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Inflammation in various stages of coronary heart disease

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2 Abbreviations

ACS	acute coronary syndrome
AMI	acute myocardial infarction
AP	angina pectoris
CABG	coronary artery bypass grafting surgery
CAM	cellular adhesion molecule
CHD	coronary heart disease
CRP	C-reactive protein
CVD	cardiovascular disease
HDL	high density lipoprotein
ICAM-1	intercellular adhesion molecule 1
IL-1β	interleukin 1β
IL-6	interleukin 6
IL-8	interleukin 8
IL-10	interleukin 10
LDL	low density lipoprotein
LVEF	left ventricular ejection fraction
mBMC	mononuclear bone marrow cells
MCP-1	monocyte chemoattractant protein 1
MMP	matrix metalloproteinase
MRI	magnetic resonance imaging
NSTEMI	non-ST-elevation myocardial infarction
PAD	peripheral arterial disease
PCI	percutaneous coronary intervention
sCD40L	soluble CD40 ligand
SMC	smooth muscle cell
STEMI	ST-elevation myocardial infarction
TGFβ	transforming growth factor β
TIMP-1	tissue inhibitor of metalloproteinases 1
TNFα	tumor necrosis factor α
VCAM-1	vascular cellular adhesion molecule 1

3 List of papers

I Solheim S, Arnesen H, Eikvar L, Hurlen M, Seljeflot I. Influence of aspirin on inflammatory markers in patients after acute myocardial infarction. Am J Cardiol 2003; 92:843-845.

II Solheim S, Pettersen AA, Arnesen H, Seljeflot I. No difference in the effects of clopidogrel and aspirin on inflammatory markers in patients with coronary heart disease. Thromb Haemost 2006; 96:660-664.

III Solheim S, Grøgaard H, Hoffmann P, Arnesen H, Seljeflot I. Inflammatory responses after percutaneous coronary intervention in patients with acute myocardial infarction or stable angina pectoris. Submitted. 2007

IV Lunde K, Solheim S, Aakhus S, Arnesen H, Abdelnoor M, Egeland T, Endresen K, Ilebekk A, Mangschau A, Fjeld JG, Smith HJ, Taraldsrud E, Grøgaard HK,Bjørnerheim R, Brekke M, Muller C, Hopp E, Ragnarsson A, Brinchmann JE, Forfang K. Intracoronary injection of mononuclear bone marrow cells in acute myocardial infarction. N Engl J Med 2006; 355:1199-1209.

V Solheim S, Seljeflot I, Lunde K, Aukrust P, Yndestad A, Grøgaard H K, Aakhus S, Forfang K, Arnesen H. Inflammatory responses after intracoronary injection of autologous bone marrow cells in patients with acute myocardial infarction. In press. 2007

4 General introduction

4.1 Atherosclerosis

4.1.1 Epidemiology and classification

Atherosclerosis is by far the most frequent underlying cause of cardiovascular disease (CVD) which accounts for about 50% of all deaths in the western world [1]. In Europe, CVDs are the most frequent cause of death in men under 65 years of age and the second most common in women [2]. Atherosclerosis is a systemic arterial disease affecting mostly the intima of large - and medium sized systemic arteries like the aorta, carotid, coronary and peripheral arteries and may lead to cardiovascular events such as acute coronary syndrome (ACS), sudden death, stroke and gangrene of lower extremity.

The atherosclerotic lesions tend to develop in specific areas in the vasculature with disturbed non-laminar flow [3]. Based on the criteria by the American Heart Association Committee on Vascular Lesions, later modified by Fuster et al, the atherosclerotic process can be divided into five pathological/clinical phases [4-6] (Figure 1).

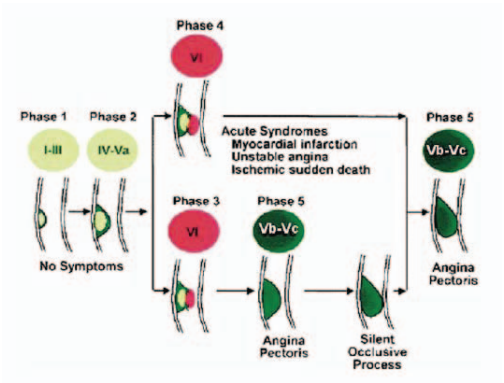

Figure 1. Clinicopathologic correlation of asymptomatic atherosclerosis leading to symptomatic atherothrombosis. Modified from Corti and Fuster [7].

Type I lesion in phase 1 is characterized by the presence of isolated groups of monocyte-derived macrophages containing lipid droplets (foam cells), the earliest stage of atherosclerosis which has been recognized in 45% of infants the first 8 months of life [8, 9]. Fatty streaks, a type II lesion, are more distinctly defined with foam cells organized in layers with the presence of smooth muscle cells (SMC) also containing lipid droplets, quite common in young children [5, 10, 11]. Type III lesions (phase 1) also include SMCs surrounded by extracellular connective tissue, fibrils and lipid materials. In phase 2 the lesions become

more advanced consisting of accumulated cellular material with more extracellular lipid intermixed with normal intima (type IV lesion) and dominated by an extracellular lipid core covered by an acquired fibrous cap (type Va lesion). Ruptured or eroded type IV or Va lesions (lesion type VI) are seen in phase 3 and may lead to thrombosis, usually clinically silent. In phase 4 lesion type VI is complicated by fixed or repetitive occlusive thrombosis leading to ACS including AMI, if apparent in a coronary artery. Lesion type Vb (calcific) and Vc (fibrotic) is categorized in phase 5 of atherosclerosis and may cause angina pectoris.
The progression of atherosclerosis in humans is partly dependent on genetic susceptibility and the presence of risk factors like smoking, hypertension, dyslipidemia (especially elevated LDL cholesterol), diabetes mellitus and obesity [12]. However, our knowledge of the links between risk factors and pathobiology is incomplete. The listed risk factors for atherothrombosis are not always present in individuals suffering acute myocardial infarction (AMI) or stroke. Clinical investigations, animal studies, population studies and cell-culture experiments have shown that inflammatory processes are deeply involved in all phases of atherosclerosis and represent a final common pathway for transducing the effects of risk factors into changes in the arterial wall [2]. Although oxidized LDL cholesterol is a key component in the atherosclerotic process, the immune response locally in the arterial wall and systemically might be more important for the progression of atherosclerosis in some individuals [13]. These considerations are supported by animal studies indicating that immunization with oxidized LDL might suppress atherosclerosis [14].

4.1.2 The atherosclerotic process

Regardless of the cause of atherosclerosis, the endothelium plays a central role in all phases of the atherosclerotic process and may be exposed to various injuries like modified LDL cholesterol, free radicals from smoking, hypertension, diabetes mellitus and disturbed flow, resulting in dysfunctional endothelium assessed clinically by impaired nitric oxide-mediated vasodilatation [15, 16]. The dysfunctional endothelium is leaky and atherogenic lipoproteins from plasma are retained in the subendothelial space, modified and oxidized exerting a broad range of pro-atherogenic effects [17]. The injured endothelium responses with expression of cellular adhesion molecules (CAMs) [2, 18] (Figure 2).

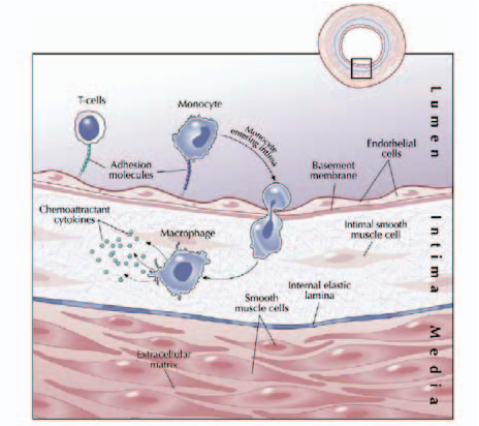

Figure 2. Transmigration of leukocytes. Adapted from P. Libby [19].

Oxidized LDL cholesterol is a key component in the activation of endothelial cells and macrophages whereby expression of different CAMs on the surface of endothelial cells take place, also mediated by cytokines like tumor necrosis factor α (TNFα) and interleukin 6 (IL-6) [17, 20-22]. CAMs like E-selectin, P-selectin, intercellular adhesion molecule 1 (ICAM-1) and vascular cellular adhesion molecule 1 (VCAM-1) are all involved in the rolling, adhesion and extravasation of monocytes and T-lymphocytes [23-26].
HDL cholesterol may reduce adhesion and transmigration of leukocytes into the subendothelial space as well as inhibit LDL oxidation and promote efflux of cholesterol from the arterial wall [27]. The recruitment of leukocytes into the arterial intima is contributed by chemokines like monocyte chemoattractant protein 1 (MCP-1) and interleukin 8 (IL-8) [28-30]. Monocytes entering the subendothelial space are differentiated into macrophages induced by macrophage colony-stimulating factor [31]. In the arterial wall macrophages express scavenger receptors mediating uptake of oxidized LDL cholesterol and apoptotic cell fragments, and toll-like receptors that can bind stress proteins like human heat shock proteins as well as oxidized LDL cholesterol and thereby activate the cell (Figure 3) [32, 33]. Once the macrophages have taken up lipid derivates and become foam cells they may replicate and elaborate a number of cytokines, chemokines, growth factors, metalloproteinases (MMPs), other hydrolytic enzymes and pro-thrombotic tissue factor [15]. Furthermore, apoptosis and necrosis of the lipid rich foam cells, endothelial cells and SMC in the atheroma may soften and destabilize the plaque core and thereby promote plaque rupture. In addition, both activated macrophages and SMC may express class II histocompatibility antigens on their cell

surface making them capable to present antigens to T-lymphocytes, mechanisms shown to be present in atherosclerosis [34]. T-lymphocytes, predominantly CD4+, are highly involved in the atherosclerotic process at all stages and are recruited to the subendothelial space by selectins and MCP-1 [14]. In advanced atherosclerotic lesions T-cells constitute up to 10-20% of the present cells, often accumulated at the rupture site and about 40% express macrophage markers, whereas most of the remainders are SMC with a small number of mast cells, B cells and dendritic cells [13, 35, 36]. When the T-cells are presented for antigens like oxidized LDL cholesterol and heat-shock protein 60, activation of the cell occurs with increased expression of cytokines, cell-surface molecules and enzymes [2]. In atherosclerosis, the type 1 helper T cell (Th1) response leads to a pro-inflammatory response with elaboration of pro-inflammatory mediators like interferon γ, lymphotoxin, CD40 ligand (CD40L), TNFα, IL-6 and C-reactive protein (CRP) [2, 37]. Activation of the Th2 pathway on the other hand may promote synthesis of anti-inflammatory cytokines like IL-10 and dampen the inflammatory process [2]. Transforming growth factor β (TGFβ), another cytokine with anti-inflammatory properties, produced by endothelial cells, SMCs, macrophages, platelets and regulatory T-cells, is in addition to IL-10 an important inhibitor of T-cell mediated immunity and may inhibit the atherosclerotic process [2, 38].

SMCs play a central role in the formation of the fibrofatty lesion, entering the arterial intima from tunica media, but may also be derived from the bone marrow (Figure 3).

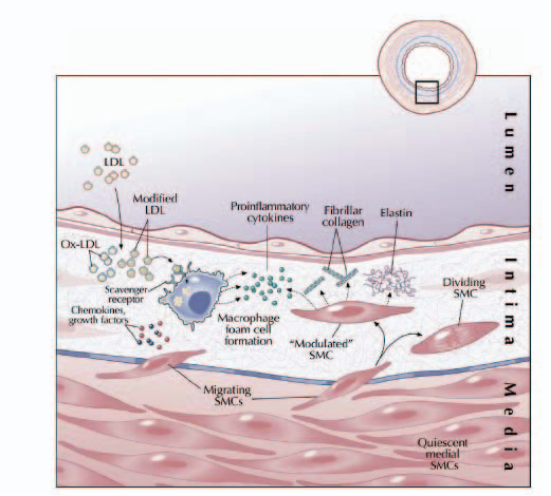

Figure 3. Formation of the fibrofatty plaque. Adapted from P.Libby [19].

They respond to a great number of signal substances including chemoattractants like platelet-derived growth factor that promotes migration of SMCs into the intima [19]. TGFβ may stimulate SMCs to produce extracellular matrix containing collagens, elastin and glycosaminoglycans contributing to lesion fibrosis and plaque stabilization [39]. However, incessant atherogenic stimuli may lead to an excessive fibroproliferative response resulting in lumen narrowing, reduced blood flow and ischemia. Although recruitment, proliferation and synthetic activities of SMC are mainly beneficial in atherosclerosis, apoptosis and necrosis of these cells are present at plaque rupture sites and thereby probably contribute to plaque destabilization.

4.1.3 Plaque disruption

During the progression of atherosclerosis the plaques may grow silently for many years until flow-limiting obstruction occurs with ischemia, or until sudden onset of thrombosis on a ruptured or eroded plaque induce more dramatic clinical events like ACS (Figure 4).

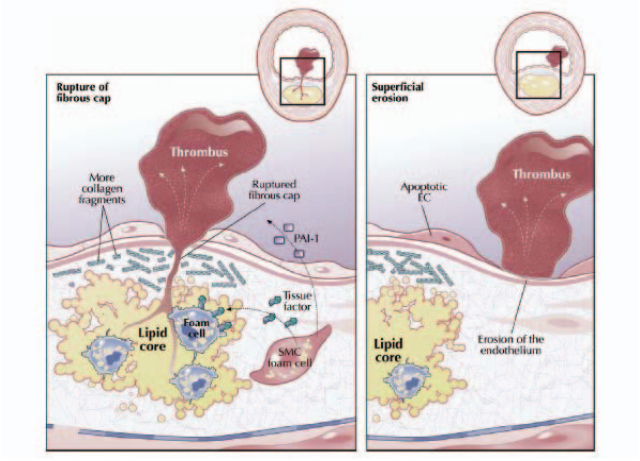

Figure 4. Two types of plaque disruption that may lead to thrombosis. Adapted from P.Libby [19].

Clearly, two types of atherosclerotic plaques exist. Atheromas may remain silent and stable throughout life without cardiovascular events, or become prone to thrombosis in response to a wide array of local and systemic influences that are patient specific [40]. Several clinical observations indicate that AMI not necessarily results from high grade stenosis, but from lesions without flow limitation [41, 42]. Inflammatory activation appears to be important, not only for progression of atherosclerosis, but also for developing vulnerable plaques with

subsequent disruption and thrombosis [18]. Unstable plaques often consist of a soft lipid-rich core with apoptotic SMCs and macrophages, a fragile and thin fibrous cap with inflammation in the cap and the plaque shoulders [43]. Activated macrophages, T cells and mast cells are present at the sites of plaque rupture and produce a large number of inflammatory cytokines like interferon γ and TNFα, proteases like MMPs and cystein proteases and prothrombotic factors like tissue factor [36, 44, 45]. These factors contribute to destabilize the plaque core and weaken the cap [2]. Eventually the fibrous cap may disrupt, leading to thrombosis mainly by two mechanisms (Figure 4). Cap fracture is the most common mechanism underlying ACS (60-70%) whereas superficial erosion of the plaque is less frequently present (25%) [46]. Plaque rupture exposes underlying pro-thrombotic material like collagen and tissue factor, to the circulating blood. Exposure of the subendothelial elements like collagen, microfibrils and laminin lead to platelet activation, platelet adhesion, mediated by von Willebrand factor and platelet aggregation by fibrinogen bridging the activated platelets, and thereby forming a platelet-rich clot. At the same time activated platelets release a large number of substances like ADP, serotonin, thromboxane A2, platelet-derived growth factor, fibrinogen, P-selectin and CD40L contributing to enhanced platelet activation and increased inflammation. Tissue factor, at the lesion site, complexes with Factor VIIa resulting in thrombin generation and fibrin formation, stabilizing the platelet-rich clot.

The CD40-CD40L system is a key system linking activated platelets to increased inflammatory response in the arterial wall [47]. CD40L (CD 154) is a transmembrane protein, a member of the TNFα superfamily, and has been identified on stimulated T-cells, mast cells, basophiles, granulocytes and platelets [47]. CD 40, a cell membrane-spanning protein, is present on B-lymphocytes, monocytes, macrophages and endothelial cells [47, 48]. CD40L is therefore a central element in the activation of many types of the CD40 expressing cells and this interaction may result in production of several inflammatory signal molecules like interleukin 1 β (IL-1β), IL-6, TNFα, IL-8 and MMPs promoting increased inflammation in the vessel wall [48].

Most plaque disruptions are however silent, without causing clinical events. Several factors both locally and systemically may influence on the faith of a plaque disruption such as plaque dependent thrombogenic substrates, rheology and systemic procoagulant activity, all influenced by inflammation, possibly balanced by antithrombotic mechanisms, endogenous or by specific therapeutics [4]. Anyway, eventually an occluding plate-rich thrombus may develop

resulting in ACS that may lead to life-threatening tissue damage (AMI). In ACS, there is compelling evidence for the presence of numerous vulnerable and active plaques in the coronary arteries beyond the culprit lesion [49]. Intravascular ultrasound studies have shown that 20-80% of the patients with ACS had multiple disrupted plaques in other areas than the culprit lesion which underscores the presence of widespread inflammatory activity in the coronary arteries [50-55].

Beyond doubt, inflammatory processes are central in all stages of atherosclerosis and represent a common pathway for the influence of a number of risk factors on the vascular wall.

4.2 Stable angina pectoris

4.2.1 Clinical manifestations

Angina pectoris (AP) is a clinical syndrome and the diagnosis is based on clinical assessment, laboratory tests and specific cardiac investigations. In the majority of cases, the AP patients have a characteristic discomfort usually located in the chest near the sternum, but can also be felt in the epigastrium, shoulder regions, either arm to the wrist and fingers, between the shoulder blades or the lower jaw or teeth. Typically, the discomfort is provoked by exertion or emotional stress and relieved by rest and/or use of nitroglycerine. Less common is myocardial ischemia without symptoms (silent ischemia). Atherosclerosis in the coronary arteries is the most common cause of myocardial ischemia and AP, but hypertrophic or dilated cardiomyopathy and aortic valve stenosis may also cause similar symptoms.

AP is caused by myocardial ischemia as a result of an imbalance between myocardial oxygen demand and supply. Adenosin from the ischemic myocardium appears to mediate chest pain through stimulation of angiotensin 1 receptor on cardiac nerve endings [56]. The oxygen supply to the myocardium is dependent on arterial oxygen saturation and myocardial oxygen extraction and coronary flow determined by the luminal area of the coronary artery and arteriolar tone [57]. Maximal flow during exercise can usually be maintained when luminal diameter reduction is $\leq 40\%$, but reduced blood flow with subsequent myocardial ischemia may occur during stress with a lumen diameter reduction of $> 50\%$ [58]. However, the individual ischemic threshold is also influenced by factors like degree of development of collateral circulation and coronary vascular tone.

4.2.2 Diagnosis and treatment

Evaluation of AP patients includes a careful history, physical examination and objective tests. Exercise electrocardiogram is frequently used as a non-invasive test for detecting myocardial ischemia. Other tests like perfusion scintigraphy and echocardiography with exercise or pharmacological stress are also used as supplementary diagnostic tools in AP patients. Computer tomography and magnetic resonance arteriography are non-invasive techniques that may give information about the likelihood for coronary artery disease, but are not recommended as routine clinical practice at the present time. Coronary arteriography is usually performed in all patients with stable AP to indentify the presence or absence of coronary lumen stenosis, define therapeutic options and determine prognosis.

The treatment of AP patients includes medical treatment and myocardial revascularization with percutaneous coronary intervention (PCI) or coronary artery bypass graft surgery (CABG) when suitable. Pharmacological treatment of stable AP includes low-dose aspirin, lipid lowering therapy and anti-ischemic therapy with betablocker and/or nitrates and/or calcium channel blockers where appropriate. After PCI with stent implantation in the coronary arteries additional anti-platelet therapy is given for a limited period of time to avoid stent thrombosis.

4.3 Acute myocardial infarction

4.3.1 Clinical manifestations, diagnosis and treatment

AMI is characterized by myocardial cell death as a consequence of prolonged myocardial ischemia. In acute coronary syndrome, which involves ST-elevation myocardial infarction (STEMI), non-ST-elevation myocardial infarction (NSTEMI) and unstable angina pectoris, plaque disruption with the formation of a thrombus in a coronary artery, is the underlying cause of myocardial ischemia. In STEMI the infarct-related epicardial coronary artery is occluded more often than in NSTEMI [59].

AMI may have different presentations in individual patients, ranging from none at all to sudden cardiac death. Most often the symptom of AMI is characterized by persistent chest pain with radiation into the jaw/teeth, shoulder, arm and/or back without pain relief of nitroglycerin. Associated symptoms like dyspnoea, nausea, vomiting, diaphoresis and sweating are often present.

The clinical diagnosis of AMI is based on a typical rise and gradual fall of biochemical markers specific for myocardial necrosis (troponin T or I, creatinin kinase MB) and either ischemic symptoms or ECG changes (development of Q-waves, ST-segment elevation or depression) or PCI [60].

In STEMI early and complete reperfusion of the occluded coronary artery is a key factor to minimize myocardial cell death. Revascularization therapy in STEMI includes either thrombolysis or PCI or the combination of both. The pharmacological long-term treatment after AMI consists of anti-thrombotics (aspirin and/or warfarin, clopidogrel for a limited period of time), lipid lowering treatment (statin), beta blocker, and when left ventricular dysfunction is present inhibitors of the renin-angiotensin-aldosteron system (angiotensin converting enzyme inhibitor, angiotensin II receptor blocker, aldosteron antagonists).

4.3.2 Pathophysiology

The hallmark for AMI is myocardial cell death due to necrosis which triggers an inflammatory reaction that results in healing and formation of a scar. The net effect of the inflammatory response in AMI can be favourable leading to healing and restoration of function (physiological), or unfavourable, leading to cardiac rupture or chronic dilatation and heart failure (pathological) [61].

Notably, most of our knowledge about the inflammatory reaction in AMI is described in animal models and may differ from humans [62]. Based on animal models, the healing process in AMI, reperfused with PCI, can be divided into three overlapping phases:

i) The inflammatory phase, the first 72 hours in AMI, is dominated by a complex and extensive inflammatory response that includes cytokine and chemokine induction with subsequent transmigration of leukocytes. Neutrophils and macrophages play an important role in removing dead tissue and contribute to further signalling with eventual healing of the infarct area.

ii) In the proliferative phase (2-7 days after AMI start) the inflammatory signalling is suppressed and replacement of dead cardiomyocytes by granulation tissue is taking place. Fibroblasts and endothelial cells infiltrate the infarct area. Extracellular matrix proteins are produced by myofibroblasts and an extensive microvascular network evolves.

iii) In the maturation phase, 7 – 21 days after AMI start, fibroblasts and vascular cells undergo apoptosis, a collagen-based matrix network evolves, and a matured scar is formed. The healing process in AMI results in profound changes in ventricular architecture and geometry

(ventricular remodeling) which may manifest clinically as increased ventricle size, altered shape of the ventricle and impaired cardiac function [62].

Studies have shown that 30-40 minutes of ischemia is sufficient to induce necrosis of cardiomyocytes [62]. Necrotic myocytes release their intracellular contents and initate an intense immune response mediated by toll-like receptor mediated pathways, complement activation and generation of reactive oxygen species. The transcriptional nuclear factor κB activation along with complement activation and free radical generation, is critical in the regulation and expression of cytokine, chemokine and adhesion molecules in both resident and blood-derived cells in AMI [63]. In rodent models of AMI there is a marked up-regulation of pro-inflammatory cytokines like IL-1β, TNFα and IL-6 in the infarct area as well as in the non-infarcted myocardium, within the the first hours [64, 65].

Early in the hyperacute phase of AMI, cardiac mast cells degranulate and release preformed TNFα which probably is a key component in initiating the cytokine cascade [66]. Studies have shown that TNFα may induce IL-6 in infiltrating mononuclear cells in the infarct area [66].

In AMI there is a loss of cardiac cells because of necrosis and apoptosis. TNFα may exert significant effects on host cells with the potential for apoptosis versus cell survival [67].

Furthermore, TNFα and IL-1β may promote fibroblast proliferation and synthesis of extracellular matrix by inducing the expression of angiotensin II type 1 receptor on fibroblasts [68].

Pro-inflammatory cytokines like IL-1β, IL-6 and TNFα are also associated with the remodeling process in AMI and may promote changes directly in cardiac myocytes like apoptosis, hypertrophy, contractility disturbances and inflammatory signal transduction [69-72]. TNFα and IL-6 may attenuate myocyte contractility directly through the immediate reduction of systolic cytosolic calcium [73].

Transmigration of leukocytes into the infarcted area is dependent on adhesion molecules and chemokines like IL-8, MCP-1 and macrophage inflammatory protein-1α (MIP-1α). IL-8 plays an important role in the recruitment of neutrophils and up-regulation of IL-8 in animal models with AMI has been documented [74]. In addition to promote recruitment of monocytes and T-cells, MCP-1 induces angiogenic effects and may promote collagen formation [75, 76].

The anti-inflammatory cytokines IL-10 and TGFβ are important factors in modulating/suppressing the inflammatory response in AMI. In animal models IL-10 mRNA expression has been demonstrated in AMI with a peak at 96-120 hours after reperfusion [77]. IL-10 inhibits the production of IL-1β, TNFα, IL-6, IL-8 and promotes synthesis of tissue

inhibitor of metalloproteinases 1 (TIMP-1) [77, 78]. TGFβ is a key regulator of the healing process in AMI with a wide range of effects including regulation of cell proliferation, differentiation, apoptosis and modulating the immune response [62]. In vitro studies have shown that TGFβ inhibits expression of pro-inflammatory cytokines and chemokines and promotes extracellular matrix deposition [62]. Although the origin of fibroblasts in AMI healing remains unclear, TGFβ plays a central role in myofibroblast differentiation [79].
The CD40-CD40L-system may also play a role in the inflammatory response in AMI, particularly by the expression of CD40L on activated platelets triggering increased production of pro-inflammatory cytokines by endothelial cells and monocytes/macrophages.

4.4 Circulating inflammatory mediators in coronary heart disease

Inflammation is present in atherosclerosis and in AMI. However, the degree of inflammatory responses is difficult to track and evaluate directly. No imaging techniques that can monitor changes of inflammation are available for clinical use and biopsies are not practical or suitable for this purpose. Inflammatory reactions in the vessel wall and in the infarcted area lead to increased production of a number of cytokines, chemokines and other signal substances partly released from the affected area, and also from circulating blood cells and other organs, especially the liver. Circulating biomarkers of inflammation measured from blood samples, may reflect the inflammatory process in atherosclerosis and AMI, providing valuable information about the extent of inflammation.
The present work is mainly focused on evaluating the levels of the circulating soluble forms of selected biomarkers in stable angina pectoris and AMI.
As described, cytokines like IL-1β, IL-6, TNFα, and CD40L are all pro-inflammatory substances linked to the progression of atherosclerosis and cardiovascular events.
Chemokines like IL-8, MCP-1 and MIP-1α are deeply involved in the recruitment of leukocytes into the injured tissue (arterial wall, infarcted area) and thereby promoting inflammation. Anti-inflammatory cytokines like IL-10 and TGFβ may on the contrary modulate and suppress the activity of the pro-inflammatory cytokines.

CD40L

CD40L is a transmembrane protein expressed by a large number of cells like T-and B-lymphocytes, SMCs, mast cells and platelets, the latters alone are the source of 95% of the circulating sCD40L [80]. Interaction between sCD40L and its receptor CD40 on cells like

endothelial cells and monocytes, may lead to elaboration of a large number of inflammatory mediators [81]. Increased levels of sCD40L have been associated with future cardiovascular events in healthy women [82]. Similarly, in patients with ACS, elevated levels of sCD40L have been related to increased risk of recurrent events [83-85].

IL-6

IL-6 is a central mediator of the acute-phase response with a broad range of humoral and cellular immune effects [86]. Up to 30% of the total circulating IL-6 originates from subcutaneous adipose tissue [87]. Studies have shown an association between elevated levels of IL-6 in men without CVD and in women with existing CVD and increased risk for cardiovascular events [88, 89]. However, limited data exist for a clear association between IL-6-levels and CVD independent of other risk factors.

CRP

CRP is the classic acute phase reactant produced by the liver in response to inflammatory stimuli like IL-6 [90]. In contrast to IL-6, CRP is released in larger amounts in response to inflammatory stimuli without diurnal variation. Clearly, CRP is, at the present time, the most robust inflammatory biomarker in CVD and large epidemiological studies have shown that elevated CRP levels predict increased risk for CVD in both healthy people and in individuals with CVD [91-106].

TNFα

TNFα was initially identified as products of lymphocytes and macrophages that caused lysis of certain cells, especially tumor cells [107]. Later research has shown that TNFα is involved in multiple human diseases including atherosclerosis and AMI. Several studies have shown that increased levels of circulating TNFα predicts coronary heart disease and other CVD in humans [108-110].

MCP-1

Chemokines like MCP-1 is involved in the recruitment of monocytes into the arterial wall as well as into the infarcted area in AMI. In addition, MCP-1 may induce production of tissue factor in the arterial wall [111]. Limited data exist, but studies have shown an association

between plasma levels of MCP-1 and traditional risk factors for atherosclerosis and clinical outcomes in patients with ACS [112, 113].

IL-8

IL-8 appears to be the primary chemokine responsible for the activation and recruitment of cytotoxic neutrophils into the ischemic myocardium and has been shown to be up regulated by oxidized LDL cholesterol in atherosclerosis [114, 115]. Elevated levels of IL-8 after PCI in patients with stable AP, have been associated with more cardiac events and restenosis [116].

IL-10

IL-10, an anti-inflammatory cytokine, tends to modulate the inflammatory cellular signalling pathways in atherosclerosis and AMI [15, 34, 39]. Clinical studies on circulating levels of IL-10 during stable coronary heart disease and ACS are inconclusive [117]. However, increased levels of circulating IL-10 have been associated with reduced risk for recurrent cardiovascular events in patients with ACS [118, 119].

TGFβ

TGFβ has a broad range of biological effects including the control of cellular proliferation and differentiation, regulation of tissue repair and extracellular matrix accumulation and modulation of immune and inflammatory responses. Low levels of TGFβ have been associated with advanced atherosclerosis and are likely to lead to an exaggerated inflammatory response [120-122].

4.5 Long-term anti-platelet therapy in coronary heart disease

Platelets are essential in the development of a thrombus on a disrupted plaque that may lead to a clinical event. In addition, platelets harbour a large number of signal molecules that may increase the inflammatory response in the arterial wall.

Acetylsalicylic acid or aspirin is widely used in patients with atherosclerotic disease including coronary heart disease. Aspirin binds to and then irreversibly acetylate the catalytic site of cyclooxygenase in platelets which is required for the metabolism of arachidonic acid to prostaglandin H2 (PGH2) [123]. PGH2 in platelets is processed to thromboxane A2 which is a potent activator of platelets and promote platelet aggregation. Aspirin, in doses of 75 to 325 mg daily, is well documented to reduce the risk for cardiovascular events in a broad range of

patients with atherosclerotic diseases [124]. In addition to its antithrombotic effects, aspirin possesses anti-inflammatory properties that may reduce vascular inflammation [125]. Clopidogrel is another anti-platelet agent, acting by blocking the binding of ADP to a specific platelet receptor P2Y12 and thereby inhibit adenyl cyclase activity, resulting in reduced activation of GP IIb/IIIa receptors and platelet aggregation [126]. Large clinical trials have shown that clopidogrel in addition to aspirin for a limited period of time reduces recurrent clinical events in patients with ACS as well as after PCI [127, 128]. However, limited data exist about the influence of clopidogrel on inflammation in patients with coronary heart disease.

4.6 Treatment of acute myocardial infarction with intracoronary injection of autologous mononuclear bone marrow cells.

In acute myocardial infarction there is a net loss of cardiomyocytes due to necrosis and apoptosis which may lead to heart failure and death. Although myocyte proliferation occurs in human hearts, this regeneration is obviously inadequate after AMI [129, 130]. Both animal and human studies have shown that bone marrow cells may home to the myocardium, but the role and function of these cells in cardiac repair are not clarified [131-134]. Animal studies have shown that intramyocardial injection of cell types like hematopoietic stem cells from the bone marrow, may improve left ventricular function in AMI, and even transdifferentiation of hematopoietic stem cells into cardiomyocytes has been reported in mice [135]. Therefore, myocardial cell-based repair has been proposed as a therapeutic option in patients with myocardial damage. Until 2003 only a few safety studies with intracoronary injection of bone marrow cells in patients with AMI had been performed, and no adverse effects of this treatment could be demonstrated [136, 137]. Even a possible beneficial effect on left ventricular function was reported, but larger studies were clearly needed [136, 137]. In addition, several issues were at that time not clarified like underlying mechanisms for potential beneficial effects of stem cell transplantation, optimal cell type and number, timing after AMI, and delivery methods. The optimal time for delivery of mBMCs in AMI is dependent on several factors like the presence of homing signals and the inflammatory environment [138]. A marked pro-inflammatory environment with rise in reactive oxygen species might be deleterious for the injected cells. Furthermore, intracoronary injection of mBMCs as well as bone marrow aspiration may also influence on the inflammatory environment affecting both the myocardium and coronary arteries.

5 Aims of the study

Inflammation is deeply involved in all phases of atherosclerosis and in AMI. The main purpose of the present work was to investigate the influence of specific treatment modalities on inflammatory responses in patients with stable coronary heart disease or AMI.
More precisely, we wanted to assess:

- … the influence of aspirin on circulating biomarkers of inflammation in patients recovered from AMI. (Paper I)

- … the effects of clopidogrel as compared to aspirin on selected circulating inflammatory markers in patients with stable angiographically verified CHD. (Paper II)

- … the profile of circulating inflammatory mediators in patients with AMI as compared to patients with stable angina pectoris, all treated with PCI and stent implantation. (Paper III)

- … the effect of intracoronary injection of autologous mBMCs on systolic left ventricular function in patients with AMI. (Paper IV)

- … the influence of intracoronary injection of autologous mBMCs on selected circulating inflammatory mediators in patients with AMI. (Paper V)

6 Material and methods

6.1 Study subjects and design

In Paper I, we studied patients participating in the Warfarin Aspirin Re-Infarction Study (WARIS II) [139]. A total of 310 patients, recovered from AMI, both gender, age about 60 years, 30% smokers, LVEF 45%, 70% on statin treatment, recruited from Ullevål University Hospital, Oslo, Norway were randomly included in the present substudy: 102 patients on warfarin alone with target International Normalized Ratio (INR) 2.8-4.2, 107 patients on aspirin 160 mg o.d. alone and 101 patients on the combination of aspirin 75 mg o.d. and warfarin with target INR 2.0-2.5.

Study medication was started during their hospital stay and mean follow-up time was 4 years. Blood samples were collected 3 months after the AMI for determination of thromboxane B_2, TNFα, IL-6, IL-10 and CRP. In 210 of the 310 study subjects, while still alive and adhering to the randomized treatment, fasting blood samples were also drawn 4 years after the AMI. During the follow-up period, while still being on the randomized treatment, relevant clinical events were recorded. The study protocol was approved by the Regional Ethics Committee and all patients gave written informed consent to participate.

In paper II, we studied a random subgroup of patients from the ASpirin non-responsiveness and Clopidogrel clinical Endpoint Trial (ASCET) which is a prospective randomized study investigating aspirin non responsiveness in relation to clinical events [140]. A total of 206 patients, age 61 years, 20% female, 50% with previous AMI, 20% current smokers and 20% with known diabetes mellitus type II were included. All patients had stable coronary heart disease verified with coronary angiography, and were randomized to either clopidogrel (Plavix® Sanofi- Synthelabo, Bristol-Myers Squibb, Paris, France) 75 mg daily (n=101) or aspirin (Albyl-E®, Nycomed, Oslo, Norway) 160 mg daily (n=105). The participants were all taking aspirin 160 mg daily for at least 7 days before randomization and continued all other medication according to general guidelines. In accordance with current guidelines 98% were on statin therapy. Medication use was similar between the groups, also after 1 year. Fasting blood samples were drawn at randomization and after 1 month and 1 year for determination of CRP, TNFα, IL-6, MCP-1, CD40L, P-selectin, IL-10 and TGFβ. The study protocol was approved by the Regional Ethics Committee and all patients gave written informed consent to participate.

In paper III, we investigated thirty men and women, age 62 years, with STEMI (n=20) or stable angina pectoris (n=10), admitted to Ullevål University Hospital, Oslo, Norway. All were treated successfully with PCI in a central coronary artery obtaining normal blood flow. Patients with previous transmural infarction, cardiogenic shock or considerable co-morbidity (i.e. short life expectancy because of malignancy, stroke, inflammatory diseases, endocrinological disturbances and lung disorders) were not enrolled. The included patients were treated in accordance with current guidelines. Blood samples were collected by standard venipuncture immediately before PCI, only in the AP group, and after 3 and 12 hours, 1, 3, 5, 7 and 14 days in both groups for determination of TNFα, IL-6, CRP, MCP-1, IL-10, TGFβ, sCD40L and IL-8. The study protocol was approved by the Regional Ethics Committee and all patients gave written informed consent to participate.

In papers IV and V, we included 100 patients, both gender, age 57 years, at Ullevål University Hospital and Rikshospitalet University Hospital between September 11, 2003 and May 4, 2005. They all had anterior wall ST-elevation myocardial infarction within 2 – 12 hours from onset of symptoms to PCI, successful PCI with stent on left anterior descending artery (LAD), culprit lesion proximal to the second diagonal branch, ≥3 hypokinetic left ventricle segments assessed by echocardiography and creatine kinase MB >3 times upper reference value. Patients with previous Q-wave myocardial infarction, cardiogenic shock, severe co-morbidity or other conditions which interfered with patient ability to comply with the protocol were excluded. All patients received medication according to current guidelines. The day of acute PCI was defined as day zero, and at day three to five, patients were randomized 1:1 to either autologous mBMC or control groups. The mBMC transplantation was performed 6 days (median) after the AMI.

In Paper IV, systolic left ventricular function was assessed by single-photon emission computed tomography (SPECT), echocardiography and MRI. Baseline SPECT and echocardiography were performed before cell treatment, and MRI recordings were obtained 2-3 weeks after the myocardial infarction. After six months, SPECT, echocardiography and MRI were repeated.

In Paper V, blood samples were obtained by standard venipuncture between 8 and 9 a.m. after a 12-hours fast the day before stem cell transplantation (baseline) (Day-1) in the mBMC group and 1 day (Day 1), 3 days (Day 3), 2-3 weeks and 3 months after stem cell transplantation (Day 0) and at corresponding time points in the control group for

determination of circulating levels of TNF α, IL-6, CRP, IL-8, MCP-1, sCD40L, IL-10, TGFβ and mRNA expression in whole blood of IL-1β, TNFα, IL-8 and MIP-1α.

6.2 Stem cell injection

The mBMC group was aspirated for 50 ml of bone marrow from the iliac crest in local anesthesia 4-7 days (baseline) after the acute percutaneous coronary intervention. The bone marrow was mixed with 10.000 IU heparin and centrifugated on a Ficoll density gradient (Axis-Shield, Oslo, Norway) for isolation of mBMC, washed, and resuspended in heparin-plasma (heparin 1000 IU/ml). The next day, a median of 6 days (interquartile range 5 to 6) after AMI (Day 0), 10 ml of the mBMC suspension containing a median number of 68 x 10^6 mononuclear cells (0.7 x 10^6 CD34+ cells) was injected in the infarct related coronary artery (Left Anterior Descending artery). After administration of heparin 100 IU/kg body weight intravenously, a 0.5 mm oversized over-the-wire balloon catheter was advanced to the proximal part of the stent on the culprit lesion and inflated with very low pressures (< 2 bar) for 90 seconds obtaining no-flow. At the same time, one third of the stem cell suspension, followed by 2 ml heparinized saline, was injected distally, followed by deflation of the balloon and re-flow for 5 minutes between a total of 3 injections (10 ml). The control group was not aspirated for bone marrow and did not go through any further coronary intervention with intracoronary injections and administration of heparin.

6.3 Evaluation of left ventricular function

Myocardial scintigraphy

Perfusion imaging was performed as ECG-gated SPECT after injection of 99mTc-tetrofosmine (Myoview™, Amersham Health, UK). The injected dose was 500MBq in baseline studies, and 250-300MBq (weight-adjusted) in 6 month studies. An Exeleris (GE Medical Systems) processing station, with the 4D-MSPECT™ software, was used for processing of all recordings and assessment of left ventricular volumes and infarct size (proportion perfusion defect). The method is described in more detail in Paper IV.

Echocardiography

Vivid 7 scanner (GE Vingmed Ultrasound, Horten, Norway) was used for echocardiography recordings. Three apical views with three consecutive cineloops were recorded for analysis on a separate work station by a single physician. EchoPAC PC software (GE Vingmed Ultrasound) was used and left ventricular volumes were computed using the modified

Simpson`s rule according to current guidelines. The method is described in more detail in Paper IV.

Magnetic resonance imaging

MRI was performed using a 1.5 tesla Siemens scanner Magnetom Vision Plus (Erlangen, Germany) for the first 18 months of the study, and Magnetom Sonata, Siemens (Erlangen, Germany), for the last 10 months. Breath-hold cine images in two-chamber, four-chamber and short axis views were acquired. Approximately 10 – 20 minutes after intravenous injection of 0.2 mmol/kg gadopentetate dimeglumine (Magnevist, Schering, Berlin, Germany), late contrast enhancement images were obtained, first in two-chamber and four-chamber views, and then with multiple short axis slices covering the entire left ventricle with a slice thickness of 7 mm and an interslice gap of 3 mm. For each slice the total area of the left ventricular wall and the area of late enhancement were manually drawn, and the areas were summed for calculation of total left ventricular wall volume and total late enhancement volume . Infarct size is presented as total late enhancement volume and as a proportion ((total late enhancement volume / total left ventricular wall volume) x 100 %). The method is described in more detail in Paper IV.

All evaluations were performed without knowledge of the patients treatment given in the randomized trial.

6.4 Laboratory analyses

Circulating levels of the inflammatory mediators in serum and plasma were measured by commercially available enzyme immunoassays, described in more detail in the papers.

Whole blood for mRNA analysis was collected in PAXgene Blood RNA Tubes (PreAnalytiX GmbH, Hombrechtikon, Switzerland) by standard venipuncture and stored at -70˚C after initially kept at room temperature for at least 2 hours. RNA was extracted using PAXgene Blood RNA Kit and reversely transcribed. Gene expression of interleukin 1β, TNFα, IL-8 and macrophage inflammatory protein-1α was assessed by use of Real-Time reverse transcription polymerase chain reaction. Results are presented relative to the gene expression of glyceraldehyde-3-phosphate dehydrogenase (house keeping gene). The method is described in detail in paper V.

6.5 Statistics

Variables are expressed as proportions, means ±SD or medians with 25, 75 percentiles when skewed data. In Paper I, II, III and V the levels of inflammatory mediators were not normally distributed and non-parametric statistical methods were used. Differences between groups were assessed by the Mann-Whitney test or the two-sample t-test when appropriate. Wilcoxon test was used to assess within group changes from baseline to later time points. In Paper V, the significance of differences in changes from baseline between groups was tested by analysis of co-variance on log-transformed data. SPSS software package for Windows and Epi Info for Windows were used for data analyses.

7 Summary of results

7.1 Paper I

We assessed the influence of aspirin on selected inflammatory markers in patients 3 months after AMI and the relation to relevant clinical events after 4 years. We also evaluated the stability of the inflammatory markers during long term aspirin treatment for 4 years.
We studied patients participating in the Warfarin Aspirin Re-Infarction Study (WARIS II), which compared the efficacy and safety of warfarin, aspirin and the two combined as secondary prophylaxis after an AMI [139]. In the present substudy a total of 310 patients, recruited from Ullevål University Hospital, Oslo, Norway were randomly included: 102 patients on warfarin alone, 107 patients on aspirin 160 mg o.d. alone and 101 patients on the combination of aspirin 75 mg o.d. and warfarin. Statistically significantly lower levels of CRP in patients treated with aspirin 160 mg o.d. as compared to the warfarin alone group could be demonstrated 3 months after AMI. The same profile appeared in the levels of TNFα and IL-6, but the differences were of borderline significance. In all treatment groups significantly lower levels of CRP and TNFα were found after 4 years (n = 210). Noteworthy, however, in general, the differences between the treatment groups were maintained. The difference in IL-6 between the aspirin 160 mg o.d. alone group and the warfarin alone group attained statistical significance after 4 years, and also in the combined group the levels were significantly reduced compared to the warfarin alone group.
Taken together, reduced levels of pro-inflammatory markers in patients treated with aspirin 160 mg o.d. compared to warfarin for up to 4 years after an acute myocardial infarction were demonstrated. These findings were, however, not reflected in the incidence of clinical end-points after 4 years.

7.2 Paper II

While aspirin is known to reduce several pro-inflammatory markers in patients with coronary heart disease (CHD), limited data exist regarding the influence of clopidogrel. The aim of this study was to assess the influence of clopidogrel as compared to aspirin on selected circulating inflammatory markers in patients with stable angiographically verified CHD. Patients on treatment with aspirin 160 mg o.d. for at least 7 days were randomized to either continuing aspirin 160 mg o.d. (n=105) or clopidogrel 75 mg o.d. (n=101) and followed for 1 year. There were no differences in any of the measured inflammatory variables including changes from baseline to 1 month and 1 year, between the groups. In the aspirin group we found significantly lower levels of TNFα and MCP-1 after 1

year. Likewise, in the clopidogrel group the level of TNFα was significantly reduced after 1 year. The present results indicate similar anti-inflammatory effects of the two drugs in patients with CHD.

7.3 Paper III

We investigated the profile of selected circulating inflammatory markers after PCI in patients with AMI or stable angina pectoris (AP). Twenty patients with AMI and 10 patients with stable AP were enrolled (age 60 years, both sexes, without previous myocardial infarction), all treated with PCI of a central coronary artery. Blood samples were drawn immediately before PCI, only in the AP group, and after 3 and 12 hours, days 1, 3, 5, 7 and 14 in both groups. The median levels of IL-6 increased in both groups to time point 12 hours with a peak on day 1, but were significantly higher in the AMI group compared to the AP group at time points 3 and 12 hours, and also at days 1 and 3. A similar profile could be demonstrated for CRP with significantly higher levels in the AMI group at days 1, 3 and 5 compared to the AP group. A slightly different pattern was shown for IL-10 with significantly higher levels in the AMI group at 3 and 12 hours, days 1 and 14 compared to the AP group.
Thus, patients with AMI treated with PCI experienced a marked short term increase in circulating levels of pro-inflammatory mediators as well as of IL-10 compared to similarly treated patients with stable angina pectoris. In addition, the PCI procedure per se also induced an increase in IL-6 levels in patients with stable angina pectoris.

7.4 Paper IV

We examined the effects of intracoronary injection of autologous mBMC on left ventricular function in AMI. One hundred patients with acute ST-elevation anterior wall myocardial infarction treated with percutaneous coronary intervention were assigned to intracoronary injection of autologous mononuclear bone marrow cells (mBMC) (n = 50) or control group (n = 50) (ASTAMI trial). End-points were changes in left ventricular ejection fraction (LVEF), end-diastolic volume and infarct size assessed by ECG-gated single-photon emission computed tomography (SPECT), echocardiography and MRI from baseline to 6 months after the infarction. In both groups a significant improvement in LVEF (SPECT) from baseline to 6 months could be demonstrated, but without between group differences.
SPECT, echocardiography and MRI did not show any effects on global left ventricular function by intracoronary injection of autologous mBMC after 6 months.

7.5 Paper V

We investigated the influence of intracoronary injection of mBMC on the pattern of inflammatory biomarkers in patients with AMI. AMI patients in the ASTAMI trial (n=100) treated with PCI were randomized to intracoronary injections of autologous mBMC (n=50) or control (n=50). Fasting blood samples were drawn the day before stem cell transplantation (baseline, 4-5 days after AMI) and at day 1 and 3, 2-3 weeks and 3 months after the transplantation for determination of circulating levels of inflammatory markers and mRNA levels in whole blood samples. From baseline to Day 1, the levels of IL-6 and the expression of TNFα mRNA increased significantly in the mBMC group compared to the control group. The decrease of IL-6 levels from baseline to 2-3 weeks in the mBMC group was less pronounced than in the controls ($p<0.05$), as was also the decrease in CRP levels from baseline to Day 1 and Day 3 in the mBMC group ($p<0.05$ for both). However, from baseline to 3 months the circulating levels of TNFα and MCP-1 increased less in the mBMC group ($p<0.05$ for both).

Intracoronary injection of mBMC in patients with AMI induces a marked short term pro-inflammatory response, but a slightly reduced inflammatory pattern after 3 months. The results may have implications for the delivery method and timing of stem cell transplantation in AMI.

8 Discussion

8.1 Methodological considerations

8.1.1 Study subjects and methods

In the present work, all the investigated patients had coronary heart disease in various stages. In Paper II, only subjects with stable angiographically verified coronary heart disease were included, whereas in paper I individuals recovered from AMI were enrolled. In paper III, patients with stable coronary heart disease were compared to subjects with AMI, both groups treated with PCI and stent implantation. In Papers IV and V only patients with AMI were included.

The influence of specific interventions on the inflammatory response is probably different in patients with stable coronary heart disease from those with AMI, which should be considered when evaluating the levels of circulating inflammatory mediators. In addition, the circulating levels of the selected inflammatory markers may not necessarily fully reflect the inflammatory process locally in the arterial wall and the myocardium.

The levels of soluble biomarkers were measured by commercially available enzyme immunoassays kits with satisfactorily inter-assay coefficients of variation below 11%. Several of the selected biomarkers have diurnal variation, and therefore blood samples were collected by standard venipuncture between 8 and 9 a.m. after 12-hours fast and before intake of medication, except for samples taken at 3 and 12 hours interval (Paper III).

In Paper V, the levels of mRNA were determined in whole blood reflecting gene expression of the selected biomarkers in circulating leukocytes and not in the myocardium and the vessel wall which could differ.

8.2 General discussion

8.2.1 Anti-inflammatory therapies in coronary heart disease

In Paper I we demonstrated significantly reduced levels of CRP in patients on aspirin 160 mg daily compared to patients on warfarin alone 3 months post AMI. A similar difference of borderline significance was observed for TNFα. In all treatment groups significantly lower levels of CRP and TNFα were found after 4 years, and the levels of IL-6 were significantly lower in the aspirin alone group compared to the warfarin group as well as between the combined group and the warfarin group. Warfarin has not been shown to exert any anti-inflammatory properties and served as a control in the present study.

Aspirin

It is now compelling evidence for anti-inflammatory effects of aspirin in patients with coronary heart disease. Our findings is in accordance with other studies that have shown reduced levels of pro-inflammatory biomarkers after treatment with aspirin [96, 141].

The reduced inflammatory activity during aspirin treatment is probably mediated by reduced phosphorylation of the inhibitor of nuclear factor κB and thereby inhibition of this transcription factor activation which is of crucial importance in inflammatory regulation [125, 142]. Although animal studies have shown that aspirin reduces atherosclerosis, the clinical benefit of reduced inflammatory activity vs inhibition of platelet aggregation by aspirin in humans with coronary heart disease remains unknown [143]. The lack of influence of aspirin on the clinical end-points in the WARIS-II trial is further discussed in para 8.2.2 (p. 30).

In Paper II we wanted to examine the anti-inflammatory effects of clopidogrel 75 mg o.d. as compared to aspirin 160 mg o.d. in patients with coronary heart disease. We did not find any between group differences in circulating markers of inflammation after 1 year treatment with clopidogrel 75 mg o.d. compared to aspirin 160 mg o.d., but in both groups lower levels of TNFα were obtained indicating similar anti-inflammatory effects.

Clopidogrel

The mechanism behind an anti-inflammatory effect of clopidogrel is different from that of aspirin. Clopidogrel inhibits binding of ADP to the specific purinergic receptor P2Y12 and thereby decreases the activation of platelets, which seems to result in reduced expression of CD40L and P-selectin and thereby less inflammatory activity [47, 144]. It has been shown that most of the effects of CD40L is confined to the membrane-bound fraction, and circulating levels may therefore not reflect the total physiologically effective expression of CD40L and P-selectin [144]. In the present study the circulating levels of sCD40L and P-selectin did not differ between the two treatment groups. However, these findings do not exclude differences between the two drugs concerning the membrane-bound fraction of CD40L and P-selectin. Beyond doubt, an anti-inflammatory effect of clopidogrel in patients with coronary heart disease is present, but the clinical effect of this anti-inflammatory action in terms of reduced cardiovascular events is not clarified.

Statins

In papers I and II we observed reduced circulating levels of several inflammatory markers during the observation time, irrespective of the study treatment. This may be partly explained

by other therapies with impact on the inflammatory responses. The majority of the patients were on statin treatment which has been shown to reduce inflammation [102, 145-148]. The clinical benefit of statin therapy has traditionally been linked to the reduction of apoB- and apoE-containing lipoproteins, LDL in particular, but also VLDL and intermediate-density lipoprotein [149]. A large number of studies have shown that statins reduce myocardial infarction, stroke, and cardiovascular and total mortality rates in both primary and secondary prevention trials [150]. However, the impact of the non-lipid-decreasing (pleiotropic) effects of statins including the anti-inflammatory properties on the clinical benefit, is not clarified.

Inhibitors of the renin-angiotensin system

A minor part of the patients in papers I and II were treated with inhibitors of the renin-angiotensin system (angiotensin converting enzyme inhibitors or angiotensin II receptor blockers). Angiotensin II promotes the activation of NF-κB, LDL oxidation and endothelial dysfunction [151-153]. Inhibitors of the renin-angiotensin system have been shown to reduce CRP and pro-inflammatory cytokines [154-156]. Usage of the inhibitors of the renin-angiotensin system may lead to reduced inflammatory activity over time which could have influenced on the levels of inflammatory mediators in papers I, II and V.

8.2.2 Inflammatory signal substances and atherosclerosis – the chicken or the egg?

Atherosclerosis is, no doubt, an inflammatory disease, but whether inflammation is causal or casual remains unknown. Inflammatory signal substances like IL-6, TNFα, MCP-1 and CD40L are all deeply involved in the inflammatory process in the arterial wall as described in the general introduction. Even CRP, mostly produced in the liver, has been shown to exert direct pro-inflammatory effects in the arterial wall like downregulation of endothelial nitric oxide synthase, increased synthesis of endothelin-1 and IL-6 by endothelial cells, and also increased expression of vascular cell adhesion molecules and MCP-1 [157-161]. Furthermore, CRP itself is a potent chemoattractant for monocytes, facilitates uptake of LDL cholesterol by macrophages, contribute to complement activation and stimulates SMCs migration, proliferation and reactive oxygen species production [162-165].

Large, prospective studies have shown that individuals with elevated levels of circulating biomarkers are at increased risk of cardiovascular events [82, 93-99, 101, 105, 145, 166-168]. Clinical studies have also shown that patients in the highest CRP quartile obtain greater reduction in cardiovascular events with statin treatment than those in the lowest CRP quartile [102, 145]. However, there are mixed results regarding the impact of reducing the circulating

inflammatory markers on cardiovascular events. In paper I, (The WARIS II population) no differences were found in the 3 months levels of CRP and TNFα between those having an event after 4 month compared to those without. When dividing the variables in quartiles for relation to future events, no associations could be found for CRP and TNFα. As discussed in paper I, the lack of association between the levels of inflammatory markers and clinical end-points could be related to superior antithrombotic effects of warfarin over aspirin in the WARIS-II trial. In accordance with our findings it has been shown that CRP levels measured at admission to hospital in patients with unstable angina pectoris did not predict risk for recurrent cardiac events the next 12 months [169]. Furthermore, in a study including patients hospitalized with chest pain, no association of serum levels of CRP, IL-6 or TNFα with atherosclerotic burden or major cardiovascular events at 6 months was detected, when adjusted for traditional risk factors [170].

It is well documented that statins reduce the levels of several circulating inflammatory markers including CRP, TNFα, IL-6 and serum amyloid A [146, 147, 171-177].

However, the direct effects of reduced inflammatory activity per se on clinical cardiovascular events are difficult to interpret because of the close pathophysiological interrelationship of inflammation with traditional risk factors and genetic susceptibility. There is evidence for association between circulating inflammatory markers and risk factors like smoking, exercise, abdominal obesity and blood pressure [178]. Aspirin, clopidogrel and statin have all been shown to reduce the risk for cardiovascular events in patients with coronary heart disease, but at the present time, the clinical benefit of the reduced levels of inflammatory markers alone remains unknown. The ultimate test for causality for inflammatory markers would be through randomized trials with long-term selective reduction of inflammatory signal substances [179].

8.2.3 Inflammation in acute myocardial infarction – is it good or bad?

Cardiac repair after AMI is a highly complex process, involving inflammatory components, extracellular matrix remodeling, release of multiple neurohormonal stimuli and adaptive responses of cardiac myocytes themselves. One specific cytokine may exert a wide range of biological effects on various cell types and similar cytokines exert similar and overlapping actions on the same cells. At the same time, the balance between the pro- and anti-inflammatory substances is crucial for the net result.

Characteristic features of the inflammatory signal substances are their functional pleiotropy and redundancy. Induction and release of the pro-inflammatory cytokines like TNFα, IL-1β

and IL-6 are always present in experimental models of AMI. In Paper III we found an increase of the circulating levels of TNFα, IL-6, CRP and IL-10 in the acute phase of AMI in patients treated with PCI. Most of previous knowledge about inflammatory responses in AMI is based on experimental models with somewhat conflicting results underscoring the complexity of the inflammatory signalling in AMI.

Animal models have shown that TNFα deficient mice undergoing infarction exhibit decreased chemokine and adhesion molecule expression and thereby reduced inflammatory response resulting in reduced infarct size [180]. However, studies in mice lacking TNF receptors (TNFR1/TNFR2 double receptor knockout mice) showed significantly higher infarct size and increased myocyte apoptosis compared to wild-type mice [181]. On the contrary, another study using treatment with sTNFR1 expression plasmid DNA that reduced TNFα activity in the myocardium demonstrated inhibition of cardiomyocyte apoptosis [182]. These findings illustrate the pleieotropic effects of TNFα which under certain circumstances may induce apoptosis, but could also promote cell survival. Furthermore, in vitro experiments have shown that TNFα is capable to induce IL-6 expression in canine mononuclear cells, and IL-6 has been shown to mediate ligand-specific adhesion of neutrophils to cardiac myocytes which may induce cytotoxic effects [183]. However, in a mice model with AMI, reduced expression of IL-6 did not affect infarct size, left ventricular function or post-infarction remodelling [184]. In fact, IL-6 null mice have demonstrated delayed cutaneous wound healing, suggesting a significant role for IL-6 in tissue repair [185]. IL-6 is also involved in inducing CRP production by the liver. In paper III we found a peak of IL-6 in the AMI group 1 day after PCI and the highest concentration of CRP was somewhat later, 3 days after PCI. Experimental studies have shown that the CRP response not only reflect tissue damage, but may also contribute to the severity of ischemic myocardial injury [186]. In humans, attempts to reduce the inflammatory response in AMI like the methylprednisolon trial and the phase II anti-CD18 study have been disappointing [187, 188].

The inflammatory mediators are necessary as regulators of cardiac repair in AMI, but they may also mediate unfavourable effects affecting also the non-infarcted myocardium. Clearly a more complete understanding of the specific molecular steps in humans with AMI is needed before specific anti-inflammatory treatment, without interfering with healing and cardiac repair, can be initiated.

8.2.4 Intracoronary injection of mBMC in acute myocardial infarction - the effects on left ventricular function and the role of inflammation.

The effects on left ventricular function

Although cardiomyocyte replication and myocardial regeneration do occur in human hearts, clearly, it is insufficient to repair the injury in AMI [129]. Substantial loss of cardiomyocytes leading to pathological left ventricular remodeling and heart failure may be seen in up to one third of patients with STEMI despite revascularization treatment [189]. Experimental studies have shown that progenitor cells from the bone marrow may improve cardiac function in AMI Therefore, cellular transplantation with autologous stem cells from bone marrow has been suggested to improve cardiac function in patients with AMI treated with PCI. Several smaller early-phase studies have shown the feasibility and safety of intracoronary injection of bone marrow cells in patients with AMI, and also a possible improvement in cardiac function has been reported [136, 137]. Therefore, larger randomized clinical trials were started with enough power to elucidate the effects of intracoronary injection of mBMC on left ventricular function. The BOOST study (Bone Marrow Transfer to Enhance ST-elevation Infarct Regeneration) included 60 patients with STEMI [190]. Half of the patients were randomized to receive intracoronary injection of autologous mBMC and the other half to the control group. After 6 months an improvement in LVEF (MRI) in the BMC group was detected from 50% (baseline) to 56.7% (6 months) compared to the control group where a surprisingly low increase from 51.3% to 52.0% in LVEF was observed. At 18 months follow-up, the between group differences in change of LVEF from baseline was not present [191]. Another study, that was double-blinded, randomized and placebo controlled, included 67 patients with STEMI (33 of the patients received intracoronary injection of mBMC) and did not show any improvement in LVEF (MRI) at 4 months follow-up in favour of the mBMC group [192]. LVEF at baseline was 46.9% in controls and 48.5% in mBMC group, and increased after 4 months to 49.1% and 51.8%, respectively (p=0.36 for treatment effect). A similar result was obtained by another randomized and controlled study from Prague that included 27 patients with large anterior wall AMI, whereof 17 patients received intracoronary injection of autologous mBMC 9 days after the acute PCI [193]. The study was prematurely terminated because of 2 deaths in the mBMC group. An improvement in LVEF (echocardiography) from baseline (39% in both groups) to 4 months could be detected in both groups (47% in controls and 45% in the mBMC group), but without any between group differences. These findings are in accordance with the present ASTAMI study (paper IV) that also showed an increase of

LVEF (SPECT) from baseline (42.6% in controls vs 41.3% in mBMC group) to 6 months (49.3% in both groups), but without differences between the groups. So far, REPAIR-AMI, is the largest clinical trial that has been performed [194]. A total of 204 patients with STEMI were randomized to undergo bone marrow aspiration (n = 101) and subsequent intracoronary injection of autologous mBMC or to a placebo group (n = 103) that underwent the same procedures, but received intracoronary injection of a cell culture medium without mBMC. After 4 months a slight increase of LVEF (angiography) of 2.5 % could be demonstrated in favour of the mBMC group. However, in the control group LVEF increased only from 46.9% at baseline to 49.9% after 4 months, compared to the mBMC group where an increase from 48.3% to 53.8% was noted. The improvement in LVEF in the control group in REPAIR-AMI is strikingly lower as compared to the control groups in Janssens study and the ASTAMI trial (paper IV) and to what would be expected in patients with STEMI treated by PCI [192]. The difference in improvement of LVEF after 4 months between the two groups in REPAIR-AMI could possibly be explained by a poor outcome in the control group. Anyway, an improvement in LVEF of 2.5% is small and would hardly influence on symptoms and survival.

So far, clinical trials have shown disappointing results regarding improvement in left ventricular ejection fraction in patients with acute ST-elevation myocardial infarction treated with intracoronary injection of bone marrow cells [190-194].

The role of inflammation

Several issues like underlying mechanisms for potential beneficial effects of stem cell transplantation, optimal cell type and number, timing after AMI, and delivery methods are not clarified. Cardiac transdifferentiation, paracrine effects, angiogenesis, and reduced apoptosis have been proposed as possible mechanisms for effects of mBMC, but are still to be proven [195]. The optimal timing for delivery of mBMC in AMI is probably dependent on the inflammatory environment. Several steps like attraction, transcapillary migration and invasion, as well as retention and survival in the infarcted area are essential for mBMC to exert their potential beneficial effects. Several critical steps like homing, engraftment, survival, differentiation and paracrine action of the mBMC are partly dependent on the inflammatory environment [138, 196-198].

Increased levels of inflammatory cytokines may reduce the survival of the injected mBMC as suggested by Suzuki et al. who showed improved survival of skeletal muscle precursor cells implanted in mouse hearts treated with anti-IL1β antibody [199, 200]. The delivery method of

mBMC may also be of importance. As discussed in paper V, we found a marked short term pro-inflammatory response the day after intracoronary injection of mBMC that could be induced by the procedure per se. Induction of apoptosis by pro-inflammatory cytokines might reduce the number of stem cells in the infarct area and thus the potential benefit of cell transplantation [201]. Bone marrow aspiration and intracoronary injection of mBMC may be an unfavourable method for treating patient with AMI by stem cells, because of the induction of a pro-inflammatory response that may have deleterious effects on the infarct process and vulnerable plaques in the coronary arteries, as well as reduced survival of the transplanted cells.

The optimal time for intracoronary injection of mBMC remains unknown, but is probably dependent on the balance between facilitating and detrimental cytokines of importance for homing and cell survival. Obviously, we need to go back to the bench and expand our knowledge about the role of mBMC in cardiac repair in patients with AMI.

8 Conclusions

The main conclusions in the present work can be summarized as follows:

- Reduced levels of pro-inflammatory markers were present in patients treated with aspirin 160 mg daily compared to warfarin for up to 4 years after an acute myocardial infarction. However, these findings were not reflected in the incidence of clinical end-points after 4 years. (Paper I)

- In patients with stable coronary heart disease 1 year treatment with aspirin 160 mg daily and clopidogrel 75 mg daily induced similar reduction in the levels of TNFα and MCP-1 (the latter only significant in the aspirin group), possibly by different mechanisms. (Paper II)

- In patients with acute ST-segment elevation myocardial infarction treated with PCI a marked short term increase in circulating levels of IL-6 and CRP as well as of IL-10 compared to similarly treated patients with stable angina pectoris appeared. The PCI procedure per se also induced an increase in IL-6 levels in patients with stable angina pectoris. The myocardial infarction induced a systemic inflammatory reaction that overwhelmed the inflammatory response induced by the PCI procedure. (Paper III).

- No effects on global left ventricular function after 6 months were obtained in patients with acute myocardial infarction treated with intracoronary injection of autologous mBMC 6 days after acute PCI. (Paper IV)

- In AMI patients treated with intracoronary injection of mBMC 6 days after the acute PCI a short-term pro-inflammatory response that may be unfavourable, and a slightly reduced inflammatory response after 3 months that may be beneficial, were noted. (Paper V)

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