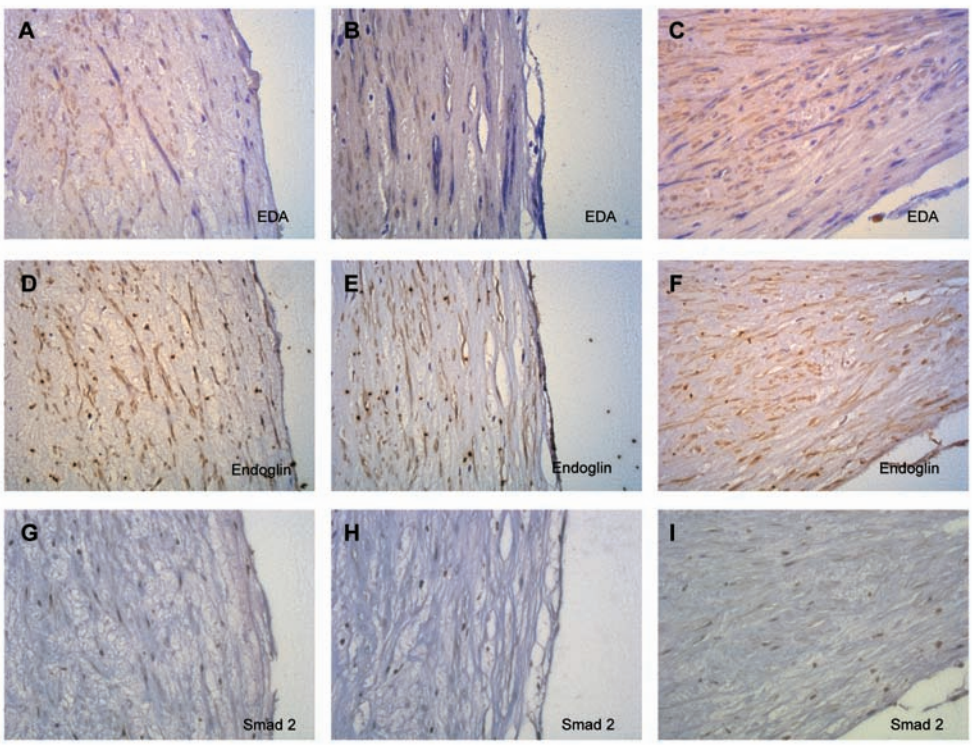
chapter 5

Figure 1. EDA expression in atherosclerotic lesions
EDA staining (1a, d, g) is predominantly co-localized with staining for macrophages (CD68, 2b), but also observed in smooth muscle cell rich areas (alpha actin, 1e) and around endothelial cells (CD31, 1h). IgG controls are negative (1c, f, i). All pictures are taken with 400x magnification.



chapter 5

Supplemental Figure 1. Expression of EDA is elevated in atherosclerotic lesions
Expression of EDA is higher in protein samples from atherosclerotic arteries compared to mammary arteries (a, n=9 and n=6 respectively, 22.9 ± 5.5 versus 6.8 ± 1.7 µg/mL, P=0.003). Immunohistochemistry showed EDA expression in the atherosclerotic plaque (b, AE-EDA, black arrows indicate EDA staining), while no staining was observed in mammary arteries (d, mam-EDA. Figure c and e are IgG1 control stainings for plaque and mammary artery respectively.)



chapter 6

Figure 1. Immunohistochemistry of EDA, Endoglin, and SMAD 2
EDA and Endoglin staining show co-localization of expression of these two proteins in the atherosclerotic plaque. p-SMAD 2 staining was shown in the nuclei of cells, in the regions positive for EDA and Endoglin staining.

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Curriculum Vitae

Karlijn van Keulen was born on February 13th, 1981 in Dordrecht. In 1999, she finished secondary school at Johan de Witt Gymnasium in Dordrecht. Biology, and in particular the human body had drawn her interest so she started in the same year with the studies Biomedical Sciences in Utrecht. She did her first internship during the master 'Biology of Disease' at the department of Experimental Cardiology in UMC Utrecht under supervision of dr. Dominique de Kleijn and prof. Gerard Pasterkamp. During this period she also worked for two months in the laboratory of dr. Sai-Kiang Lim in Singapore. She did her second internship in the research group Functional Genomics at the Hubrecht Laboratory in Utrecht, under supervision of dr. Nadine Vastenhouw and prof. Ronald Plasterk. She also visited the laboratory of prof. Cordeiro in Coimbra, Portugal for an internship of 8 weeks. In 2004, Karlijn obtained her Master's degree in Biomedical Sciences. After graduation, she started her PhD at the department of Experimental Cardiology in UMC Utrecht, of which the results are described in this thesis. During her PhD, the Netherlands Heart Foundation awarded her a travel grant for a five-month work visit in the research group of dr. Sai-Kiang Lim in Singapore. She was also awarded a travel grant from The Netherlands Organisation for Scientific Research which allowed her to work at the University of California, in Davis, California, in the group of dr. John Peters. Part of her research has been acknowledged with a fellowship from the Dutch Atherosclerosis Society.
Since October 2008 Karlijn is working at the department of Experimental Cardiology as education coordinator and researcher in the biomedical company Cavadis.