The mast cell as a regulator of atherosclerotic plaque stability

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ACADEMIC DISSERTATION

To be presented for public examination with the permission of the Faculty of Biological and Environmental Sciences of the University of Helsinki in the auditorium 1041 at Viikki Biocenter 2, Viikinkaari 5, Helsinki, on 1st October 2010 at 12 noon.

Helsinki 2010
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ISBN 978-952-10-6429-6 (PDF)
http://ethesis.helsinki.fi
Helsinki University Print
Helsinki 2010
To my family
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ORIGINAL PUBLICATIONS
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* equal contribution


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<thead>
<tr>
<th>Abbreviation</th>
<th>Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABCA1</td>
<td>ATP (adenosine-triphosphate)-binding cassette transporter A1</td>
</tr>
<tr>
<td>apoB-100</td>
<td>apolipoprotein B-100</td>
</tr>
<tr>
<td>bFGF</td>
<td>basic fibroblast growth factor</td>
</tr>
<tr>
<td>BMCMC</td>
<td>bone marrow-derived cultured mast cell</td>
</tr>
<tr>
<td>CCL</td>
<td>CC-chemokine ligand</td>
</tr>
<tr>
<td>CETP</td>
<td>cholesteryl ester transfer protein</td>
</tr>
<tr>
<td>CPA</td>
<td>carboxypeptidase A</td>
</tr>
<tr>
<td>CR</td>
<td>complement receptor</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>CTMC</td>
<td>connective tissue mast cell</td>
</tr>
<tr>
<td>CXC1</td>
<td>CXC-chemokine ligand</td>
</tr>
<tr>
<td>CXC2</td>
<td>CXC-chemokine receptor</td>
</tr>
<tr>
<td>FAK</td>
<td>focal adhesion kinase</td>
</tr>
<tr>
<td>FcεR1</td>
<td>Fc epsilon receptor 1; high affinity IgE receptor</td>
</tr>
<tr>
<td>HDL</td>
<td>high-density lipoprotein</td>
</tr>
<tr>
<td>IDL</td>
<td>intermediate-density lipoprotein</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>intercellular adhesion molecule-1</td>
</tr>
<tr>
<td>IgE</td>
<td>immunoglobulin E</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>interferon-gamma</td>
</tr>
<tr>
<td>LDL</td>
<td>low-density lipoprotein</td>
</tr>
<tr>
<td>LRP</td>
<td>LDL receptor-related protein</td>
</tr>
<tr>
<td>MC-CPA</td>
<td>mast cell carboxypeptidase A</td>
</tr>
<tr>
<td>MCP-1</td>
<td>monocyte chemotactic protein-1</td>
</tr>
<tr>
<td>MC_T</td>
<td>human mast cell containing tryptase (T)</td>
</tr>
<tr>
<td>MC_TC</td>
<td>human mast cell containing tryptase (T) and chymase (C)</td>
</tr>
<tr>
<td>MMC</td>
<td>mucosal mast cell</td>
</tr>
<tr>
<td>mMCP</td>
<td>mouse mast cell protease</td>
</tr>
<tr>
<td>MMP</td>
<td>matrix metalloproteinase</td>
</tr>
<tr>
<td>NF-κB</td>
<td>nuclear factor-kappaB</td>
</tr>
<tr>
<td>NGF</td>
<td>nerve growth factor</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>PAI</td>
<td>plasminogen activator inhibitor</td>
</tr>
<tr>
<td>PAF</td>
<td>platelet activating factor</td>
</tr>
<tr>
<td>PAR</td>
<td>protease-activated receptor</td>
</tr>
<tr>
<td>PI3K</td>
<td>phosphatidylinositol 3-kinase</td>
</tr>
<tr>
<td>PLTP</td>
<td>phospholipid transfer protein</td>
</tr>
<tr>
<td>rMCP</td>
<td>rat mast cell protease</td>
</tr>
<tr>
<td>sICAM-1</td>
<td>soluble intercellular adhesion molecule-1</td>
</tr>
<tr>
<td>SR</td>
<td>scavenger receptor</td>
</tr>
<tr>
<td>TGF-β</td>
<td>transforming growth factor-beta</td>
</tr>
<tr>
<td>TIMP</td>
<td>tissue inhibitor of MMPs</td>
</tr>
<tr>
<td>TLR</td>
<td>toll-like receptor</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumor necrosis factor-alpha</td>
</tr>
<tr>
<td>TNFR</td>
<td>TNF receptor</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>vascular cell adhesion molecule-1</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>VLDL</td>
<td>very low-density lipoprotein</td>
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</tbody>
</table>
SUMMARY

Atherosclerosis is an inflammatory disease progressing over years via the accumulation of cholesterol in arterial intima with subsequent formation of atherosclerotic plaques. The stability of a plaque is determined by the size of its cholesterol-rich necrotic lipid core and the thickness of the fibrous cap covering it. The strength and thickness of the cap are maintained by smooth muscle cells and the extracellular matrix produced by them. A plaque with a large lipid core and a thin cap is vulnerable to rupture that may lead to acute atherothrombotic events, such as myocardial infarction and stroke. In addition, endothelial erosion, possibly induced by apoptosis of endothelial cells, may lead to such clinical events. One of the major causes of plaque destabilization is inflammation induced by accumulated and modified lipoproteins, and exacerbated by local aberrant shear stress conditions. Macrophages, T-lymphocytes and mast cells infiltrate particularly into the plaque’s shoulder regions prone to atherothrombotic events, and they are present at the actual sites of plaque rupture and erosion. Two major mechanisms of plaque destabilization induced by inflammation are extracellular matrix remodeling and apoptosis.

Mast cells are bone marrow-derived inflammatory cells that as progenitors upon chemotactic stimuli infiltrate the target tissues, such as the arterial wall, differentiate in the target tissues and mediate their effects via the release of various mediators, typically in a process called degranulation. The released preformed mast cell granules contain proteases such as tryptase, chymase and cathepsin G bound to heparin and chondroitin sulfate proteoglycans. In addition, various soluble mediators such as histamine and TNF-α are released. Mast cells also synthesize many mediators such as cytokines and lipid mediators upon activation. Mast cells are capable of increasing the level of LDL cholesterol in the arterial intima by increasing accumulation and retention of LDL and by decreasing removal of cholesterol by HDL in vitro. In addition, by secreting proinflammatory mediators and proteases, mast cells may induce plaque destabilization by inducing apoptosis of smooth muscle and endothelial cells. Also in vivo data from apoE−/− and ldlr−/− mice suggest a role for mast cells in the progression of atherosclerosis. Furthermore, mast cell-deficient mice have become powerful tools to study the effects of mast cells in vivo.

In this study, evidence suggesting a role for mast cells in the regulation of plaque stability is presented. In a mouse model genetically susceptible to atherosclerosis, mast cell deficiency (ldlr−/−/KitW-sh/W-sh mice) was associated with a less atherogenic lipid profile, a decreased level of lipid accumulation in the aortic arterial wall and a decreased level of vascular inflammation as compared to mast-cell competent littermates. In vitro, mast cell chymase-induced smooth muscle cell apoptosis was mediated by inhibition of NF-κB activity, followed by downregulation of bcl-2, release of cytochrome c, and activation of caspase-8, -9 and -3. Mast cell-induced endothelial cell apoptosis was mediated by chymase and TNF-α, and involved chymase-mediated degradation of fibronectin and vitronectin, and inactivation of FAK- and Akt-mediated survival signaling. Subsequently, mast cells induced inhibition of NF-κB activity and activation of caspase-8 and -9. In addition, possible mast cell protease-mediated mechanisms of endothelial erosion may include degradation of fibronectin and VE-cadherin. Thus, the present results suggest a role for mast cells in destabilization of atherosclerotic plaques.
REVIEW OF THE LITERATURE

1. Atherosclerosis

Atherosclerosis is an inflammatory disease progressing over years via accumulation of cholesterol in arterial intima with subsequent formation of atherosclerotic plaques. These typically develop at certain predilection sites in large- and medium-sized arteries (Fuster et al., 2005). The disease is silent for decades, and complications typically arise when the plaque has grown to an advanced level (Stary et al., 1995) and shows signs of vulnerability (Naghavi et al., 2003a). Typical complications of atherosclerosis, due to atherothrombosis, are acute coronary syndromes such as myocardial infarction, unstable angina and sudden cardiac death (Virmani et al., 2006), as well as stroke (Badimon and Vilahur, 2007). Atherosclerosis is the leading cause of death worldwide causing 19 million deaths annually, and it is the major cause of coronary artery disease (Halvorsen et al., 2008; Lopez et al., 2006; Naghavi et al., 2003a). In Finland, coronary artery disease accounted for 23% of all deaths during 2008 (Statistics Finland, www.stat.fi, 18.12.2009). Typical risk factors for atherosclerosis include high serum levels of low-density lipoprotein (LDL) and low serum levels of high-density lipoprotein (HDL), hypertension, smoking, diabetes and aging (Kovanen, 2008).

1.1. Pathobiology of the disease

1.1.1. Lesion progression

The site for lesion progression, the arterial intima, is the region of the arterial wall between the vessel lumen and the media (see Figures 1 and 3). The intima is lined with endothelial cells on the luminal side, and an internal elastic lamina, regarded as part of the media, serves as a border between intima and media (Stary et al., 1992). The intima contains a substantial amount of extracellular matrix produced by endothelial and smooth muscle cells, and it can be separated into two layers. The proteoglycan layer on the luminal side contains an abundance of proteoglycans as well as isolated macrophages and smooth muscle cells of both synthetic and contractile phenotypes. The musculoelastic layer adjacent to the media is rich in smooth muscle cells of the contractile phenotype as well as elastic fibers and collagen. The thickness of the intima in normal human arteries, expressed as the intima:media ratio, may vary from about 0.1 to 1.0 or more. As a result of physiological adaptations to changes in mechanical stresses like blood flow and vascular wall tension, all human subjects regardless of their age have thicker segments of the intima. Intimal thickenings may form particularly in areas with reduced wall shear stress and/or increased wall tensile stress. These adaptive intimal thickenings may be focal (eccentric, Figure 1) or more extensive (diffuse), depending on the distribution of the mechanical stresses. In addition, at vascular transitions such as bifurcations, thickening induced by normal structural reorganizations of the arterial wall may overlap or fuse with adaptive intimal thickenings (Stary et al., 1992). Regions of adaptive intimal thickening differ functionally from adjacent thinner regions of vasculature by having increased turnover of endothelial cells and smooth muscle cells as well as increased concentrations of plasma components such as LDL (Schwenke and Carew, 1988). In addition, it has been proposed that these sites may contain extracellular enzymes such as lipases and proteases capable of retaining lipoproteins (Tabas et al., 1993; Virmani et al., 2000; Öörni et al., 2000). These factors make the sites of adaptive intimal thickening prone to atherosclerosis (Stary et al., 1992).
The changes in the arterial wall during the progression of atherosclerosis, as defined by American Heart Association, begin with an increase in the number of intimal macrophages and the appearance of small, isolated groups of macrophages filled with lipid droplets. These cells are called macrophage foam cells, and plaques with these changes are defined as type I lesions (Figure 1) (Stary et al., 1994). Type II lesions contain grossly visible fatty streaks with layers of macrophage foam cells, and a small amount of lipid droplets in smooth muscle cells and extracellular space. These are further subdivided into progression-prone (IIa) and progression-resistant (IIb) types based on the location of the lesions in the arterial tree. Type III lesions, also known as intermediate lesions, contain separate pools of extracellular lipid droplets among the layers of smooth muscle cells in the musculoelastic part of the intima (Stary et al., 1994). Advanced lesions (types IV, V, VI) are associated with structural disorganization, repair and thickening of the intima, as well as deformity of the arterial wall (Stary et al., 1995). These lesions may narrow the arterial lumen and produce clinical manifestations of atherosclerosis. Type IV lesions are characterized by a dense accumulation of extracellular lipid as well as of a well-defined region of the intima, the lipid core. The area of the intima between the lipid core and the lumen contains mainly proteoglycans and macrophage foam cells, and thus is susceptible to fissures. In type V lesions, this area contains thick layers of newly formed fibrous connective tissue, and the plaque is called a fibroatheroma. These lesions may be multilayered with several lipid cores and fibrous tissue between them. Subgroups with calcification (Vb, or type VII) and without lipid core (Vc, or type VIII) have also been defined. Advanced lesions (type IV and V) with disruptions of the lesion surface, hematomas or hemorrhages, and/or thrombotic deposits are defined as type VI lesions (Stary et al., 1995). Type I and II lesions are present already in young children, and type III lesions are typical for young adults in their third decade (Stary et al., 1994). Type I-III lesions may still regress to normal (Stary, 2000). Beginning around the fourth decade of life, type V and VI lesions may be present (Stary et al., 1995). Other classifications have been presented as well, particularly regarding the final steps in the progression of atherosclerosis (Fuster et al., 2005; Virmani et al., 2000). Pathological intimal thickenings and thick cap fibroatheromas may erode, whereas thin cap fibroatheromas predispose the plaque to rupture (Figure 2). In addition, calcified nodules may disrupt a thin fibrous cap and protrude into the
lumen with subsequent luminal thrombus. Furthermore, fibrocalcific plaque may result in significant stenosis without thrombosis (Virmani et al., 2000; Virmani et al., 2006).

1.1.2. Atherothrombosis

Vulnerable atherosclerotic plaque has a high short-term risk to become complicated by thrombosis (Schaar et al., 2004; Thim et al., 2008). Thrombotic complications result from physical disruption of the plaque mainly by erosion of luminal endothelial cells or by rupture of the plaque’s fibrous cap (Figure 1) (Schaar et al., 2004; Virmani et al., 2006). The most frequent cause of arterial thrombosis is plaque rupture, which has been estimated to account for approximately 75% of coronary thrombi that result in myocardial infarction or death (Falk, 2006; Kubo et al., 2007). Plaque erosion is responsible for 20-25% of coronary thrombosis in myocardial infarctions (Arbustini et al., 1999; Virmani et al., 2006). Of thrombi seen in sudden cardiac death, plaque erosion has been estimated to account for 30-35% of cases (Virmani et al., 2006). Of thrombosed carotid plaques leading to ischemic stroke, 90% are caused by plaque rupture and 10% by plaque erosion (Spagnoli et al., 2004). Besides rupture and erosion, atherothrombosis may be induced by calcified nodules (Virmani et al., 2000; Virmani et al., 2006).

Plaque erosion is defined as a loss and/or dysfunction of the luminal endothelial cells, leading to thrombosis (Schaar et al., 2004). Except for the endothelial injury, there are no structural defects or gaps in plaques that are often rich in smooth muscle cells and proteoglycans (Schaar et al., 2004). The plaque types typically prone to erosion are a pathologic intimal thickening or a thick cap fibroatheroma (Figure 2) containing minimal (or absent) lipid pools deep within the plaque and without contact with the thrombus (Farb et al., 1996; Virmani et al., 2000; Virmani et al., 2006). There are usually few if any macrophages and lymphocytes present in plaque erosions, and calcification is rare (Farb et al., 1996). The most typical location for erosion, as well as for rupture, is the proximal left anterior descending artery followed by the right and left circumflex coronaries (Virmani et al., 2006). Plaque erosion is highly correlated with smoking (Burke et al., 1998) and is typically seen in younger patients and in women (Farb et al., 1996). Indeed, plaque erosion accounts for 80% of coronary thrombi occurring in women <50 of age (Burke et al., 1998).

Plaque rupture is a deep injury containing an actual defect or gap in the fibrous cap, thus exposing the thrombogenic lipid core to blood flow, and is the most common cause of coronary thrombosis (Schaar et al., 2004). The plaque type most prone to rupture is a thin cap fibroatheroma that contains large necrotic lipid core covered by a thin fibrous cap, usually <65 μm thick (Figure 2) (Burke et al., 1997; Kolodgie et al., 2001; Virmani et al., 2000). As a marker of active inflammation, the levels of macrophages, T-lymphocytes, and mast cells are all increased (Farb et al., 1996; Kaartinen et al., 1994a; Kovanen et al., 1995; Virmani et al., 2000). In addition, these plaques show loss or scarcity of smooth muscle cells and extracellular matrix such as collagen (Falk et al., 1995; Farb et al., 1996; Virmani et al., 2000). Furthermore, calcification and intraplaque hemorrhage are often present (Burke et al., 1999; Farb et al., 1996; Virmani et al., 2000). Intraplaque hemorrhage may derive from ruptures in the neovessels formed upon neovascularization (Ribatti et al., 2008). For eccentric plaques, the characteristics described above are often concentrated at junctions between the plaque and the adjacent less-diseased vessel wall, the shoulder regions of the plaque (Falk et al., 1995; Richardson et al., 1989). Thin
cap fibroatheromas and ruptures typically occur in the proximal portions of the major coronary arteries, mostly in the left anterior, but also in the left circumflex or the right coronary arteries, and to a lesser extent also in the middle and distal portions of these arteries (Kolodgie et al., 2001; Virmani et al., 2006). The typical risk factors for plaque rupture are high serum levels of total cholesterol, total cholesterol/HDL cholesterol ratio, and high-sensitivity C-reactive protein (CRP) as well as low levels of HDL cholesterol (Burke et al., 1997; Burke et al., 2002; Virmani et al., 2006).

**Figure 2. Characteristics of unstable/vulnerable plaques.** The plaque type prone to erosion is a pathologic intimal thickening or a thick cap fibroatheroma (Virmani et al., 2006) (fibrous cap atheroma (Virmani et al., 2000)), while the plaque type prone to rupture is a thin cap fibroatheroma (Kolodgie et al., 2001; Virmani et al., 2006) (thin fibrous cap atheroma (Virmani et al., 2000)). Data derived from (Halvorsen et al., 2008; Virmani et al., 2000) and references mentioned in the text.

The characteristics of the plaque itself play a major role in predisposing it to atherothrombotic events. Vulnerable plaques include plaques prone to erosion and rupture, but also plaques containing calcified nodules, intraplaque hemorrhage, critical stenosis or non-occlusive thrombus due to erosion or rupture (Naghavi et al., 2003a). Besides active inflammation, a thin fibrous cap with a large lipid core, endothelial denudation with superficial platelet aggregation and plaque fissure, the main criteria for defining vulnerable plaques include a stenosis level of >90% (Naghavi et al., 2003a). The characteristics of vulnerable plaques prone to erosion and rupture are
listed in Figure 2. In terms of plaque rupture, the thickness of the cap and the size of the lipid core below it are critical in determining the stability of the plaque (Thim et al., 2008). Without a core there is no fibrous cap, and, consequently no plaque rupture. When the core and the cap have formed, the thinning of the cap renders the plaque unstable (Thim et al., 2008). Two major intrinsic factors that affect a plaque’s vulnerability, lipid accumulation and inflammation, as well as the mechanisms that are thought to predispose vulnerable plaques to erosion or rupture, matrix remodeling and apoptosis, will be discussed in sections below.

Besides the intrinsic factors of the plaque itself, possible clinical manifestations depend on various systemic factors affecting the susceptibility for thrombus formation. In acute coronary syndromes, proinflammatory and prothrombotic conditions involving prolonged elevation of fibrinogen, CRP and plasminogen activator inhibitor (PAI) levels have been reported (Hoffmeister et al., 1999; Naghavi et al., 2003b). Excess fibrinogen can increase thrombus formation, elevated PAI decreases endogenous fibrinolysis activity, and increased CRP reflects systemic inflammation. These factors, among others, could serve as markers predicting the vulnerability of the patient to clinical complications (Naghavi et al., 2003b). Interestingly, high LDL cholesterol, diabetes and smoking are atherosclerotic risk factors that have been associated with increased blood thrombogenicity and high levels of circulating tissue factor (Fuster et al., 2005; Sambola et al., 2003). Besides factors in the blood, the vulnerability of the patient may also be affected by shear stress and characteristics of the myocardium such as its sensitivity to fatal arrhythmias (Fuster et al., 2005; Naghavi et al., 2003b). Thus, the term ‘vulnerable patient,’ including vulnerable plaque, blood and myocardium, is a more precise term to be used in determining the risk for fatal atherothrombotic events (Fuster et al., 2005; Naghavi et al., 2003a; Naghavi et al., 2003b; Schaar et al., 2004). Furthermore, extrinsic forces acting on vulnerable plaques may serve as triggers for actual erosion or rupture (Falk et al., 1995). These local stresses include mechanical forces induced by blood and pulse pressures such as wall tension, compression, bending and stretching as well as hemodynamic forces (Falk et al., 1995), and they are typically seen at sites of plaque disruption (Cheng et al., 1993).

Thrombus formation is initiated upon exposure to thrombogenic substrates such as subendothelial extracellular matrix, lipids and tissue factor (Badimon and Vilahur, 2007; Libby, 2008), the latter of which is considered to be the major regulator of coagulation, hemostasis and thrombosis (Fuster et al., 2005; Libby, 2008). The outcome of an atherothrombotic event may range from its healing without clinical symptoms to sustained and occlusive thrombus causing death of the patient. Advanced atherosclerotic lesions have been reported to contain thrombi or remnants of thrombi, the size of which may range from microscopic to grossly visible deposits (Stary et al., 1995). Frequently the thrombus may not show any clinical symptoms and may heal by fibrotic response (Fuster et al., 2005; Libby, 2008; Virmani et al., 2000). Plaques exhibiting a multilayering of lipid and necrotic core may reflect previous thrombi that have already healed (Burke et al., 2001; Virmani et al., 2000). Repeated formation of small and healing thrombi over months or years may gradually narrow the lumen of the vessel (Burke et al., 2001; Stary et al., 1995). On the other hand, some thrombi may enlarge and occlude the lumen of a medium-sized artery within hours or days (Stary et al., 1995). Thrombi may also detach and occlude a smaller distal vessel, a phenomenon known as thromboembolism (Bennett et al., 2009).
1.2. Lipid accumulation in atherosclerosis

Cholesterol-rich lipoproteins can cross the endothelium and enter the vascular subendothelial intima (Vasile et al., 1983). Lipoproteins, the carriers of lipids in plasma, are particles that are filled with cholesteryl esters and triglycerides, and covered by phospholipids, cholesterol and apolipoprotein(s), making the particles hydrophilic (Brown and Goldstein, 1986). Lipoproteins are classified based on their density. In the order of increasing density, there are chylomicrons, very low-density lipoproteins (VLDL), intermediate-density lipoproteins (IDL), LDL and HDL (Berg et al., 2007) (see Table 1 and Figure 3 for more details on lipoproteins). Apolipoprotein (apo) B-100-containing particles VLDL, IDL and LDL are considered atherogenic, since they transport lipids, mainly cholesterol, into the arterial intima (Tulenko and Sumner, 2002). Conversely, HDL is regarded atheroprotective as it delivers cholesterol from the intima to the liver (referred as reverse cholesterol transport) (Schmitz and Grandl, 2009). Cholesterol is delivered to peripheral cells mainly by LDL via LDL receptor-mediated endocytosis (Brown and Goldstein, 1986). The importance of LDL receptors is highlighted by familial hypercholesterolemia (FH), a genetic disorder caused by the absence or deficiency of functional LDL receptors (Brown and Goldstein, 1986). Patients that are homozygotes for the defect have LDL cholesterol levels of 20-30 mmol/l in plasma, as compared to the desirable level of < 3 mmol/l (Kovanen, 2008). In these homozygotes, cholesterol accumulates in the arteries and as nodules called xanthomas in other tissues such as skin and tendons, and patients often die of coronary artery disease while still in childhood (Berg et al., 2007).

Table 1. Characteristics of lipoproteins.

<table>
<thead>
<tr>
<th>Lipoprotein</th>
<th>Major core lipids</th>
<th>Major apolipoproteins</th>
<th>Function</th>
<th>Lipid delivery by</th>
<th>Receptors in liver</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chylomicron</td>
<td>TG</td>
<td>B-48, C-II, E*</td>
<td>Dietary lipid transport</td>
<td>LPL**, LPL**</td>
<td>LDLr, LRP1</td>
</tr>
<tr>
<td>VLDL</td>
<td>TG</td>
<td>B-100, C-II, E</td>
<td>Endogenous lipid transport</td>
<td>LPL</td>
<td>-</td>
</tr>
<tr>
<td>IDL</td>
<td>CE, TG</td>
<td>B-100, E</td>
<td>LDL precursor</td>
<td>receptor-mediated</td>
<td>LDLr, VLDLr, LRP1</td>
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<tr>
<td>LDL</td>
<td>CE</td>
<td>B-100</td>
<td>Cholesterol transport*</td>
<td>receptor-mediated</td>
<td>LDLr</td>
</tr>
<tr>
<td>HDL</td>
<td>CE</td>
<td>A, C-II, E</td>
<td>Reverse cholesterol transport##</td>
<td>CETP</td>
<td>SR-B1</td>
</tr>
</tbody>
</table>

*ApoE and apoB-100 serve as ligands for cell-surface lipoprotein receptors such as LDL receptor (LDLr).

**Lipoprotein lipase (LPL) in the capillary endothelium is activated by apoC-II and delivers fatty acids from triglycerides (TG) to muscle and other tissues using fatty acids as fuels. As a result, chylomicrons are converted to chylomicron remnants and VLDL to IDL. 

*Cells outside the liver and intestine obtain cholesterol from the plasma rather than by synthesizing it de novo. 

##HDL delivers cholesteryl esters (CE) to VLDL, IDL and LDL by cholesteryl ester transfer protein (CETP) and finally to the liver by receptor-mediated uptake. LRP, LDL receptor-related protein; VLDLr, VLDL receptor; SR-B1, scavenger receptor B1. Data derived from (Berg et al., 2007; Daniels et al., 2009; Tulenko and Sumner, 2002) and references mentioned in the text.
Figure 3. An overview of lipoprotein metabolism and accumulation of lipids in atherosclerosis. Dietary lipids are transported in chylomicrons to peripheral tissues or adipose tissue where chylomicron triglycerides release fatty acids (FA). Cholesterol-rich chylomicron remnants are delivered to the liver through a receptor-mediated process. Synthesis of triglycerides, cholesterol and apolipoproteins happens mainly in the liver and small intestine. The liver secretes synthesized or excess dietary triglycerides and cholesteryl esters in the form of VLDL that delivers fatty acids from triglycerides to peripheral tissues. Subsequently VLDL is transformed into IDL and LDL. Cholesteryl esters are transported to peripheral cells by LDL via the LDL receptor (LDLr) mediated endocytosis. ApoB-100 serves as a ligand for LDL receptors. HDL delivers cholesteryl esters from peripheral tissues ultimately to the liver to be converted to bile acids for elimination from circulation (Schmitz and Grandl, 2009). Cholesterol in plasma lipoproteins accumulates in the arterial wall. In the arterial wall, the apoB-100-containing lipoproteins VLDL, IDL and LDL enter the intima, are retained by proteoglycans, are modified by oxidation, proteolysis and lipolysis, and are aggregated or fused to form lipid droplets. Modified and aggregated lipoproteins are internalized in macrophages and smooth muscle cells via binding to receptors of the scavenger receptor (SR) family such as SR-A and CD36 (Linares et al., 2006; Rader and Pure, 2005), and the cells are then transformed to foam cells containing cholesteryl esters as cytoplasmic droplets. Besides by SRs, VLDL receptors and LDL receptor-related protein (LRP) may also serve as receptors for internalization of cholesterol in the atherosclerotic arterial wall where they are expressed (Hiltunen et al., 1998; Luoma et al., 1994). LDL receptors, however, are not expressed in atherosclerotic lesions (Luoma et al., 1994). HDL removes cholesterol from the intima to be carried ultimately to liver either directly by SR-B1, or indirectly by VLDL, IDL and LDL after cholesteryl ester transfer protein (CETP) has transferred cholesteryl esters from HDL to these particles. EC, endothelium; ABCA1, ATP (adenosine triphosphate) -binding cassette (ABC) transporter A1. Data derived from (Berg et al., 2007; Daniels et al., 2009; Tulenko and Sumner, 2002) and references mentioned in the text unless otherwise stated.
Review of the literature

In the arterial intima, apoB-100 containing lipoproteins accumulate first in the extracellular space where they are retained by binding to extracellular proteoglycans (Figure 3) (Skålen et al., 2002; Williams and Tabas, 1995). This extends the exposure time of lipoproteins to local agents that modify them by oxidation, proteolysis and lipolysis (Pentikäinen et al., 2000). In addition, the arterial intima contains potent bridging molecules such as lipoprotein lipase that may serve as a bridge between proteoglycans and lipoproteins and thus enhance the retention of lipoproteins and their subsequent modification (Gustafsson and Boren, 2004; Pentikäinen et al., 2002a; Saxena and Goldberg, 1994). Modifications in vitro induce physical changes in the lipoprotein particles such as their aggregation and fusion (Öörni et al., 2000). These larger lipoprotein particles/aggregates resemble extracellular lipid droplets found in atherosclerotic plaques (Guyton and Klemp, 1994). Aggregation and fusion increase the binding of lipoprotein particles to proteoglycans (Pentikäinen et al., 2000), and as larger particles they may be trapped more easily within the tight extracellular matrix and may lose their ability to egress via the arterial wall (Khalil et al., 2004; Öörni and Kovanen, 2006). On the other hand, small LDL particles bind to proteoglycans more strongly than do larger ones (Khalil et al., 2004). Interestingly, areas of acidic pH have been reported in atherosclerotic plaques (Naghavi et al., 2002), and acidic pH may enhance extracellular lipid accumulation by increasing the binding of lipoproteins to proteoglycans and their modifications by acidic enzymes (Öörni and Kovanen, 2006).

Modifications of lipoproteins, such as oxidation of LDL, make them chemotactic for monocytes that then start to accumulate in the intima and differentiate into macrophages (Steinberg, 1997). Modified atherogenic cholesterol ester-rich lipoproteins are bound and internalized in macrophages via scavenger receptors (SRs) such as SR-A and CD36 (Rader and Pure, 2005). In macrophages, cholesterol esters are hydrolyzed into free cholesterol that can either be utilized in the cell, or re-esterified by acyl:cholesterol acyltransferase 1 (ACAT1) (Brown et al., 1980). These cholesterol esters form cytoplasmic lipid droplets with a foamy appearance that is characteristic of the foam cell (Stary et al., 1994). Besides macrophages, smooth muscle cells can also internalize lipoproteins and transform to foam cells, and they have been reported to internalize mainly aggregated LDL via LDL receptor-related protein (LRP) (Llorente-Cortes et al., 2006). Interestingly, macrophages in atherosclerotic lesions seem, by producing fibronectin, cytokines and growth factors, to contribute to the phenotypic modulation, migration and/or proliferation of smooth muscle cells. These are prerequisites for the ability of smooth muscle cells to take up lipids (Takahashi et al., 2002). Both macrophage and smooth muscle cell-derived foam cells tend to die by apoptosis (Akishima et al., 2005; Hegyi et al., 1996) which contributes to the formation of the lipid core (Geng and Libby, 2002). In addition to cholesterol esters stored in foam cells, free cholesterol can accumulate in atherosclerotic lesions as extracellular deposits (Sarig et al., 1995). Cholesterol crystals may derive from extracellularly trapped lipids (Bocan et al., 1986) or from the hydrolysis of cholesterol esters in foam cells (Kellner-Weibel et al., 1999). Interestingly, free cholesterol has been reported in association with plaque instability (Felton et al., 1997), and ruptured plaques often contain large numbers of cholesterol crystals in the lipid-rich core (Virmani et al., 2000).

The efflux of cholesterol from intimal foam cells is promoted by HDL. It is secreted from the intestine and liver as apoA-I with subsequent formation of pre-β-HDL, a nascent discoidal HDL particle containing only a small amount of phospholipids and a single apoA-I (Daniels et al., 2009; Tulenko and Sumner, 2002). Phospholipid transfer protein (PLTP) can generate pre-β-
HDL particles by transferring phospholipids from remnant lipoproteins to the lipid poor apoA-I, thus enhancing the removal of cellular cholesterol and phospholipids by HDL (Huuskonen et al., 2001; Wolfbauer et al., 1999). Cholesterol efflux may proceed via passive diffusion of cholesterol from cell membranes to mature α-HDL in all types of cells (Yancey et al., 2003). Cholesterol efflux from macrophage foam cells into HDL particles is facilitated by interaction with ATP (adenosine triphosphate) -binding cassette (ABC) transporters such as ABCA1 and ABCG1 (Oram and Vaughan, 2006) or SR-B1 (de la Llera-Moya et al., 1999). Cholesterol efflux via ABCA1 into pre-β-HDL particles is followed by their maturation, during which free cholesterol on the surface of pre-β-HDL is esterified by lecithin-cholesterol acyltransferase (LCAT), thus generating spherical α-HDL with a cholesteryl ester-containing core (Daniels et al., 2009; Tulenko and Sumner, 2002). PLTP is able to induce fusion of two α-HDL particles, thereby generating large α-HDL and at the same time releasing precursors for pre-β-HDL (Lusa et al., 1996). From α-HDL, lipids can be removed directly to the liver by selective SR-B1-mediated cholesteryl ester uptake pathway or by uptake of the entire HDL particle (Robichaud et al., 2009; Tulenko and Sumner, 2002). In addition, cholesteryl esters can be transferred from α-HDL to the apoB-100-containing lipoproteins VLDL, IDL and LDL by cholesteryl ester transfer protein (CETP) (Stein and Stein, 2005). These particles may then be taken up by the liver through LDL receptors (Tulenko and Sumner, 2002). Thus, HDL particles help to protect against the formation of foam cells, or even induce their regression (Rader and Pure, 2005). The importance of reverse cholesterol transport is highlighted by the fact that patients with Tangier disease, a genetic disorder characterized by the virtual absence of plasma HDL because of homozygous mutations in the ABCA1 gene, tend to develop premature atherosclerosis (Bodzioch et al., 1999; Brooks-Wilson et al., 1999).

1.3. Inflammation in atherosclerosis

Inflammation has an important role during all stages of the progression of atherosclerosis (Hansson and Libby, 2006; Libby et al., 2010). The initiation of atherosclerosis involves endothelial cell injury, which leads to endothelial dysfunction, a condition characterized by reduced levels of endothelium-derived nitric oxide (NO). Physiological anti-atherogenic functions of NO such as inhibition of cell growth, inhibition of leukocyte adhesion, and inhibition of platelet adherence and aggregation (Cooke and Dzau, 1997; Kubes et al., 1991) are thus reduced. In addition, NO protects endothelial cells from apoptosis (Dimmeler et al., 1997). Endothelial dysfunction may be caused by turbulent blood flow, hypertension, hypercholesterolemia, diabetes, smoking and oxidative stress (Cooke and Dzau, 1997; Karafilou et al., 2008). Besides reduced function of NO, endothelial dysfunction is associated with increased expression of proinflammatory cytokines, cell adhesion molecules such as vascular cell adhesion molecule (VCAM)-1 and intercellular adhesion molecule (ICAM)-1, and chemoattractant cytokines such as monocyte chemotactic protein (MCP)-1 and interleukin (IL)-8 (Karafilou et al., 2008; Tedgui and Mallat, 2006). Adhesion molecules induce adherence of monocytes and T-lymphocytes to the surface of the endothelium and their migration through cell-cell junctions into the intima (Karafilou et al., 2008; Lawson and Wolf, 2009). Transmigration of leukocytes seems to also involve proteases capable of degrading cell-cell junctions and basement membrane proteins (Hermant et al., 2003). The recruitment of inflammatory cells is induced by cytokines produced by smooth muscle cells and macrophages in the arterial wall (Takahashi et al., 2002; Tedgui and Mallat, 2006).
Besides macrophages (Aqel et al., 1984) and T-lymphocytes (Jonasson et al., 1986), atherosclerotic lesions have been reported to contain mast cells (Kaartinen et al., 1994a; Kaartinen et al., 1994b; Pollak, 1957), neutrophils (Naruko et al., 2002), dendritic cells (Bobryshev and Lord, 1998) and natural killer (NK) cells (Jonasson et al., 1986) (Table 2). In addition, a few B-lymphocytes are present but they are more numerous in the adventitia of the lesions (Jonasson et al., 1986; Vanderlaan and Reardon, 2005). Although macrophages remove the proinflammatory modified lipoproteins from the extracellular matrix, and so can be considered to be antiatherogenic, they also contribute to plaque destabilization by the formation of the lipid core by their apoptotic death, and by secretion of matrix metalloproteinases (MMPs) and a variety of inflammatory mediators (Halvorsen et al., 2008; Seimon and Tabas, 2009; Takahashi et al., 2002). Furthermore, inflammation in the plaque is promoted by secondary necrosis of foam cells (Seimon and Tabas, 2009). The role of lymphocytes in atherosclerosis has been highlighted in atherosclerotic apoE-deficient (apoE\(^{-/-}\)) mice crossbred with scid/scid immunodeficient mice lacking T- and B-lymphocytes (Zhou et al., 2000). In these mice, lesion sizes were reduced by 70% compared to immunocompetent apoE\(^{-/-}\) mice, and when they received CD4\(^+\) T helper cells from apoE\(^{-/-}\) mice, their lesions dramatically increased in size. T-lymphocytes are considered to have an important role in plaque destabilization by producing interferon-gamma (IFN-\(\gamma\)) (Halvorsen et al., 2008). Mast cells and their role in atherosclerosis are discussed in later sections. In addition to inflammatory cells, also smooth muscle cells and endothelial cells are capable of secreting inflammatory mediators (Doran et al., 2008; Tedgui and Mallat, 2006). Overall, the effects of inflammation are broad and include activation of proteases, regulation of matrix remodelling and induction of apoptosis.

### Table 2. Inflammatory cells and their suggested functions in atherosclerotic plaque.

<table>
<thead>
<tr>
<th>Cell Type</th>
<th>Main Functions</th>
<th>Main Mediators</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Macrophages</strong></td>
<td>clearance of accumulated/modified lipoproteins</td>
<td>MMPs, cytokines such as TNF, IL-1, reactive oxygen species, NO, tissue factor, myeloperoxidase</td>
</tr>
<tr>
<td></td>
<td>clearance of apoptotic cells</td>
<td></td>
</tr>
<tr>
<td></td>
<td>secretion of inflammatory mediators</td>
<td></td>
</tr>
<tr>
<td></td>
<td>presentation of antigens</td>
<td></td>
</tr>
<tr>
<td><strong>T-lymphocytes</strong></td>
<td>recognition of local antigens</td>
<td>IFN-(\gamma), TNF, CD40L, IL-4,5,10</td>
</tr>
<tr>
<td>(mainly CD4+)</td>
<td>Th1 responses: secretion of proinflammatory mediators</td>
<td></td>
</tr>
<tr>
<td></td>
<td>induction of immunoglobulin production in B-lymphocytes</td>
<td></td>
</tr>
<tr>
<td><strong>Mast cells</strong></td>
<td>secretion of inflammatory mediators</td>
<td>neutral proteases chymase, tryptase, caphepsin G and MC-CPA, histamine, TNF-(\alpha), IL-6, IFN-(\gamma)</td>
</tr>
<tr>
<td></td>
<td>proteolysis of apolipoproteins and pericellular matrix</td>
<td></td>
</tr>
<tr>
<td></td>
<td>apoptosis of smooth muscle cells, endothelial cells and macrophages</td>
<td></td>
</tr>
<tr>
<td></td>
<td>recruitment of other inflammatory cells</td>
<td></td>
</tr>
<tr>
<td><strong>Dendritic cells</strong></td>
<td>antigen presentation</td>
<td>chemokines CCL19, CCL22, type 1 IFNs</td>
</tr>
<tr>
<td><strong>Neutrophils</strong></td>
<td>endocytosis of foreign material</td>
<td>reactive oxygen species, myeloperoxidase, elastase, MMPs</td>
</tr>
<tr>
<td></td>
<td>production of inflammatory mediators</td>
<td></td>
</tr>
<tr>
<td><strong>B-lymphocytes</strong></td>
<td>production of immunoglobulins</td>
<td>immunoglobulins</td>
</tr>
</tbody>
</table>

Order of cells in the table represents their relative abundancies in plaque. TNF-\(\alpha\), tumor necrosis factor-alpha; MC-CPA, mast cell carboxypeptidase A; CCL, CC-chemokine ligand. Data derived from (Hansson and Libby, 2006; Hansson, 2009; Libby et al., 2009; Libby et al., 2010; Weber et al., 2008) and references mentioned in the text.
1.4. Mechanisms of plaque destabilization

1.4.1. Extracellular matrix remodeling
The formation and thickness of the fibrous cap are regulated mainly by smooth muscle cells and extracellular matrix produced by them. Smooth muscle cells respond to various inflammatory cell cytokines and growth factors by proliferation, migration and production of matrix proteins (Halvorsen et al., 2008). Although endothelial cells and macrophages also participate in the production of the extracellular matrix, smooth muscle cells are the major producers of connective tissue both in the healthy and in the atherosclerotic vessel (Falk, 2006). As compared to the extracellular matrix in healthy arteries containing mostly type I and type III fibrillar collagen, atherosclerotic lesions typically contain mostly proteoglycans with scattered type I collagen fibrils and fibronectin (Doran et al., 2008; Ross, 1999). Interestingly, when bound to fibrillar collagen or laminin, smooth muscle cells become arrested in the G1 phase of the cell cycle, whereas their attachment to fibronectin and proteoglycans in atherosclerotic lesions promotes their proliferation (Doran et al., 2008). Increased influx and proliferation of smooth muscle cells stabilizes the plaque, at the same time still narrowing the lumen (Halvorsen et al., 2008). Collagen is the major extracellular matrix protein of the fibrous cap and contributes to the tensile strength of the plaque (Burleigh et al., 1992; Falk et al., 1995; Libby, 2008). Thus, its increased degradation may destabilize the plaque (Libby, 2008). Overall, the degradation of the extracellular matrix may destabilize the plaque, whereas its increased content may be associated with plaque stability (Bäck et al., 2010; Falk et al., 1995). The level of extracellular collagen, as well as other matrix proteins, is regulated by the balance of its synthesis and degradation by proteases (Halvorsen et al., 2008). In vulnerable atherosclerotic plaques, the balance is on the side of matrix degradation (Halvorsen et al., 2008). MMPs are required for the initiation of collagen triple helix degradation (Libby, 2008). In atherosclerotic plaques, the levels of MMP-1, -3, -8, -9, -11 and -14 are increased in the rupture-prone shoulder regions (Newby, 2007), and colocalization of MMP-1 and MMP-13 with cleaved collagen has been reported in vulnerable plaques (Sukhova et al., 1999). MMPs are produced mainly by macrophages, smooth muscle cells and endothelial cells. MMPs are capable of degrading also other extracellular proteins such as elastin, fibronectin, vitronectin, laminin, proteoglycans, as well as other MMPs to induce their activation (Bäck et al., 2010) or cytokines to enhance their bioactivity (Halvorsen et al., 2008). The activity of MMPs is regulated by proteolytic activation of pro-protease and by tissue inhibitors of MMPs (TIMPs) (Newby, 2007). In mice, TIMP-2 has been reported to decrease plaque size and markers of plaque instability in brachiocephalic arteries (Johnson et al., 2006), and TIMP-1 to decrease plaque size and macrophage content at the aortic root (Rouis et al., 1999), suggesting that TIMPs may stabilize the plaque (Newby, 2007). The net effect of MMPs, with some increasing and some decreasing the vulnerability of the plaque (Johnson et al., 2005), may depend on the spectrum of active MMPs and the stage of the plaque (Newby, 2007).

Besides MMPs, other proteases are also present in atherosclerotic lesions. Cathepsin cysteine proteases are produced by macrophages, smooth muscle cells and endothelial cells, and are differentially expressed during the progression of atherosclerosis (Lutgens et al., 2007). Elastolytic cathepsins S and K have been reported in advanced atherosclerotic lesions where they localize mainly in macrophages and the smooth muscle cells of the fibrous cap (Sukhova et al., 1998). They are strong elastases at neutral pH, and cathepsin K is also a collagenase at acidic pH (Lutgens et al., 2007). Deficiency of cathepsin S and K in atherosclerotic mouse models has been
associated with reduced plaque size, although in cathepsin K-deficient mice only advanced atherosclerotic plaques were reduced in size (Lutgens et al., 2006; Rodgers et al., 2006; Sukhova et al., 2003). In addition, the cathepsin S- and K-deficient mice showed decreases in elastin breaks or content, and, in cathepsin S-deficient mice, decreases in the numbers of plaque ruptures as well. These data suggest destabilizing roles for cathepsin S and K in advanced atherosclerotic lesions. Furthermore, the level of cystatin C, the natural inhibitor of cathepsins, is decreased in atherosclerotic plaques (Shi et al., 1999). Atherosclerotic plaques show also enhanced expression of ‘a disintegrin and metalloproteinases’ (ADAMs) and plasmin-generating urokinase and tissue plasminogen activators (uPA and tPA) of the fibrinolytic system, the action of which may result in degradation of extracellular matrix proteins such as fibronectin (Garcia-Touhard et al., 2005; Oksala et al., 2009; Toriseva and Kähäri, 2009). Cytotoxic T-lymphocytes and natural killer (NK) cells may also have a role in extracellular matrix remodeling by the production and secretion of granzymes that have been reported to degrade type IV collagen, laminin, fibronectin and proteoglycans (Edwards et al., 1999). T-lymphocytes may also induce MMP expression in smooth muscle cells by a CD40-mediated mechanism (Schönbeck et al., 1997). Furthermore, neutrophils have been detected at sites of plaque erosion and rupture (Naruko et al., 2002), and they are capable of releasing neutrophil elastase, neutrophil proteinase 3 (PR3), and granzymes that all have the ability to degrade extracellular matrix proteins (Wagner et al., 2004; Yang et al., 1996). Interestingly, macrophages may also produce neutrophil elastase in atherosclerotic plaques (Dolley et al., 2003). Thus, the destabilizing role of inflammation may be at least partly explained by matrix degradation induced by proteases derived from inflammatory cells. The possible role of mast cell-derived proteases will be discussed in section 3. Studies showing how manipulation of proteases capable of remodeling extracellular matrix may affect atherosclerotic plaque stability in mouse models of atherosclerosis are listed in table 4.

1.4.2. Apoptosis

Inflammation may also have a central role in regulating the level of cell death in atherosclerotic lesions. Indeed, apoptotic cells have been detected at sites of inflammation in atherosclerotic lesions (Björkerud and Björkerud, 1996; Crisby et al., 1997; Kockx et al., 1998). Besides apoptosis, cell death may also proceed via necrosis, autophagy or pyroptosis (Duprez et al., 2009). Apoptosis is an intrinsic programmed suicide of the cell characterized by its controlled breakdown into apoptotic bodies that are then recognized and engulfed by phagocytes and surrounding cells. Necrosis proceeds via cytoplasmic and organelle swelling, which eventually releases cellular contents into the extracellular space (Duprez et al., 2009). Secondary necrosis can result when the clearance of apoptotic bodies is disturbed (Seimon and Tabas, 2009). Cell death may also result from autophagy although autophagy can be accompanied by other forms of death (Duprez et al., 2009). Interestingly, there seems to be cross-talk between apoptotic and autophagic mechanisms, allowing fine tuning of the fate of a cell in different cellular settings (Eisenberg-Lerner et al., 2009). Pyroptosis has been reported in infected monocytes, macrophages and dendritic cells, and leads to activation of inflammatory caspase-1 with subsequent pyroptotic cell death usually accompanied by release of proinflammatory cytokines IL-1β and IL-18 (Duprez et al., 2009).

Apoptotic cell death is characterized by the activation of cysteinyl aspartate-specific proteases, known as caspases, that are responsible for the degradation of cellular substrates such as actin and nuclear lamin proteins, and thus for the execution of apoptosis (Duprez et al., 2009; Pop and
Salvesen, 2009). Caspases are classically activated by intrinsic or extrinsic apoptotic pathways, although perforin/granzyme pathway and caspase-2 activation by PIDDosome have also been reported (Elmore, 2007; Pop and Salvesen, 2009). Intrinsic or mitochondrial pathways are activated by various developmental cues and cytotoxic insults such as DNA damage, and mitochondria and Bcl-2 family proteins have central role in these pathways (Youle and Strasser, 2008). In normal conditions, mitochondrial integrity is maintained by anti-apoptotic Bcl-2 protein family members, such as Bcl-2 and Bcl-XL, which inhibit pro-apoptotic Bcl-2 family members Bax and Bak (Youle and Strasser, 2008). During cellular stress, activation of Bcl-2-homology 3 (BH3)-only proteins such as Bim, Bid and Bad, leads to their ligation with anti-apoptotic Bcl-2 proteins, and subsequent promotion of apoptosis via relief of the pro-apoptotic Bax and Bak inhibition (Duprez et al., 2009; Youle and Strasser, 2008). Bax and Bak then induce the release of cytochrome c and other proteins from mitochondria into the cytosol (Youle and Strasser, 2008). Cytochrome c associates with Apaf-1 with subsequent formation of apoptosomes that activate initiator caspase-9 (Riedl and Salvesen, 2007). The activated caspase then cleaves and activates effector caspases-3, -6 and -7 (Duprez et al., 2009). Extrinsic or death-receptor pathways are activated by the ligation of death receptors such as tumor necrosis factor (TNF) receptor 1 (TNFR1) and Fas (Duprez et al., 2009; Youle and Strasser, 2008). Induction of apoptosis by this pathway proceeding through Fas (CD95), involves formation of death-inducing signaling complex (DISC), and results, by Fas-associated death domain (FADD), in the recruitment and activation of initiator caspase-8 and/or -10 (Wilson et al., 2009). TNF stimulation via TNFR1 proceeds, via TNFR associated death domain (TRADD), to activation of nuclear factor-kappaB (NF-κB) and subsequent transcription of anti-apoptotic genes (Duprez et al., 2009; Wilson et al., 2009). In addition, endocytosis of TNFR1 leads to formation of a complex that activates caspase-8 and -10 (Duprez et al., 2009). Caspase-8 and -10 then activate downstream effector caspases (Duprez et al., 2009). In addition, caspase-8-mediated cleavage of pro-apoptotic BH3-only protein Bid induces translocation of this protein to mitochondria and amplification of apoptosis by activation of the mitochondrial pathway (Duprez et al., 2009; Youle and Strasser, 2008).

The level of apoptosis in a normal vessel wall containing laminar shear stress is very low (Dimmeliner et al., 1996; Lutgens et al., 1999). In atherosclerotic lesions, the amount of potent inducers of apoptosis increases, including modified lipoproteins such as oxidized LDL (Hardwick et al., 1996; Li et al., 1998a; Nishio et al., 1996), a broad repertoire of various inflammatory mediators (Geng and Libby, 2002) and reactive oxygen species (Dimmeliner and Zeiher, 2000; Napoli et al., 2001), as well as altered mechanical and hemodynamic forces such as turbulent blood flow (Tricot et al., 2000). As a result, the overall level of apoptosis in advanced atherosclerotic lesions is increased (Björkerud and Björkerud, 1996; Geng and Libby, 1995; Han et al., 1995; Isner et al., 1995; Lutgens et al., 1999). In advanced lesions, macrophage apoptosis and secondary necrosis are considered to account for the formation of the necrotic acellular lipid core of the plaque, increasing the plaque vulnerability (Seimon and Tabas, 2009; Thim et al., 2008). In addition, apoptotic macrophages have been reported to colocalize with sites of plaque rupture (Kolodgie et al., 2000). These sites also exhibit reduced smooth muscle cell content suggesting that apoptosis of smooth muscle cells may promote plaque rupture (Bennett, 1999; Geng and Libby, 1995). Indeed, genetically induced increased smooth muscle cell apoptosis has been reported to associate with thinning of the fibrous cap, decreased matrix content and increased necrotic core volume (Clarke et al., 2006). Endothelial cell apoptosis is one possible cause of plaque erosion (Durand et al., 2004), and indeed, circulating endothelial cell-derived
apoptotic microparticles have been reported in patients with unstable angina or myocardial infarction (Mallat et al., 2000). Endothelial cell apoptosis has been reported in oxidative stress (Dimmeler and Zeiher, 2000), in lack of hemodynamic forces (Kaiser et al., 1997) and induced by various inflammatory mediators (Karaflou et al., 2008). Loss of cell-matrix interactions may induce apoptosis in endothelial and smooth muscle cells (Michel, 2003), a phenomenon known as anoikis (Frisch and Francis, 1994). Degradation of the pericellular matrix, close to cell membrane-associated adhesive glycoproteins such as fibronectin, vitronectin and laminin (Bosman and Stamenkovic, 2003; Ekmekci and Ekmekci, 2006), may induce changes in intracellular signaling via cell membrane protein integrins (Stupack and Cheresh, 2002). For the survival of anchorage-dependent cells, integrin-mediated activation of focal adhesion kinase (FAK) and its downstream signaling pathways is critical (Stupack and Cheresh, 2002). FAK has been reported to regulate anti-apoptotic Bcl-2 family members, as well as caspase inhibitors of the inhibitor of apoptosis (IAP) family via phosphatidylinositol 3-kinase (PI3K)/Akt-mediated activation of the NF-κB pathway (Matter and Ruoslahti, 2001; Sonoda et al., 2000). Thus, disruption of FAK signaling pathways leads to apoptosis of the cell. As described above, many inflammatory cells present in atherosclerotic lesions may secrete proteases capable of degrading pericellular matrix proteins, and thus have the potential to induce apoptosis of vascular resident cells by the process of anoikis. The possible role of mast cells in this process will be discussed in section 3.

1.5. Mouse models of atherosclerosis

Most widely used atherosclerotic animal models are genetically manipulated apoE- and LDL receptor-deficient, apoE−/− (Piedrahita et al., 1992; Plump et al., 1992) and ldlr−/− (Ishibashi et al., 1993) mice fed a Western-type diet (Table 3). Wild-type mice are quite resistant to the development of atherosclerosis since their lipoprotein profile exhibits a high level of HDL and low levels of LDL and VLDL (Zadelaar et al., 2007; Zhang et al., 1992a). When compared to human normal intima containing smooth muscle cells and connective tissue (Stary et al., 1992), mouse normal intima is very thin and contains only endothelium and scattered dendritic cells (Bentzon and Falk, 2010). Even when fed with pro-atherogenic diet (15% fat, 1.25% cholesterol, and 0.5% cholic acid), wild-type mice develop only very simple and small lesions (Paigen et al., 1985). Since apoE serves as a ligand for cell-surface lipoprotein receptors, its targeted homozygous deletion leads to severe hypercholesterolemia with highly increased levels of LDL and VLDL in plasma (Zadelaar et al., 2007; Zhang et al., 1992a). ApoE−/− mice develop atherosclerosis spontaneously even on a standard chow diet (Zhang et al., 1992a), with plaques ranging from fatty streaks to advanced lesions that show similarity to human plaques (Nakashima et al., 1994). The progression of atherosclerosis in apoE−/− mice can be strongly accelerated by a Western-type diet, such as one containing 21% fat and 0.15% cholesterol but without cholate, that elevates plasma cholesterol from about 10 mmol/l to the level of >40 mmol/l (Plump et al., 1992). On a high cholesterol diet, such as one containing 15% fat and 1.25% cholesterol, i.e. an approximately 8-fold higher content of cholesterol, even levels of about 70 mmol/l can be seen (Scalia et al., 2001; Zadelaar et al., 2007). Disadvantages of the apoE−/− model are cholesterol accumulation in VLDL instead of LDL as in humans (Zhang et al., 1992a), the ability of apoE to modulate immune activation such as inhibition of T-cell proliferation (Tenger and Zhou, 2003) and the antiatherogenic properties of apoE as an antioxidant or antiproliferative, antiinflammatory, antiplatelet and NO-generating factor (Davignon, 2005). Ldlr−/− mice lack the gene for LDL receptors, and, on a chow diet, have modestly elevated plasma cholesterol with
only slow development of atherosclerosis (Ishibashi et al., 1993; Ishibashi et al., 1994). On a Western-type diet, such as one containing 10% coconut oil and 0.2% cholesterol, plasma cholesterol elevates to the level of >10 mmol/l, whereas on a high-fat diet that includes cholate, such as one containing 7.5% cocoa butter, 1.25% cholesterol and 0.5% sodium cholate, plasma cholesterol levels can increase to >25 mmol/l (Ishibashi et al., 1993; Ishibashi et al., 1994; Wouters et al., 2005; Zadelaar et al., 2007). Thus, on a diet containing fat and cholesterol atherosclerosis develops rapidly in ldlr-/− mice (Ishibashi et al., 1994). The major advantage of ldlr-/− mice is that their lipoprotein profile resembles that of humans, that is, plasma cholesterol is confined mainly to LDL (Ishibashi et al., 1993; Zadelaar et al., 2007). Morphology of the lesions in ldlr-/− mice is comparable to that in apoE-/− mice. Furthermore, when ldlr-/− mice are combined with an apoB-editing deficiency (ldlr-/−/apoBEC-/− mice) (Powell-Braxton et al., 1998) or with human apoB-100 transgenic mice (ldlr-/−/apoB+/+) (Sanan et al., 1998), a large increase in plasma LDL cholesterol and development of atherosclerosis on a low-fat diet are seen (Zadelaar et al., 2007).

**Table 3. Comparison of wild-type, apoE-/− and ldlr-/− mouse models of atherosclerosis.**

<table>
<thead>
<tr>
<th>Mouse</th>
<th>Serum cholesterol in lipoproteins</th>
<th>Serum total cholesterol</th>
<th>Susceptibility to atherosclerosis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>HDL</td>
<td>LDL</td>
<td>VLDL</td>
</tr>
<tr>
<td></td>
<td>↑↑</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td></td>
<td>on chow diet</td>
<td>on Western diet</td>
<td></td>
</tr>
</tbody>
</table>

Arrows indicate relative amounts of lipoproteins, ↑↑↑ high, ↑↑ and ↑ moderate, ↓ low. Data derived from (Ishibashi et al., 1993; Plump et al., 1992) and references mentioned in the text.

An ideal animal model comparable to human plaque rupture has yet to be developed (Ni et al., 2009). In mice, advanced atherosclerotic lesions and plaque rupture have been reported in the brachiocephalic artery (Johnson et al., 2005; Johnson and Jackson, 2001; Rosenfeld et al., 2000), and also in the aortoiliac bifurcation and the left and right common carotid bifurcations (Jackson et al., 2007). Nevertheless, plaque rupture and superimposed thrombosis are only very rarely seen in mice (Bentzon and Falk, 2010; Schwartz et al., 2007). For vulnerable plaques in mice, similar histological markers as for humans may be used (Ni et al., 2009). These include a large lipid core, a thin fibrous cap, increased macrophages and inflammatory mediators and decreased extracellular matrix (Ni et al., 2009). In mice, however, the fibrinolytic function seems to be more pronounced compared to humans, and thus the presence of a luminal thrombosis should not be regarded as a defining characteristic of plaque rupture (Jackson et al., 2007; Ni et al., 2009). Actually, luminal thrombus is not included in the definition for human plaque rupture either (Schaar et al., 2004) even though it is the result of plaque rupture. Instead, buried caps, as a suggestive of remnants of previous ruptured caps, have been reported in mice at sites where plaque rupture occurred (Johnson et al., 2005), and may be used as an indicator of plaque rupture.
Table 4. Studies showing how manipulation of proteases capable of remodeling extracellular matrix may affect atherosclerotic plaque stability in mouse models of atherosclerosis.

<table>
<thead>
<tr>
<th>The factor studied / treatment</th>
<th>Genetic background</th>
<th>Diet (cholesterol %)</th>
<th>Time</th>
<th>Artery studied</th>
<th>Characteristics measured*</th>
<th>Effect on plaque stability</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMPs</td>
<td>apoE−/−</td>
<td>high-fat diet (0.21%)</td>
<td></td>
<td>proximal aorta</td>
<td>at 16 weeks: plaque area↓; at 10 weeks: collagen↓</td>
<td>↓</td>
<td>(Lemaitre et al., 2001)</td>
</tr>
<tr>
<td>Human macrophage MMP-1 expression (transgenic)</td>
<td>apoE−/−</td>
<td>high-fat diet (0.21%)</td>
<td>10/16/25 weeks</td>
<td>proximal aorta</td>
<td>plaque area↓, SMC↓; in aortic arch: collagen↑</td>
<td>↑↓</td>
<td>(Kuzuya et al., 2006)</td>
</tr>
<tr>
<td>MMP-2 deficiency</td>
<td>apoE−/−</td>
<td>Western diet (0.15%)</td>
<td>8 weeks</td>
<td>aortic root and arch</td>
<td>plaque area↑, buried fibrous layers↑, SMC↓</td>
<td>↓</td>
<td>(Johnson et al., 2005)</td>
</tr>
<tr>
<td>MMP-3 deficiency</td>
<td>apoE−/−</td>
<td>high-fat diet (0.15%)</td>
<td>8 weeks</td>
<td>BCA</td>
<td>plaque area↑, buried fibrous layers↑, SMC↓</td>
<td>↓</td>
<td>(Johnson et al., 2005)</td>
</tr>
<tr>
<td>MMP-7 deficiency</td>
<td>apoE−/−</td>
<td>high-fat diet (0.15%)</td>
<td>8 weeks</td>
<td>BCA</td>
<td>SMC↑</td>
<td>↑</td>
<td>(Johnson et al., 2005)</td>
</tr>
<tr>
<td>MMP-8 deficiency</td>
<td>apoE−/−</td>
<td>Western diet (1.25%)</td>
<td>12 weeks</td>
<td>aortic root</td>
<td>plaque area↑, MØ↑, collagen↑, VCAM-1 expression↑, leukocyte recruitment↓</td>
<td>↑↑</td>
<td>(Laxton et al., 2009)</td>
</tr>
<tr>
<td>MPP-9 deficiency</td>
<td>apoE−/−</td>
<td>atherogenic diet (1.25%) with cholate</td>
<td>15/25 weeks</td>
<td>descending aorta</td>
<td>at 15 weeks: plaque area↓; at 25 weeks: plaque area↑, collagen↑, MØ↑, elastin degradation↓</td>
<td>↓↑</td>
<td>(Luttun et al., 2004)</td>
</tr>
<tr>
<td>MMP-9 deficiency</td>
<td>apoE−/−</td>
<td>high-fat diet (0.15%)</td>
<td>4 weeks (3 weeks after ligation)</td>
<td>carotid artery</td>
<td>plaque area↑, foam cell area↑, MØ↑, SMC↑, collagen↓</td>
<td>↓↑</td>
<td>(Choi et al., 2005)</td>
</tr>
<tr>
<td>MMP-9 deficiency</td>
<td>apoE−/−</td>
<td>high-fat diet (0.15%)</td>
<td>8 weeks</td>
<td>BCA</td>
<td>plaque area↑, buried fibrous layers↑, SMC↑, MØ↑</td>
<td>↓</td>
<td>(Johnson et al., 2005)</td>
</tr>
<tr>
<td>Retroviral overexpression of active MMP-9</td>
<td>apoE−/−</td>
<td>normal chow</td>
<td>treatment at 41 weeks, for 11-12 weeks</td>
<td>BCA, aortic arch</td>
<td>fibrous cap disruption↑, intraplaque hemorrhage↑, fibrin deposition↑, fibrous cap breaks↑↑***</td>
<td>↓</td>
<td>(Gough et al., 2006)</td>
</tr>
<tr>
<td>MMP-9 overexpression (adenovirus in collar)</td>
<td>apoE−/−</td>
<td>high-fat diet (0.25%)</td>
<td>4/6 weeks</td>
<td>carotid artery</td>
<td>cap thickness↓; at 4 weeks: intima:lumen ratio↑, media area↑; at 6 weeks: intraplaque hemorrhage↑</td>
<td>↓</td>
<td>(de Nooijer et al., 2006)</td>
</tr>
<tr>
<td>Human proMMP-9 expression in macrophages</td>
<td>apoE−/−, apoE−/−, Timp−/−</td>
<td>Western diet (0.21%)</td>
<td>16 weeks</td>
<td>aortic root</td>
<td>collagen↑ in apoE−/−, Timp−/− background</td>
<td>↑</td>
<td>(Lemaitre et al., 2009)</td>
</tr>
<tr>
<td>MMP-12 deficiency</td>
<td>apoE−/−</td>
<td>high-fat diet (0.15%)</td>
<td>8 weeks</td>
<td>BCA</td>
<td>plaque area↑, buried fibrous layers↑, SMC↑, MØ↑</td>
<td>↑</td>
<td>(Johnson et al., 2005)</td>
</tr>
</tbody>
</table>
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<table>
<thead>
<tr>
<th>The factor studied / treatment</th>
<th>Genetic background</th>
<th>Diet (cholesterol %)</th>
<th>Time</th>
<th>Artery studied</th>
<th>Characteristics measured*</th>
<th>Effect on plaque stability</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-13 deficiency</td>
<td>apoE&lt;−/−</td>
<td>atherogenic diet (1.25%)</td>
<td>diet for 5/10 weeks</td>
<td>aortic root</td>
<td>at 10 weeks: collagen↑, collagen structure organization↑, thin collagen fibers in the cap region↓</td>
<td>↑</td>
<td>(Deguchi et al., 2005)</td>
</tr>
<tr>
<td>Bone marrow MMP-14 deficiency</td>
<td>ldlr&lt;−/−</td>
<td>high-cholesterol diet (1.25%)</td>
<td>diet for 8/16 weeks</td>
<td>aortic root</td>
<td>at 16 weeks: collagen↑</td>
<td>↑</td>
<td>(Schneider et al., 2008)</td>
</tr>
<tr>
<td>Cathepsins</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Cathepsin S deficiency</td>
<td>ldlr&lt;−/−</td>
<td>atherogenic diet (1.37%)</td>
<td>diet for 12/26 weeks</td>
<td>aortic arch</td>
<td>plaque area↓, elastin breaks↓, SMC↓, MØ↑, T-cells↓; at 12 weeks: collagen↓, at 26 weeks: fibrous cap thickness↓</td>
<td>↑↓</td>
<td>(Sukhova et al., 2003)</td>
</tr>
<tr>
<td>Cathepsin S deficiency</td>
<td>apoE&lt;−/−</td>
<td>high-fat diet (0.15%)</td>
<td>diet for 12 weeks</td>
<td>BCA</td>
<td>plaque rupture↓, buried fibrous caps↓, plaque area↓, fibrous cap thickness↑</td>
<td>↑</td>
<td>(Rodgers et al., 2006)</td>
</tr>
<tr>
<td>Leukocyte cathepsin S deficiency</td>
<td>ldlr&lt;−/−</td>
<td>high-fat diet (0.25%)</td>
<td>diet for 12 weeks</td>
<td>aortic root</td>
<td>MØ↑, SMC↓, collagen↓, necrotic area↓, apoptosis↓, elastic lamina rupture↓</td>
<td>↓</td>
<td>(de Nooijer et al., 2009)</td>
</tr>
<tr>
<td>Cathepsin K deficiency</td>
<td>apoE&lt;−/−</td>
<td>normal chow</td>
<td>26 weeks</td>
<td>aortic arch with branch points</td>
<td>plaque area↑, number of advanced plaques↓, number of elastin breaks↓, MØ foam cell size↑; in advanced plaques: collagen↑</td>
<td>↑</td>
<td>(Lutgens et al., 2006)</td>
</tr>
<tr>
<td>Cathepsin K deficiency</td>
<td>apoE&lt;−/−</td>
<td>high fat diet (1%) with cholate</td>
<td>diet for 8/16 weeks</td>
<td>BCA</td>
<td>apoptosis↑, elastin breaks↓; at 8 weeks: collagen↑, cap thickness↑, buried caps↑, MØ↑; at 16 weeks: MØ↓</td>
<td>↑↓</td>
<td>(Samokhin et al., 2008)</td>
</tr>
<tr>
<td>Leukocyte cathepsin K deficiency</td>
<td>ldlr&lt;−/−</td>
<td>Western diet (0.25%)</td>
<td>diet for 12 weeks</td>
<td>aortic root</td>
<td>necrotic area↑, MØ↑, collagen↓, apoptosis↑, elastin fragmentation↓</td>
<td>↓</td>
<td>(Guo et al., 2009a)</td>
</tr>
<tr>
<td>Cathepsin L deficiency</td>
<td>ldlr&lt;−/−</td>
<td>Western diet (0.15%)</td>
<td>diet for 12/26 weeks</td>
<td>aortic arch</td>
<td>plaque area↑, collagen↑, elastin degradation↓; at 12 weeks: SMC↓, MØ↓, T cells↓, serum LDL and TG↓; at 26 weeks: core area↓</td>
<td>↓↑</td>
<td>(Kitamoto et al., 2007)</td>
</tr>
</tbody>
</table>

*Only characteristics measured showing effects (P<0.05) in the plaque are listed. **direction of an arrow is based on assumptions that plaque stability increases when SMC↑, collagen↑, inflammation↓. ***no P-values reported. BCA, brachiocephalic artery; SMC, smooth muscle cells; MØ, macrophages. In addition, evidence suggesting the involvement of mast cell chymase in atherosclerotic plaque stability in vivo has been reported in a hamster model of atherosclerosis (Guo et al., 2009b). At least when connected with cap disruption (Ni et al., 2009). Studies showing how manipulation of proteases capable of remodeling extracellular matrix may affect atherosclerotic plaque stability in mouse models of atherosclerosis are listed in Table 4.

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2. Mast cells

Mast cells are inflammatory cells participating in immediate host defence. As porters at the interfaces of the body, such as skin, airways, gastrointestinal tract and vasculature, mast cells are able to function against foreign invaders very rapidly, i.e. within minutes (Heib et al., 2008). This property originates from liberation of cytoplasmic granules containing preformed mediators and gives mast cells their unique ability in innate immunity. Although they are best known for their actions in allergic reactions, mast cells have diverse roles in host defence and tissue homeostasis (Rao and Brown, 2008). The various roles of mast cells immunity, mainly in innate immunity and some also in adaptive immunity, include amplification of immunological reaction by activation of mast cells with other immunological molecules such as complement proteins, and by recruitment of other cells such as neutrophils (Benoist and Mathis, 2002; Heib et al., 2008). Furthermore, mast cells play a role in different pathophysiological conditions, such as asthma, autoimmune diseases and atherosclerosis.

2.1. Mast cell origin and distribution

Mast cells originate from hematopoietic stem cells in the bone marrow (Kirshenbaum et al., 1991; Kitamura et al., 1977), where their differentiation begins. Multipotent stem cells become mast cell-committed progenitors expressing CD34, c-kit and CD13 but not Fc epsilon receptor I (FcεRI) (Jamur et al., 2005). These progenitors enter the circulation (Kirshenbaum et al., 1999; Rottem et al., 1994) and migrate to their residential peripheral tissue locations where they complete their differentiation and maturation. Integrin β7 has been detected in mast cell-commited progenitors in the bone marrow (Chen et al., 2005), and, as a heterodimer α4β7 on the mast cell membrane, it plays a role in homing of mast cell progenitors to the small intestine (Gurish et al., 2001). In contrast, homing to the lungs involves both α4β1 and α4β7 integrins (Abonia et al., 2006) implying that there are specific molecular interactions for specific tissue locations. In the endothelium of the target tissue, mast cell-committed progenitors interact with VCAM-1 (Abonia et al., 2006), mucosal addressin cellular adhesion molecule 1 (MAdCAM-1) (Gurish et al., 2001), and/or E-selectin (Boyce et al., 2002). In addition to adhesive interactions, migration is induced by different chemotactic interactions, such as anaphylatoxins C3a and C5a (Nilsson et al., 1996), and interactions of CXC-chemokine receptor 2 (CXCR2) (Abonia et al., 2005) and c-kit with their respective ligands. The critical role for stem cell factor (SCF, also called Kit ligand, steel factor or mast cell growth factor) and its receptor c-kit (also called CD117) (Galli et al., 1993) in mast cell development is highlighted by the fact that mice with mutations in either of the proteins resulting in defective c-kit signaling virtually lack mature mast cells (Kitamura et al., 1978; Kitamura and Go, 1979). On the other hand, patients with gain-of-function mutations for increased c-kit expression have mastocytosis, i.e. increased numbers of mast cells in various tissues (Nagata et al., 1998). Interestingly, the homing of mast cell progenitors to mucosal tissues has been shown to be regulated by transcription factor T-bet expressed in dendritic cells (Alcaide et al., 2007). Mast cell-committed progenitors infiltrate the target tissue where their proliferation and differentiation continues in the presence of local growth factors, such as SCF, IL-3, IL-4, IL-6, IL-9 and nerve growth factor (NGF) (Lindstedt et al., 2007) secreted by various resident tissue cells, such as fibroblasts (Fujita et al., 1988). 

In vertebrates, mast cells are widely distributed throughout vascularized tissues, and are present especially in the vicinity of blood vessels, lymphatic vessels and nerves, as well as in boundaries between the outside world and the internal milieu (Galli et al., 2005a; Sacchi et al., 2003). These
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include mucosal surfaces such as those in the intestines, lungs, conjunctiva and the genital tract, as well as connective tissues such as skin, and, at least in murine rodents, mesothelium-lined cavities such as the peritoneal cavity (Galli et al., 2005a; Schwartz and Huff, 1998). Parenchymatous organs such as the liver, brain, kidneys and adrenals have modest numbers of mast cells, and bone and cartilage have few, if any (Schwartz and Huff, 1998). Mast cells are long-lived cells and some of them may live perhaps even more than a year (Kitamura, 1989). Not all mature mast cells in tissues are at an endstage of differentiation, since they can re-enter the cell cycle and, following appropriate stimulation, even proliferate (Galli et al., 1992). Besides proliferation, the number of mature mast cells may increase by recruitment of new progenitors and by their local differentiation into mature mast cells (Galli et al., 2008). Interestingly, after Trichinella spiralis infection, integrin β7 positive progenitor cells have been shown to increase first in bone marrow, then in blood, and finally in the small intestine (Pennock and Grencis, 2004).

2.2. Mast cell activation

Mast cells may interact with a repertoire of agents from the environment or agents generated by the host during immune reactions. In immune defence, diverse stimuli such as invading pathogens, environmental antigens, allergens, and toxins (Galli and Tsai, 2008) are first encountered by mast cells and dendritic cells, and induce mast cell activation, the release of mast cell mediators into their surroundings (Galli et al., 2008). This is a prerequisite for an efficient and directed action of mast cells in a specific tissue location.

The most widely known activator of mast cells, and the one which gave the mast cell its reputation as an allergy cell, is immunoglobulin E (IgE). In addition, IgG1 is able to activate mast cells in mice. Human mast cells are also activated by IgG1 after treatment with IFN-γ (Okayama et al., 2000). The IgE/IgG-induced activation of mast cells begins with the interaction of the Fc portions of the immunoglobulin molecule with their specific receptors expressed on the mast cell membrane. FcεRI and FcγRI are high-affinity receptors for IgE and IgG, respectively, and FcγRII and FcγRIIΙ are low-affinity receptors for IgG (Rao and Brown, 2008). IgE-mediated mast cell activation is important in immunity against helminths and parasites such as Trichinella spiralis (Gurish et al., 2004), and activation through FcγRI and FcγRIIΙ has a role in IgG-dependent allergic reactions and in immunological defence against pathogens producing superantigens such as protein A from Staphylococcus aureus (Genovese et al., 2000; Genovese et al., 2000; Rao and Brown, 2008). In innate immunity and bacterial infections, mast cells are activated independently of IgE through their cell membrane receptors including toll-like receptors (TLRs), complement receptors (CRs), and CD48 which binds the FimH subunit of type-1 fimbriated Escherichia coli (Heib et al., 2008). TLRs expressed in mast cells include TLR 1, 2, 3, 4, 6 and 9, and TLR 2, TLR 4 and TLR 3 have been demonstrated to bind bacterial cell wall components peptidoglycan, lipopolysaccharide (LPS), and viral dsRNA, respectively (Rao and Brown, 2008; Supajatura et al., 2002). CRs include CR3, CR4, CR5, receptors for anaphylatoxins C3a and C5a, and integrin αβ1 that can bind complement factor C1q (Edelson et al., 2006; Heib et al., 2008; Rao and Brown, 2008). In addition, peptidoglycan from Gram-positive bacteria has recently been shown to activate mast cells via complement in vivo (Jawdat et al., 2006). Mast cells can also be activated without receptors on the mast cell membrane by a variety of substances, such as bacterial toxins and polycationic compounds like defensins (Heib et al., 2008). Furthermore, mast cell activation may also be induced by nonimmunological molecules such as endothelins, a group
of endogenous peptides produced by endothelial cells (Heib et al., 2008). Interestingly, mast cell proteases are able to degrade these peptides and so neutralize the toxicity induced by high concentration of endothelins (Maurer et al., 2004; Metsärinne et al., 2002). Furthermore, mast cell carboxypeptidase A has been shown to detoxify sarafotoxins, peptides with high homology to endothelins, which are found in snake venom (Metz et al., 2006). Also calcium ionophores, basic biomolecules such as compound 48/80, various neuropeptides such as substance P (Schwartz and Huff, 1998) and neuromedin U (NMU) (Moriyama et al., 2005), some cytokines such as tumor necrosis factor-alpha (TNF-α) and MCP-1 (Benoist and Mathis, 2002), and components of mosquito saliva (Demeure et al., 2005) are able to activate mast cells.

Traditionally mast cell activation proceeds to degranulation, a process involving liberation of preformed cytosolic granules. Paul Ehrlich, who first described mast cells in 1877 and chose the name *mastzellen* to refer to the possible overfeeding of the granules, described the process of degranulation for the first time (Ehrlich, 1879; Schwartz and Huff, 1998). Mast cell activation and degranulation by IgE has been extensively studied (Blank and Rivera, 2004). Briefly, mast cell degranulation induced by IgE includes bridging of IgE-FcεRI complexes to form dimeric or trimeric aggregates with multivalent allergens. This crosslinking initiates intracellular signal transduction cascades that involve phosphorylation of the FcεRI receptors as well as of many downstream molecules, and leads to activation of phospholipase C-γ (PLC-γ), mitogen-activated protein kinase (MAPK), and PI3K pathways. Degranulation is also associated with the activation of small G proteins causing polymerization and relocation of actin, as well as generation of diacylglycerol that releases calcium from the endoplasmic reticulum (Schwartz and Huff, 1998). Eventually, the granules swell, their membranes fuse with the plasma membrane, and mediators are released via exocytosis (Lawson et al., 1977; Schwartz and Huff, 1998). Various mast cell activators may induce degranulation through different signaling cascades (Theoharides et al., 2007). In addition, mast cells may also liberate mediators such as cytokines without abrupt degranulation. Piecemeal degranulation involves the release of granule contents in a slow, progressive manner with only partial degranulation (Dvorak and Kissell, 1991). Furthermore, the secretion of mast cell mediators may proceed without any signs of degranulation (Fischer et al., 2006; Kandere-Grzybowska et al., 2003). Interestingly, the release of the mediators in the absence of degranulation has been shown to involve small vesicles, unrelated to the large secretory granules with a diameter of approximately 1 μm (Heib et al., 2008; Theoharides et al., 2007). The nature and dose of the stimulus may modify the forms of activation. For instance, low concentration of antigen or low occupancy of mast cells with IgE favours the release of leukocyte-attracting chemokines without considerable degranulation (Gonzalez-Espinosa et al., 2003). The flexibility of mast cells to respond at different magnitudes to stimuli with different natures and doses suggests that these cells may be life-saving via induction of inflammatory reactions involving both innate and adaptive immunity, and may also be the cause of fatal anaphylactic responses triggered by IgE (Heib et al., 2008).

Under different specific activation conditions, the constituents in the released mast cell mediator cocktail may vary (Galli et al., 2005b; Theoharides et al., 2007). For instance, the release of TNF-α from bone marrow-derived cultured mast cells (BMCMCs) is huge when activated by crosslinking of IgE, whereas when activated by peptidoglycan, it is negligible (Jawdat et al., 2006). In addition, mast cells can participate in multiple cycles of activation (Xiang et al., 2001). Interestingly, synergistic activation of mast cells through TLR and FcεRI pathways has been
demonstrated in an experimental model of asthma, suggesting that bacterial infection could increase the severity of asthma (Nigo et al., 2006). Overall, the duration, intensity, and tissue distribution of a particular response are affected by various characteristics of the activator, immunologic sensitivity of the host, the target tissue involved, and any underlying pathology (Schwartz and Huff, 1998). In addition, the complexity of the response is determined by the type of mast cell and its possible priming to become more responsive to the activator (Schwartz and Huff, 1998).

2.3. Mast cell mediators
Mast cells in tissues have round, spindly, or spiderlike shapes, and with toluidine blue they stain metachromatically, i.e. reddish-purple as compared to basic tone blue, based on the content of highly charged heparin and chondroitin sulfate proteoglycans that are stored in association with cationic histamine and proteases in their granules (Trivedi and Caughey, 2010). The contents of the granules can be modulated by the tissue microenvironment, which leads to mast cell heterogeneity (as will be discussed in next section). At the time of activation, mast cells release their granule contents, which induce immediate response (in minutes, peaking in 15-30 minutes), and start production of de novo synthesized mediators responsible for a sustained response (in hours, peaking in 6-12 hours) which amplifies the immediate response (Benoist and Mathis, 2002; Metcalfe et al., 1997).

2.3.1. Preformed mast cell mediators
Upon degranulation, mast cells release preformed mediators that are critical in initiating mast cell-mediated innate immune responses (Rao and Brown, 2008). These mediators include the neutral proteases tryptase, chymase, cathepsin G and carboxypeptidase A that are stored in active form in association with heparin and/or chondroitin sulfate proteoglycans (Pejler et al., 2009). In addition, the mediators include histamine, growth factors and cytokines such as TNF-α. Interestingly, TNF-α can also be synthesized rapidly after activation stimulus and thus, similarly to vascular endothelial growth factor (VEGF), it can be liberated from both preformed and newly synthesized pools of mast cell mediators (Galli et al., 2005b; Gordon and Galli, 1990).

Mast cell proteases
In mature mast cells, the expression of proteases is very high, ~2.5% of total transcripts bearing for chymase (rat mast cell protease (rMCP)-1), ~0.4% for tryptase (rMCP-6) and ~0.5% for mast cell carboxypeptidase A (MC-CPA) in rat peritoneal mast cells (Lutzelschwab et al., 1997; Pejler et al., 2009). For comparison, the levels for the housekeeping genes actin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) are in the range of 0.03-0.4%, and the levels of transcripts for immunoglobulins in antibody-producing B lymphocyte plasma cells may be in the range of 5-10% of all transcripts (Lutzelschwab et al., 1997). Also the levels of mature proteins are high: for example 10⁶ human foreskin mast cells contain 4.5 μg chymase and 11.5 μg tryptase (Schwartz et al., 1987). Indeed, approximately 50% of the weight of mast cell consists of neutral proteases stored in association with proteoglycans (Stevens and Adachi, 2007). Mast cells can be identified based on their protease composition (Pejler et al., 2007). Still, these proteases are expressed, even though at much lower levels, by some other cells such as basophils, which express α-tryptase, β-tryptase, and chymase (Jogie-Brahim et al., 2004; Li et al., 1998b). In addition, neutrophils and monocytes express cathepsin G (Gerber et al., 1974; Senior and Campbell, 1984), and chymase expression has also been detected in rat smooth muscle cells (Guo et al., 2001).
Tryptase is a neutral serine protease present in all human mast cells, which has a strong preference for cleaving substrates at the C-terminal side of Arg and Lys residues, i.e. it shows trypsin-like specificity (Hallgren and Pejler, 2006; Kam et al., 1995; Schwartz et al., 1981). Tryptase is the major type of protease stored in human mast cell granules (Schwartz et al., 1987), and there is actually a group of tryptases in human and other species (Table 5) (Hallgren and Pejler, 2006). Two classes of secreted tryptases exist in human mast cells, constitutively released α-tryptase, and β-tryptase that is released only upon degranulation (Schwartz et al., 1995). α-tryptase is present at low levels in circulation even without mast cell degranulation, but β-tryptase only appears after mast cell activation during extreme inflammatory conditions (Schwartz et al., 1995). Of human tryptases, only β-tryptase seems likely to play an important role outside the cell since other tryptases have defects in catalytic domains and therefore only low activity (Caughey, 2007). Tryptases expressed in the mouse and rat include mouse mast cell protease (mMCP)-6 and -7, and rMCP-6 and -7, respectively (Pejler et al., 2007). The substrate specificity of mMCP-6 closely resembles that of β-tryptase (Hallgren and Pejler, 2006; Huang et al., 1998), and it is released from mast cells only upon mast cell degranulation (Hallgren and Pejler, 2006) into the vicinity of the degranulated mast cell (Ghildyal et al., 1996). mMCP-7, instead, is released into circulation upon degranulation (Ghildyal et al., 1996). Interestingly, C57BL/6, a commonly used mouse strain, lacks the expression of mMCP-7 because of a premature stop codon (Ghildyal et al., 1994; Hunt et al., 1996). Human β-tryptase is proteolytically active as a tetrameric form and stabilized by heparin proteoglycan (Pereira et al., 1998; Schwartz and Bradford, 1986), although an active monomeric form has also been described (Addington and Johnson, 1996). There are no known natural inhibitors of the tetrameric form of β-tryptase (Pejler et al., 2007; Smith et al., 1984) but lactoferrin has the ability to inactivate tryptase by inducing its monomerization (Elrod et al., 1997). In addition, endogenous inhibitors such as antithrombin III and α-2-macroglobulin can inhibit the reassembly and activity of monomers (Fukuoka and Schwartz, 2004).

After its release from mast cells, tryptase can activate neighboring cells by cleaving and activating protease-activated receptor (PAR)-2 and thrombin receptors (Molino et al., 1997). In addition, tryptase is capable of degrading the pericellular matrix fibronectin (Lohi et al., 1992) and type IV collagen (Kielty et al., 1993), fibrinogen (Schwartz et al., 1985), pro-urolase plasminogen activator (pro-uPA) (Stack and Johnson, 1994), apolipoproteins in HDL (Lee et al., 2002b), complement protein C3 (Schwartz et al., 1983), IL-6 (Mallen-St Clair et al., 2004), denatured collagen (gelatin) (Fajardo and Pejler, 2003) and neuropeptides, such as vasoactive intestinal peptide (VIP) (Caughey et al., 1988; Caughey et al., 1988). In addition, tryptase may activate MMP-3 (Gruber et al., 1989; Johnson et al., 1998; Lees et al., 1994), increase leukocyte recruitment (He et al., 1997; Meyer et al., 2005), induce collagen synthesis (Cairns and Walls, 1997; Gruber et al., 1997), and increase vascular permeability by activation of prekallikrein and by direct release of bradykinin from kininogens (Imamura et al., 1996). Nevertheless, the in vivo substrates for tryptase are still unresolved with the exception of PAR-2 (Hallgren and Pejler, 2006), and may contain peptides from pathogens (Trivedi and Caughey, 2010). After challenge with Klebsiella pneumoniae (Thakurdas et al., 2007) or Trichinella spiralis (Shin et al., 2008) mice deficient in mMCP-6 have lower survival than wild-type mice, suggesting a role for tryptases in defence against bacteria and parasites.
Chymase is a neutral serine protease present in one subtype of mast cells, and it has a preference for cleaving substrates at the C-terminal side of aromatic or Leu residues, i.e. chymotrypsin-like specificity (Powers et al., 1985). Chymase released from mast cell granules is usually bound to heparin proteoglycans that protect it from endogenous protease inhibitors such as α1-antichymotrypsin, α2-macroglobulin and α1-protease inhibitor (Lindstedt et al., 2001; Pejler and Berg, 1995). In addition, this association with heparin proteoglycans may contribute to the ability of chymase to reach heparin-binding proteins such as thrombin or apoB-100 as a substrate (Kokkonen and Kovanen, 1987b; Pejler and Sadler, 1999). Human mast cells express only one chymase, α-chymase (Schechter et al., 1983). In mice, mMCP-1, -2, -4, -5, and -9 are regarded as chymases, and rats contain chymases rMCP-1, -2, -3, -4, -5 (Pejler et al., 2007) (Table 5). Rodent α-chymases, mMCP-5 and rMCP-5, have lost their chymase activities but have instead acquired elastase activities (Karlson et al., 2003; Kunori et al., 2002). The other chymases in rodents are β-chymases. α- and β-chymases are discriminated by their abilities to degrade different peptide bonds in angiotensin I leading either to its activation, i.e. the formation of a potent vasoconstrictor angiotensin II, or to its degradation, respectively (Pejler et al., 2007; Reilly et al., 1982; Sanker et al., 1997). Nevertheless, this does not apply to all chymases since β-chymases, such as mMCP-4, may cleave angiotensin I at both activating and degrading peptide bonds (Caughhey et al., 2000). The mouse chymase most similar to human chymase is mMCP-4, which is chymotryptic, highly cationic, heparin-binding, angiotensin II generating and present in similar tissue locations as human chymase (Caughhey et al., 2000; Tchougounova et al., 2003; Trivedi and Caughhey, 2010) even though the amino acid sequence of the functionally dissimilar mMCP-5 closely resembles that of human chymase (Caughhey, 2007).

Table 5. Neutral mast cell proteases in human, mouse and rat.

<table>
<thead>
<tr>
<th>Human</th>
<th>Mouse</th>
<th>Rat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tryptases</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-tryptase (αI, αII)</td>
<td>mMCP-7</td>
<td>rMCP-7</td>
</tr>
<tr>
<td>β-tryptase (βI, βII, βIII)</td>
<td>mMCP-6</td>
<td>rMCP-6</td>
</tr>
<tr>
<td>γ-tryptase (γI, γII) / hTMT</td>
<td>mTMT</td>
<td>rTMT</td>
</tr>
<tr>
<td>δ-tryptase (δI, δII)</td>
<td>mMCP-11/mastin</td>
<td>rMCP-11</td>
</tr>
<tr>
<td>Chymases</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α-chymase</td>
<td>mMCP-4 (β)</td>
<td>rMCP-1 (β)</td>
</tr>
<tr>
<td></td>
<td>mMCP-5 (α)*</td>
<td>rMCP-5 (α)*</td>
</tr>
<tr>
<td></td>
<td>mMCP-1 (β)</td>
<td>rMCP-2 (β)</td>
</tr>
<tr>
<td></td>
<td>mMCP-2 (β)</td>
<td>rMCP-3 (β)</td>
</tr>
<tr>
<td></td>
<td>mMCP-9</td>
<td>rMCP-4 (β)</td>
</tr>
<tr>
<td>Cathepsin G</td>
<td>cathepsin G</td>
<td>cathepsin G</td>
</tr>
<tr>
<td>Carboxypeptidase A</td>
<td>MC-CPA</td>
<td>MC-CPA</td>
</tr>
<tr>
<td>Other</td>
<td>mMCP-8</td>
<td>rMCP-8, -9, -10</td>
</tr>
</tbody>
</table>

Corresponding proteases are on the same line, proteases showing similar functionality to human proteases in bold, group other, specificity unknown. TMT, transmembrane tryptase. *mMCP-5 and rMCP-5 have lost their chymotryptic activities and have instead acquired elastolytic activities. Data derived from (Caughhey, 2007; Hallgren and Pejler, 2006; Pejler et al., 2007; Trivedi and Caughhey, 2010).
Besides angiotensin I (Reilly et al., 1982), the substrates for chymase include extracellular matrix proteins type I procollagen (Kofford et al., 1997), type IV collagen (Kielty et al., 1993), fibronectin (Vartio et al., 1981), and vitronectin (Banovac and De Forteza, 1992), apoB-100, apoE, apoA-I, apoA-II (Kokkonen et al., 1986; Lee et al., 2002a; Lee et al., 2003a; Lindstedt et al., 1996), PLTP (Lee et al., 2003b), and many inflammatory substances and bioactive peptides such as IL-1β and substance P (Pejler et al., 2007). In addition, human chymase is able to activate human interstitial collagenase (MMP-1) and human gelatinase B (MMP-9) (Furubayashi et al., 2008; Saarinen et al., 1994), and chymase may also, in different species, activate stromelysin (MMP-3) and gelatinases A and B (MMP-2 and -9) by cleaving their inactive propeptides (for more details see Table 6) (Fang et al., 1996; Kishi et al., 2007; Lees et al., 1994; Suzuki et al., 1995; Tchougounova et al., 2005). Chymase may also degrade TIMP-1 (Frank et al., 2001), release and activate latent transforming growth factor-beta (TGF-β) I from the extracellular matrix (Lindstedt et al., 2001; Taipale et al., 1995), inhibit smooth muscle cell growth (Wang et al., 2001), and induce apoptosis of smooth muscle cells and endothelial cells (Leskinen et al., 2001; Lätti et al., 2003). Furthermore, chymase seems to recruit inflammatory cells (He and Walls, 1998a), increase vascular permeability possibly through degradation of cell-cell or cell-basement membrane contacts (Ebihara et al., 2005a; He and Walls, 1998b; Scudamore et al., 1998), and may play a role in angiogenesis (Muramatsu et al., 2002; Russo et al., 2005), fibrosis (Kakizoe et al., 2001; Tomimori et al., 2003), aortic aneurysms (Tsunemi et al., 2004), myocardial infarctions (Jin et al., 2003), intimal hyperplasia after balloon injury (Takai et al., 2003), atherosclerosis (Uehara et al., 2002), and defence against parasites (Knight et al., 2000).

Cathepsin G is a serine endopeptidase present in chymase-containing mast cells (Schechter et al., 1990), and in humans it has both trypsin and chymotryptic specificity (Polanowska et al., 1998). Mouse cathepsin G may only have chymotryptic specificity (Trivedi and Caughey, 2010), whereas cathepsin G expression in rat mast cells has not been detected (Lutzelschwab et al., 1997). Human cathepsin G has structural similarity to chymase (Salvesen et al., 1987), and there are inhibitors capable of inhibiting both cathepsin G and chymase (de Garavilla et al., 2005). Human cathepsin G has similar activities to chymase, such as the ability to convert angiotensin I to II (Reilly et al., 1982), to degrade fibronectin (Vartio et al., 1981), vitronectin (Rao et al., 1991) and type IV collagen (Kielty et al., 1993), and to increase the level of active MMP-I (Son et al., 2009).

Carboxypeptidase A (CPA) is a metallocarboxypeptidase, a zinc-dependent exoprotease with a cleaving specificity for C-terminal aromatic (referring to A) and branched aliphatic residues (Everitt and Neurath, 1980; Pejler et al., 2009). The CPA expressed in mast cells (Goldstein et al., 1987), MC-CPA or CPA3 (Pejler et al., 2009; Trivedi and Caughey, 2010), is found in mast cells containing both tryptase and chymase (Irani et al., 1991). Pejler and coworkers presented a model of cooperative action for chymase and MC-CPA, in which chymase (or another endopeptidase) cleaves the substrate internally and MC-CPA subsequently trims the cleavage products C-terminally (Pejler et al., 2007). This kind of action has been suggested for cleavage of apoB in LDL (Kokkonen et al., 1986) and angiotensin I (Lundequist et al., 2004). In addition, MC-CPA seems to have a role in protection against the toxicity of endothelin-1 (Metsärinne et al., 2002) and snake venom toxins (Metz et al., 2006; Schneider et al., 2007).
In addition to the neutral proteases described above, preformed mast cell mediators may also include granzyme B (Pardo et al., 2007; Strik et al., 2007), MMPs, such as MMP-1 (Di Girolamo and Wakefield, 2000), MMP-2 and MMP-9 (Baram et al., 2001; Fang et al., 1999), and other enzymes such as arylsulfatases, pro-caspase 3 and 4, β-hexosaminidase, kininogenases, NO synthase, peroxidases and phospholipases (Theoharides et al., 2007). The presence of acid hydrolases and other destructive enzymes in mast cell granules indicates that the granules are modified lysosomes developed from the Golgi apparatus (Church et al., 1998).

**Proteoglycans** are critical for mast cell granule organization and storage of proteases and histamine (Matsumoto et al., 1995; Ringvall et al., 2008). They contain highly anionic glycosaminoglycans (GAGs) such as heparin and chondroitin sulfates attached covalently to core protein, such as serglycin (Åbrink et al., 2004; Church et al., 1998; Kjellen et al., 1989; Stevens and Adachi, 2007). Heparin is a glycosaminoglycan rich in trisulfated disaccharide and thus the most negatively charged molecule in the body (Stevens and Adachi, 2007). Human mast cells contain heparin and chondroitin sulfates A and E (Stevens et al., 1988; Welle, 1997). These are also found in rodents, in addition to chondroitin sulfate di-B in rats (Razin et al., 1982; Stevens et al., 1986; Welle, 1997). In mast cell granules, anionic proteoglycans form a scaffold where cationic mast cell proteases bind ionically (Stevens and Adachi, 2007; Uvnäs et al., 1970). In addition, heparin binds histamine (Rabenstein et al., 1998; Uvnäs et al., 1970) as well as
cytokines and growth factors such as basic fibroblast growth factor (bFGF) (Reed et al., 1995). Upon degranulation, proteases are released in association with proteoglycans (Goldstein et al., 1992; Serafin et al., 1986; Serafin et al., 1987) while histamine diffuses away in the neutral pH of the extracellular environment (Church et al., 1998). The differences in protease release either in the vicinity of the degranulated mast cell or even to circulation may be due to differences in their affinities for proteoglycans (Pejler et al., 2007). Heparin plays an important role in the activity of mast cell proteases by stabilizing trypase tetramers and by protecting chymase and cathepsin G from their natural inhibitors (Ermolieff et al., 1994; Lindstedt et al., 1998; Pejler and Berg, 1995; Schwartz and Bradford, 1986).

**Histamine** is one of the main inflammatory mediators in conditions such as allergic reaction and asthma (Thurmond et al., 2008). In mast cell granules, histamine is associated with heparin proteoglycans (Church et al., 1998; Rabenstein et al., 1998; Uvnäs et al., 1970). Interestingly, the lack of histamine has been shown to result in defective storage of mast cell proteases in the granules of peritoneal mast cells (Ohtsu et al., 2001). In addition to increased secretion after mast cell activation, histamine is also secreted at low levels by resting mast cells (Church et al., 1998). The biological effects of histamine are mediated via histamine receptors H1, H2, H3 and H4 that are expressed differently in various cells and tissues (Thurmond et al., 2008). Histamine induces vasodilatation, increased vascular permeability, and P-selectin-mediated leukocyte rolling (Jones et al., 1993; Thurmond et al., 2008). In skin, this results in a ‘triple response’ or ‘wheal and flare response,’ immediate redness due to vasodilatation, a wheal or swelling due to increased vascular permeability, and a flare or redness due to indirect vasodilation via the nerve stimulation (Thurmond et al., 2008). In addition, histamine induces the expression of TLRs 2 and 4 in endothelial cells (Talreja et al., 2004) and the expression of tissue factor in smooth muscle and endothelial cells (Steffel et al., 2005). In addition to histamine, rodents also produce serotonin (Galli et al., 2005b).

**Preformed cytokines**
Mast cells release upon secretion several growth factors such as VEGF and bFGF and TGF-β that regulate inflammation and angiogenesis (Marshall, 2004). Other preformed cytokines include IL-8, MCP-1, MCP-3, MCP-4, and ‘regulated on activation, normal T cell expressed and secreted’ (RANTES) that have a role in chemotraction and recruitment of leukocytes (Theoharides et al., 2007). In addition, mast cells have a unique ability to store and acutely release preformed TNF-α (Gordon and Galli, 1990), and therefore play an important role in initiating inflammation, since TNF-α induces the fast recruitment of neutrophils (Gamble et al., 1985). This is mediated via endothelial cell adhesion molecules P-selectin, E-selectin and VCAM-1 induced by TNF-α (Norman et al., 2005). In addition, TNF-α seems to increase vascular permeability (Folli et al., 1993). Two peaks of TNF-α accumulation in the peritoneal fluid have been detected in a mouse model of immune complex-mediated peritonitis, first at 5 minutes after the challenge and a second one with greater magnitude after 4-8 hours (Zhang et al., 1992b). In mast cell-deficient mice, the first peak was absent and the second reduced by 60%, and, in addition, both peaks could be restored by reconstitution of mast cells. This suggests that mast cells have a major role in the first peak and also play a role in the second one.
2.3.2. Newly generated mast cell mediators

**Lipid mediators**

Eicosanoids are derivatives of arachidonic acid, and include prostaglandins, thromboxanes, and leukotrienes. Arachidonic acid is metabolized via cyclooxygenase into prostanoids, such as prostaglandins D$_2$ and E$_2$ and thromboxane A$_2$, and via lipoxygenase into leukotrienes, such as leukotrienes B$_4$ and C$_4$. Human mast cells contain prostaglandin D$_2$ and leukotriene C$_4$. Prostaglandin D$_2$ is a coronary vasoconstrictor, peripheral vasodilator, inhibitor of platelet aggregation, and chemoattractant to neutrophils (Church et al., 1998). Leukotrienes increase vascular permeability and induce arterial, arteriolar and intestinal constriction, and a prolonged ‘wheal and flare’ response in skin (Metcalfe et al., 1997). A precursor for platelet activating factor (PAF) is formed when phospholipase A$_2$ generates arachidonic acid from phosphatidylcholine (Church et al., 1998; Metcalfe et al., 1997). PAF aggregates platelets, increases vascular permeability, and is a chemoattractant for neutrophils. All described lipid mediators are also bronchoconstrictors (Church et al., 1998). In addition to prostaglandin D$_2$ and leukotriene C$_4$, mast cells may also contain prostaglandin E$_2$, leukotriene B$_4$, and PAF (Galli et al., 2005b).

**Cytokines and chemokines**

Mast cells are able to release a large variety of newly synthetized cytokines and chemokines. Cytokines include proinflammatory mediators TNF, IL-1$\alpha$, IL-1$\beta$, IL-6, IL-18, granulocyte-macrophage colony-stimulating factor (GM-CSF), leukemia inhibitory factor (LIF), IFN-\(\alpha\) and IFN-\(\beta\); T helper 2-type cytokines IL-3, IL-4, IL-5, IL-9, IL-13, IL-15, and IL-16; T helper 1-type cytokines IL-12 and IFN-\(\gamma\); and anti-inflammatory, immunomodulatory or angiogenesis-regulating IL-10, TGF-\(\beta\), and VEGF (Marshall, 2004). In addition, mast cells are able to release macrophage inflammatory factor (MIF), SCF, gonadotropin-releasing hormone (GnRH-I), bFGF and NGF (Theoharides et al., 2007). Chemokines released from mast cells include CC-chemokine ligands CCL-2, CCL-3, CCL-4, CCL-5, CCL-11, and CCL-20 as well as CXC-chemokine ligands CXCL-1, CXCL-2, CXCL-8, CXCL-9, CXCL-10, and CXCL-11, and these mediators have a role in recruiting other effector cells and in regulating immune response (Marshall, 2004).

Overall, the mediators released from mast cells, notably various cytokines, exhibit variation in their inflammatory properties. Some mediators may possess proinflammatory properties, and others may have anti-inflammatory properties. It is difficult to predict, in a particular response, what will be the net effect of mast cell activation. Actually, it could well be that both effects may be seen but at different stages of the response (Galli and Tsai, 2008).

2.4. Heterogeneity of mast cells

Mast cells in different tissue locations exhibit substantial heterogeneity. It is the microenvironment and its associated growth and differentiation factors, including interactions with the tissue matrix and resident cells such as fibroblasts, that are considered to define the phenotype of a mast cell (Bradding, 2009; Metcalfe et al., 1997). Mast cells may show variation according to their content of mediators, ultrastructure, size and shape, expression of receptors, sensitivity to various activators and pharmacological responsiveness (Bradding, 2009). This heterogeneity is obvious across the species, between different organs in the same species and even within the same organ (Bradding, 2009). Human mast cells are classified based on their
content of proteases as MC₇ type, containing only tryptase, and MC₇C type, containing tryptase, chymase, cathepsin G, and MC-CPA (Irani et al., 1986; Irani et al., 1991; Schechter et al., 1990). In addition, a rare phenotype containing only chymase, MC₇C, has been reported (Weidner and Austen, 1991). Mucosal surfaces such as the mucosa of the lungs and intestines typically contain mast cells of the MC₇ phenotype, and mast cells of the MC₇C phenotype are located to connective tissues such as the skin and peritoneal cavity. The mast cells in a particular tissue location, however, are usually a mixture of both types. As an example, human skin contains >99% MC₇C and <1% MC₇S whereas alveolar tissue contains 93% MC₇S and 7% MC₇C (Irani et al., 1989). In rodents, the nomenclature of mast cell subtypes is derived from the location of mast cells. Mucosal mast cells (MMCs) in mice and rats, at least in terms of tissue localization, closely resemble human MC₇ type, whereas connective tissue mast cells (CTMCs) in mice and rats resemble MC₇C type (Metcalfe et al., 1997). Interestingly, among the mast cells, CTMCs are long-lived cells with a life time of perhaps more than one year while MMCs have a much shorter life span of perhaps only 1 to 2 weeks (Kitamura, 1989; Welle, 1997). Furthermore, rat CTMCs and human MC₇C (skin mast cells) are readily activated by compound 48/80 while rat MMCs and human MC₇S (lung mast cells) are relatively insensitive to it (Church et al., 1982; Metcalfe et al., 1997). Similarly, rat CTMCs and human MC₇S (lung mast cells) are responsive to the mast cell stabilizer sodium chromoglycate whereas rat MMCs and human MC₇C (skin mast cells) are not (Church and Young, 1983; Church and Young, 1983; Metcalfe et al., 1997; Pearce et al., 1974).

Due to the presence of large amounts of heparin in their granules, murine CTMCs can be distinguished from murine MMCs by safranin staining (Enerbäck, 1966). Many other differences in mediator contents between phenotypes have been reported and are summarized in table 7. The distribution of proteases determines the subtype in human mast cells. Also in mice and rats, differences in protease content exist between mast cell subtypes. Thus, MMCs contain only chymases whereas CTMCs contain tryptases, chymases, MC-CPA, and, in mice, also cathepsin G (Jippo et al., 1999; Pejler et al., 2007). All human mast cells seem to contain heparin sulfate and chondroitin sulfate A and E proteoglycans (Metcalfe et al., 1997; Welle, 1997). In contrast, rodent heparin sulfate proteoglycans are restricted to CTMCs (Church et al., 1998), and chondroitin sulfates are found in MMCs as well as in rat CTMCs (Katz et al., 1986; Welle, 1997). Histamine is contained in all types of mast cells in humans, mice, and rats but concentrations in mouse and rat mast cells differ in different phenotypes, the values being higher for CTMCs (Table 7). Rodents also produce serotonin that is present in the CTMCs (Welle, 1997).

The phenotypes described here give only a rough estimation of various types of mast cells across the species. Actually, it may well be more like a spectrum of different phenotypes (Marshall, 2004) depending on the external microenvironment of a particular mast cell. Interestingly, mast cells seem to have plasticity for changing their phenotype after their transfer from one anatomical site to another one (Kitamura et al., 1987; Nakano et al., 1985). Furthermore, local inflammation may alter the nature of residential mast cells (Marshall, 2004). These findings even further highlight the potential of mast cells for substantial heterogeneity based on their surrounding tissue microenvironment.
Table 7. Main mast cell mediators in different types of mast cells.

<table>
<thead>
<tr>
<th></th>
<th>Protease(s)</th>
<th>Proteoglycans</th>
<th>Histamine (and serotonin)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MC_T</td>
<td>tryptase</td>
<td>HS, CS-A, CS-E</td>
<td>1.5 pg/cell*</td>
</tr>
<tr>
<td>MC_TC</td>
<td>tryptase, chymase, cathepsin G</td>
<td>HS, CS-A, CS-E</td>
<td>1.9 pg/cell*</td>
</tr>
<tr>
<td>Mouse</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMC</td>
<td>chymase (mMCP-1, -2)</td>
<td>CS-E</td>
<td>0.1 pg/cell**</td>
</tr>
<tr>
<td>CTMC</td>
<td>tryptase (mMCP-6, -7), chymase (mMCP-4, -5) cathepsin G</td>
<td>HS</td>
<td>10 pg/cell serotonin</td>
</tr>
<tr>
<td>Rat</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMC</td>
<td>chymase (rMCP-2, -3, -4), other (rMCP-8, -9, -10)</td>
<td>CS-di-B, CS-A, CS-E</td>
<td>1-2 pg/cell</td>
</tr>
<tr>
<td>CTMC</td>
<td>tryptase (rMCP-6, -7), chymase (rMCP-1, -5)</td>
<td>HS</td>
<td>15 pg/cell serotonin</td>
</tr>
</tbody>
</table>

*data from human lung and foreskin (Schwartz et al., 1987), also values of 3-8 pg histamine/cell are reported for mast cells isolated from lung, skin, lymphoid tissue, and small intestine (Metcalfe et al., 1997). **data from BMCMCs (Nakano et al., 1985). Data derived from (Jippo et al., 1999; Kitamura, 1989; Metcalfe et al., 1997; Pejler et al., 2007; Schwartz et al., 1987; Welle, 1997).

2.5. Function of mast cells

Mast cells are involved in many physiological processes of the body, and besides their diverse roles in host defence, they participate in processes like wound healing, tissue remodeling and homeostasis (Rao and Brown, 2008). In innate immunity, mast cells are able to phagocytose and kill bacteria (Malaviya et al., 1994), produce antimicrobial peptides such as cathelicidins (Di Nardo et al., 2003) as well as NO and superoxide radicals (Rao and Brown, 2008). They participate in immune defence against parasites by, for instance, releasing proteases (Knight et al., 2000; McDermott et al., 2003; Shin et al., 2008), and against viruses by releasing specific mediators for recruitment of antiviral effector cells (Burke et al., 2008). In addition to important roles in innate immunity, mast cells are regarded as part of, or at least as important modulators of adaptive immunity. Interestingly, mast cells may be considered masquerading as cells of the adaptive immune system since they are constantly coated with antigen-specific IgE because of the unusually high affinity (10^{-10} M) of the FceR for IgE (Benoist and Mathis, 2002). Mast cells may present antigens from engulfed bacteria to T cells (Malaviya et al., 1996), contribute to the migration of antigen presenting cells (APCs) to lymph nodes (Bryce et al., 2004; Heib et al., 2008), participate in the recruitment of naïve T cells to lymph nodes (Wang et al., 1998) or sites of inflammation (Sayed and Brown, 2007), and enhance T cell proliferation and activity (Nakae et al., 2006). In addition, mast cells may participate in immune tolerance, by, for instance, suppressing immune responses directly by releasing anti-inflammatory cytokines (Rao and Brown, 2008). Besides host defence, mast cells have a role in the maintenance of tissue integrity
and function by inducing the proliferation of epithelial cells (Cairns and Walls, 1996) and fibroblasts (Ruoss et al., 1991), by participating in wound healing (Weller et al., 2006), and by contributing to bone remodeling (Silberstein et al., 1991) and hair follicle cycling (Maurer et al., 1997). Furthermore, the cooperation of mast cells with the nervous system is important in many processes such as wound healing (Gottwald et al., 1998) and stress response (Singh et al., 1999).

In addition, mast cells participate in many pathological conditions such as allergy and asthma, rheumatoid arthritis, atherosclerosis, and cancer, and they may even have a role in controlling diet-induced obesity (Liu et al., 2009). The best known consequences of mast cell action are type I hypersensitivity reactions induced by IgE-mediated allergic inflammation (Rao and Brown, 2008). Anaphylaxis is an acute and systemic severe allergic reaction involving the release of mediators from mast cells and basophils that, at its worse, leads to death (Peavy and Metcalfe, 2008). Mast cells contribute to allergic disorders such as asthma, allergic rhinitis, and atopic dermatitis (Brown et al., 2008). In addition, mast cells participate in chronic inflammatory and autoimmune disorders that are induced by dysregulated activation of mast cells (Rao and Brown, 2008). The activation of mast cells is mediated through autoantibodies in bullous pemphigoid and rheumatoid arthritis, and by deposited immune complexes in glomerulonephritis (Rao and Brown, 2008). Mast cells have important roles in heart diseases such as heart failure and atherosclerosis (the latter will be discussed in more detail in next section) (Reid et al., 2007) as well as in stroke (Strbian et al., 2009) and aortic aneurysms (Sun et al., 2009). In addition, the role of mast cells in cancer seems to include promotion of angiogenesis with subsequent tumour growth and/or formation of metastasis (Ribatti et al., 2001). Interestingly, the chemotactic factors for the recruitment and activation of mast cells in cancer may derive from the tumor itself (Conti et al., 2007). Mast cell accumulation, however, may also have detrimental effects on tumor growth, such as induction of tumor cell death by IL-4 or TNF-α (Conti et al., 2007). Moreover, mast cells may serve as a reservoir for human immunodeficiency virus (HIV) through latent infection with mast cell activation through TLRs 2, 4, and 9 triggering viral replication (Sundstrom et al., 2004).

In conclusion, while the effects of mast cells in host defence and tissue homeostasis are beneficial to the host, in chronic inflammatory conditions, however, mast cells may be seen as enemies rather than friends, and the actions of mast cells, when unregulated, may become harmful or even lethal to the host, as in anaphylaxis.

2.6. Mast cell deficiency

Mast cell-deficient mice have become powerful tools for studying the effects of mast cells in different biological contexts in vivo. Most of these genetically mast cell-deficient mice have spontaneous mutations in the alleles of the dominant white spotting (W) locus coding for the protein c-kit, a tyrosine kinase receptor located on the surface of mast cells and ready to bind its ligand SCF. Several murine models with defective c-kit signaling have been described, including Kit\(^{W-s/W-s}\) rats and Kit\(^{W-f/W-f}\) mice, but the Kit\(^{W-v/W-v}\) and Kit\(^{W-sh/W-sh}\) mice have become the most commonly used models for mast cell deficiency (Grimbaldeston et al., 2005) (Table 8).

Mast cell-deficient Kit\(^{W-v/W-v}\) mice are generated by crossing the strains WB/Re-W/+ and C57BL/6-W/+ (Kitamura et al., 1978). In addition to white W/W and black +/+ mice, the litter contains heterozygotes for the W locus, namely W/+ and W/+; both of which contain white spots on their
ventral skin (Kitamura et al., 1978). Kit\textsuperscript{W/W-v} mice, also referred as (WB/Re x C57BL/6)F\textsubscript{1}-
Kit\textsuperscript{W/W-v}, or WBB6F\textsubscript{1}- Kit\textsuperscript{W/W-v} mice, are sterile due to lack of germ cells, and their production
requires maintenance of parental strains (Galli and Tsai, 2008; Heib et al., 2008; Metz et al., 2007). The W mutation contains a 78-amino acid deletion in the transmembrane domain of c-kit protein, and results in the lack of c-kit cell surface expression and kinase activity (Nocka et al., 1990). The W\textsuperscript{v} mutation is a missense mutation in the kinase domain of c-kit resulting in decreased kinase activity (Nocka et al., 1990). As a result, Kit\textsuperscript{W/W-v} mice have decreased kinase activity of their c-kit protein (Nocka et al., 1989). Kit\textsuperscript{W/W-v} mice contain no mast cells in the stomach, caecum, mesentery, bone marrow, spleen, thymus, heart, lung, kidney, liver, brain, urinary bladder, ileum, peritoneal cavity, hindlimb skeletal muscle, and uterus (Galli and Kitamura, 1987; Kitamura et al., 1978). Nevertheless, in the skin, a minor presence of mast cells is observed, the actual amounts being below 1% of the amounts observed in other genotypes of the litter (Kitamura et al., 1978). Besides mast cell deficiency, Kit\textsuperscript{W/W-v} mice are sterile and anemic (Kitamura et al., 1978), and virtually lack melanocytes, thus giving rise to the white colour of the skin. Furthermore, adult Kit\textsuperscript{W/W-v} mice lack interstitial cells of Cajal in the intestines and intraepithelial lymphocytes in the small intestine, exhibit bile reflux, and develop stomach papillomas and ulcers, spontaneous dermatitis, and dilatation of the duodenum (Grimbaldeston et al., 2005). On the other hand, adult Kit\textsuperscript{W/W-v} mice have normal levels of neutrophils and macrophages in bone marrow, spleen, and peritoneal cavity, normal levels of basophils and B cells in bone marrow and spleen, normal levels of dendritic cells and natural killer cells in spleen, and normal levels of T cells in thymus and spleen (Grimbaldeston et al., 2005).

Mast cell-deficient Kit\textsuperscript{W-sh/W-sh} mice contain W-sash (W\textsuperscript{sh}) mutation, an inversion mutation in the transcriptional regulatory elements upstream of the c-kit transcription start site (Nagle et al., 1995). This mutation results in the blocking of c-kit expression in specific tissue locations of adult mice such as mast cell progenitors present in the bone marrow (Duttlinger et al., 1993; Tono et al., 1992; Yamazaki et al., 1994), as well as its ectopic expression during embryogenesis, i.e. expression not seen in Kit\textsuperscript{+/+} mice, that may affect melanogenesis (Duttlinger et al., 1993). The phenotype of the heterozygotes, from which the name of the mutant mouse is derived, is characterized by a white band or sash in the lumbar region (Lyon and Glenister, 1982). This phenotype was spontaneously born among the progeny of a cross between two inbred strains, C3H/HeH and 101/H (Lyon and Glenister, 1982). Homozygotes are white with black eyes and small pigmented patches around the ears and eyes (Lyon and Glenister, 1982). Adult Kit\textsuperscript{W-sh/W-sh} mice are mast cell-deficient at multiple anatomical sites, such as tongue, lung, spleen, trachea, heart, stomach, jejunum, ileum, colon, kidney, bladder, tail, liver, brain, lymph nodes, mesentery, peritoneum (Grimbaldeston et al., 2005; Stevens and Loutit, 1982; Wolters et al., 2005). The reports on mast cell amounts in the skin of Kit\textsuperscript{W-sh/W-sh} mice show some discrepancy. Grimbaldeston and coworkers have shown that mast cells decline to the level of 1.2% of the level seen in Kit\textsuperscript{+/+} mice by the age of 12 weeks, Yamazaki and coworkers found a level of 0.6% at the age of 90 days, whereas Wolters and coworkers did not see any mast cells in the skin of 10 week old mice (Grimbaldeston et al., 2005; Wolters et al., 2005; Yamazaki et al., 1994). In any case, both Kit\textsuperscript{W-sh/W-sh} and Kit\textsuperscript{W/W-v} mice are virtually mast cell-deficient. Kit\textsuperscript{W-sh/W-sh} mice have two major benefits over Kit\textsuperscript{W/W-v} mice: they are not anemic, and they are fertile (Lyon and Glenister, 1982) enabling the studies that cross these mice to other genetically modified mice. Other effects of the W\textsuperscript{sh} mutation include impairment of skin pigmentation, deficiency of interstitial cells of Cajal in the intestines, and bile reflux into the stomach (Grimbaldeston et al., 2005). More importantly,
these mice have normal levels of other differentiated hematopoietic and lymphoid cells, namely neutrophils and macrophages in bone marrow, spleen, and peritoneal cavity, basophils and B cells in bone marrow and spleen, dendritic cells and natural killer cells in spleen, and T cells in thymus and spleen (Grimbaldeston et al., 2005). Unlike KitW/W-v mice, KitW-sh/W-sh mice do not have either deficiency in intraepithelial lymphocytes in the small intestine, or a high incidence of spontaneous pathology in skin, stomach or duodenum (Grimbaldeston et al., 2005).

In addition, mast cell deficiency may arise from mutations in the alleles of the Steel (Sl) locus, coding for the c-kit ligand, SCF (Table 8). In these mice, mast cell deficiency arises not because of abnormality of bone marrow-derived mast cell precursors as in the mutations at the W locus but because of abnormality in the microenvironmental factors that promote mast cell differentiation and maturation in the tissues (Galli and Kitamura, 1987). This is also likely to be the reason for their inability to repair mast cell deficiency after injection of wild-type bone marrow or mast cells (Galli and Kitamura, 1987). Sl mutation is a deletion mutation resulting in the absence of most, if not all of the coding region (Heib et al., 2008). Sl<sup>d</sup> (Steel-Dickie) mutation is a spontaneous deletion mutation resulting in removal of the transmembrane and intracellular domains of the SCF protein and lack of its cell surface expression (Flanagan et al., 1991). Mast cell-deficient Sl/Sl<sup>d</sup> mice, also referred as WCB6F<sub>I</sub>-Sl/Sl<sup>d</sup>, are generated by crossing the strains WC/Re-Sl<sup>+/+</sup> and C57BL/6-Sl<sup>+/+</sup>, and their progeny can also be distinguished according to coat colour. Sl/Sl<sup>d</sup> mice are white with black eyes, their congenic +/+ mice are black, and the heterozygotes, Sl<sup>+/+</sup> and Sl<sup>d</sup>/+, have white spotting (Galli and Kitamura, 1987). The deficiency of mast cells in Sl/Sl<sup>d</sup> mice is similar to KitW/W-v mice; minor amounts are seen in the skin of adult mice (Galli and Kitamura, 1987; Kitamura and Go, 1979). Sl/Sl<sup>d</sup> mice have other similarities to KitW/W-v mice: they are sterile, have severe anemia, lack interstitial cells of Cajal, and have decreased numbers of bone marrow granulocytes and megakaryocytes (Galli and Kitamura, 1987; Heib et al., 2008).

Mast cell deficiency is also seen to variable degrees in other mice with single or double doses of mutant alleles at the W, Sl, Ph or mi locus. These mutants either have not as severe mast cell deficiencies as the KitW/W-v, KitW-sh/W-sh and Sl/Sl<sup>d</sup> mice described above, or they die soon after they are born (Galli and Kitamura, 1987).

Mast cells can be reconstituted in mast cell-deficient mice either by bone marrow transplantation (Grimbaldeston et al., 2005; Kitamura et al., 1977; Kitamura et al., 1978) or by injection of cultured in vitro differentiated mast cells. In the bone marrow transplantation, the recipient mouse is irradiated and cells from donor whole bone marrow are injected into the recipient mouse. In this case, other hematopoietic lineages from the transplanted bone marrow are reconstituted as well (Grimbaldeston et al., 2005), which results in challenges in the interpretation of the effects of the mast cells. These problems are avoided by reconstitution with cultured mast cells that, in addition, enable studies on specific mast cell proteins. The cultured mast cells for reconstitution are derived either from bone marrow (BMCMCs), from other sources of hematopoietic cells (such as fetal liver), or directly from embryonic stem cells (embryonic stem cell-derived cultured mast cells, ESCMCs) (Galli and Tsai, 2008; Metz et al., 2007). The source of cells may be wild-type mice or mice with genetic alteration(s), or, wild-type embryonic stem cells or embryonic stem cells with genetic alteration(s). Furthermore, the expression of specific proteins in BMCMCs can be reduced with short hairpin RNA (shRNA). Cultured mast cells can be
administered into mast cell-deficient recipient mice by intravenous, intraperitoneal, or intradermal injection, or by injection into the anterior wall of the stomach (Galli and Tsai, 2008; Metz et al., 2007).

### Table 8. Comparison of the characteristics of the most commonly used mast cell-deficient mice, *Kit*<sup>W/W-v</sup>, *Kit*<sup>W-sh/W-sh</sup> and *Sl/Sld* mice.

<table>
<thead>
<tr>
<th>Mouse</th>
<th><em>Kit</em>&lt;sup&gt;W/W-v&lt;/sup&gt;</th>
<th><em>Kit</em>&lt;sup&gt;W-sh/W-sh&lt;/sup&gt;</th>
<th><em>Sl/Sld</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Genetic background</td>
<td>WB/Re-W/+ x C57BL/6-W/+</td>
<td>C57BL/6</td>
<td>WC/Re-Sl/+ x C57BL/6-Sl/+</td>
</tr>
<tr>
<td>Locus</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>in chromosome</td>
<td>White spotting (W)</td>
<td>White spotting (W)</td>
<td>Steel (Sl)</td>
</tr>
<tr>
<td>coding for protein</td>
<td>c-kit</td>
<td>c-kit</td>
<td>SCF</td>
</tr>
<tr>
<td>mutation</td>
<td>W: deletion</td>
<td>W*: inversion</td>
<td>Sl: deletion</td>
</tr>
<tr>
<td></td>
<td>W*: missense</td>
<td></td>
<td>Sl*: deletion</td>
</tr>
<tr>
<td>Phenotype</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Coat colour</td>
<td>white</td>
<td>white</td>
<td>white</td>
</tr>
<tr>
<td>Virtual lack of melanocytes</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Sterility</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Anemia</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Mast cells in adult mice (10-12 weeks)*</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Lack of interstitial cells of Cajal</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Reconstitution of mast cells</td>
<td>by whole bone marrow</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>by injection of cultured mast cells</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>by injection of SCF</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>

+ indicates characteristics are seen, - indicates characteristics are not seen, * minor amounts seen in skin, **after bone marrow transplantation in *Kit*<sup>W-sh/W-sh</sup> mice, the numbers of mast cells are significantly higher than those found in *Kit*<sup>W/w</sup> (wild-type) mice. In addition, other hematopoietic lineages of donor origin are developed as well (Grimbaldeston et al., 2005). Data derived from (Grimbaldeston et al., 2005) and references mentioned in the text.

Even though this reconstitution technology is elegant and gives new insights into mast cell biology in vivo, there are some points of concern. After the injection of cultured mast cells, phenotypic characteristics, anatomical distribution and numbers of the cells originating from the injected cells, may change depending on the interval after injection (Metz et al., 2007). In addition, depending on the anatomical site, the level of mast cell reconstitution may vary. Indeed, after reconstitution with BMCMCs, no mast cells were observed in the brain, spinal cord, lymph nodes and heart tissues of *Kit*<sup>W/W-v</sup> mice (Tanzola et al., 2003) or in the tongue, trachea or skin of *Kit*<sup>W-sh/W-sh</sup> mice (Wolters et al., 2005), tissues that in wild-type mice contain significant amounts of native mast cells. Furthermore, the phenotypic characteristics of native and in vitro differentiated reconstituted mast cells have differences, and even though reconstituted mast cells gradually come to resemble the native ones, it is probably impossible to prove that, at a specific anatomical site, the reconstituted mast cells are ‘identical’ to the native mast cells in wild-type mice (Metz et al., 2007).

### 3. Mast cells and atherosclerosis

The number of mast cells is highly elevated in the atherosclerotic vessel wall compared to the level in normal healthy arteries (Atkinson et al., 1994; Jeziorska et al., 1997; Kaartinen et al.,
Review of the literature

1994a). In the arterial intima, normal human coronary arteries contain only few mast cells (on average, 1 mast cell/mm², 0.1% of total intimal cells) while fivefold higher values (on average, 5 mast cells/mm², 0.9% of total intimal cells) have been reported in fatty streaks (Kaartinen et al., 1994a). In the human aorta, however, even healthy intima contains considerable numbers of subendothelial mast cells (on average, 15 mast cells/mm²) (Kaartinen et al., 1994b). Thus, the number of mast cells in the intima of coronary as well as carotid arteries, but not of aorta, increases as the atherosclerotic lesions become more advanced (Jeziorska et al., 1997; Kaartinen et al., 1994a; Kaartinen et al., 1994b; Kovanen et al., 1995). In advanced lesions of coronary arteries, the number of mast cells in the regions of cap and core is on average 2 mast cells/mm² (0.5% of total intimal cells), while the shoulder regions of the lesions contain on average 6 mast cells/mm² (1.1% of total intimal cells) (Kaartinen et al., 1994a). Interestingly, the actual sites of plaque erosion and rupture are highly infiltrated with mast cells (28 mast cells/mm², 6% of total intimal cells) suggesting a role for mast cells in these atherothrombotic events (Kovanen et al., 1995). In the medial layer, both healthy and atherosclerotic arteries are mainly devoid of mast cells (Jeziorska et al., 1997; Kaartinen et al., 1994b) although occasional mast cells have been reported in atherosclerotic arteries (Atkinson et al., 1994). In the outermost layer of the vessel, the adventitia, high numbers of mast cells are seen even in healthy coronary arteries (19 mast cells/mm²), and their numbers are increased in atherosclerotic arteries both in non-ruptured plaques (41 mast cells/mm²) and in ruptured plaques (98 mast cells/mm²) (Laine et al., 1999). Also numbers of macrophages and T-lymphocytes are increased both in the shoulder regions of the intima, and in the adventitial layer (Kaartinen et al., 1994a; Laine et al., 1999).

In the normal aortic intima, 60% of mast cells are of the MCₜ subtype, and 40% of the MCₜc subtype (Kaartinen et al., 1994b) even though the proportion of chymase-containing mast cells, whether in aorta or coronary of healthy or atherosclerotic intima, shows high variation between subjects (Kovanen, 2007). In carotid intima, levels of 80-95% have been reported for MCₜc (Jeziorska et al., 1997). Of the adventitial mast cells, the majority seems to be of the MCₜc subtype, the proportion of them being 66%, 80%, and 88% in arteries containing normal intima, no rupture or rupture, respectively (Laine et al., 1999). The accumulation of mast cells in the arteries is considered to be mediated by SCF expressed in the arterial endothelial and smooth muscle cells (Miyamoto et al., 1997), and by eotaxin expressed in activated vascular smooth muscle cells (Haley et al., 2000). Indeed, mast cells expressing CC-chemokine receptor (CCR)-3, a chemokine receptor for eotaxin, have been reported in atherosclerotic lesions (Haley et al., 2000). In mice, the number of mast cells in atherosclerotic intima is small or even negligible (Bot et al., 2008; Lindstedt et al., 2007; Sun et al., 2007) whereas in the adventitia, mast cells are numerous and so may have the potential to promote the progression of atherosclerosis and destabilization of plaques (Bot et al., 2007).

Mast cell activation is also increased in the atherosclerotic arteries. The number of activated mast cells is especially high in the shoulder regions prone to rupture (Kaartinen et al., 1994a), and at the actual sites of erosion or rupture in patients who died of myocardial infarction (Kovanen et al., 1995). At these sites, the proportion of degranulated mast cells is approximately 85% as compared to the 17-28% levels seen in the normal intima (Kaartinen et al., 1994a; Kovanen et al., 1995). The mechanisms of mast cell activation in atherosclerotic plaques are largely unknown. Potential mediators include inflammatory mediators such as oxidized LDL (Kelley et al., 2006), complement anaphylatoxin C5a (el-Lati et al., 1994; Laine et al., 2002; Oksjoki et al., 2007) and
inflammatory mediators from macrophages and T-lymphocytes that are localized in the same arterial regions as mast cells (Kaartinen et al., 1994a; Kovanen et al., 1995; Lindstedt et al., 2007). Mast cell activation may also proceed upon direct contact with activated T-lymphocytes (Baram et al., 2001). Bacteria and various microbes present in atherosclerotic plaques also seem to have the potential to activate mast cells (Oksaharju et al., 2009). In addition, nicotine from cigarette smoke (Helske et al., 2006) and stress in a mouse model of acute restraint stress (Huang et al., 2002), have been reported to activate mast cells, both smoking and psychological stress being known risk factors for acute myocardial infarction (O'Keefe et al., 2009). Furthermore, adventitial mast cells that contain high amounts of mast cell-nerve contacts may be activated by neuropeptides such as substance P and calcitonin gene-related peptide (CGRP) (Laine et al., 2000). Indeed, substance P has been reported to activate adventitial mast cells in mice (Bot et al., 2010).

During the early events of atherosclerosis, mast cells may increase the accumulation of LDL in the intima. Histamine released from locally activated mast cells is capable of increasing endothelial permeability and thus the transendothelial transport of LDL into tissues, e.g. the skin (Ma and Kovanen, 1997). Heparin proteoglycans from activated mast cell granules are capable of binding LDL (Kokkonen and Kovanen, 1987a), thus facilitating granule-bound chymase, together with carboxypeptidase A, to proteolyze apoB-100 on LDL (Kokkonen et al., 1986). This results in the fusion of LDL and the formation of larger lipid droplets on mast cell granules (Kokkonen and Kovanen, 1989b). These particles containing fused LDL and granule remnants are then taken up by macrophages (Kokkonen and Kovanen, 1989b; Kokkonen, 1989) and smooth muscle cells (Wang et al., 1995) resulting in the formation of foam cells (Kaartinen et al., 1995). Furthermore, this uptake of LDL by macrophages proceeds via SRs (Lindstedt et al., 1992). On the other hand, mast cells have been reported to inhibit macrophage-induced oxidation of LDL which may lead to atheroprotection (Lindstedt et al., 1993; Lindstedt, 1993). Mast cells may also have inhibiting effects on reverse cholesterol transport. Chymase is capable of degrading apoA-I in pre-β-HDL as well as apoA-II, apoA-IV and apoE, and thus can reduce the removal of cholesterol by HDL from macrophage foam cells (Lee et al., 1992; Lee et al., 1999; Lee et al., 2002a; Lindstedt et al., 1996). The mechanism involved seems to include the inhibition of the ABCA1 efflux pathway (Favari et al., 2004). Furthermore, tryptase is also capable of degrading apolipoproteins in HDL thus blocking its function as a cholesterol acceptor (Lee et al., 2002b). In addition, chymase is also capable of inhibiting the activities of PLTP and CETP (Lee et al., 2003b; Lee-Rueckert et al., 2008).

As described above, activated mast cells are capable of secreting a wide variety of inflammatory mediators, many of which have pro-inflammatory properties. The mast cell mediator histamine has the ability to induce adhesion molecule expression in endothelial cells, and thus contributes to the recruitment of other inflammatory cells into the plaque (Burns et al., 1999). In addition, mast cell-derived TNF-α and TGF-β are capable of inducing the production of MCP-1 (Gordon, 2000), and tryptase can induce expression of MCP-1 and IL-8 in endothelial cells (Kinoshita et al., 2005), suggesting that mast cells may recruit monocytes into the plaque. Mast cells may also have a role in recruitment of neutrophils (Chen et al., 2002). In addition, mast cell-derived TNF-α is able to enhance the activation of T-lymphocytes (Nakae et al., 2005). Finally, mast cell-derived IFN-γ and IL-6 have been shown to mediate the in vivo effects of mast cells on the progression of atherosclerosis in ldlr-/- mice (Sun et al., 2007).
Mast cells may also more directly participate in the destabilization of atherosclerotic plaques through remodeling of the extracellular matrix. Mast cells are able to synthesize and release MMP-1 (Di Girolamo and Wakefield, 2000) that has been reported in atherosclerotic plaques (Nikkari et al., 1995). Mast cell-derived TNF-α present at sites of plaque rupture (Kaartinen et al., 1996) is capable of inducing synthesis and release of MMP-9 in autocrine fashion, and in paracrine fashion from macrophages (Baram et al., 2001; Saren et al., 1996). Mast cell protease chymase is able to activate pro-MMP-1 and pro-MMP-9, and may also activate pro-MMP-2, pro-MMP-3 and pro-MMP-9 (Fang et al., 1996; Furubayashi et al., 2008; Kishi et al., 2007; Lees et al., 1994; Saarinen et al., 1994; Suzuki et al., 1995; Tchougounova et al., 2005), while mast cell tryptase has been reported to activate pro-MMP-3 (Gruber et al., 1989; Lees et al., 1994). Also cathepsin G has been reported to activate pro-MMP-1 (Son et al., 2009). Indeed, MMP activation induced by activated mast cells has been reported in atherosclerotic human carotid arteries in vitro (Johnson et al., 1998). In addition, chymase can degrade MMP inhibitor TIMP-1 (Frank et al., 2001). Chymase, tryptase and cathepsin G are also capable of degrading extracellular matrix proteins such as fibronectin and type IV collagen (Kielty et al., 1993; Lohi et al., 1992; Vartio et al., 1981). In addition, vitronectin has been reported to be degraded by chymase and cathepsin G (Banovac and De Forteza, 1992; Rao et al., 1991). Furthermore, chymase can reduce the synthesis of extracellular components by inhibiting the expression of collagen in smooth muscle cells (Wang et al., 2001). Mast cells may also regulate the cellular content in atherosclerotic plaques. Mast cell-derived heparin proteoglycans have been reported to inhibit the proliferation of smooth muscle cells (Wang and Kovanen, 1999). In addition, mast cell chymase and TNF-α have been reported to possess pro-apoptotic properties (Ebihara et al., 2005b; Hara et al., 1999; Leskinen et al., 2001; Lätti et al., 2003; Wallach, 1997). Chymase is able to induce apoptosis of smooth muscle cells by degrading pericellular fibronectin with subsequent disruption of focal adhesions (Leskinen et al., 2003). TNF-α is capable of inducing apoptosis in endothelial cells by a mechanism involving downregulation of bcl-2 (Lätti et al., 2003). Furthermore, in the carotid arteries of atherosclerotic apoE−/− mice, targeted activation of adventitial/perivascular mast cells increases the incidence of macrophage apoptosis, intraplaque hemorrhage, vascular leakage and CXCR2/very late antigen (VLA)-4-mediated recruitment of leukocytes to the plaque (Bot et al., 2007). Thus, mast cells may contribute to the vulnerability of the plaque and its subsequent erosion or rupture.
AIMS OF THE STUDY

Atherosclerosis is an inflammatory disease involving the accumulation of mast cells both in fatty streaks (Atkinson et al., 1994; Kaartinen et al., 1994b) and in the shoulder regions of plaques prone to atherothrombotic events (Kaartinen et al., 1994a). In addition, the number of mast cells increases with the severity of unstable coronary syndromes (Kaartinen et al., 1998). Furthermore, activated mast cell infiltrates have been reported at the actual sites of plaque erosion and rupture (Kovanen et al., 1995; Laine et al., 1999). In our laboratory, it has been previously shown that mast cells induce apoptosis of smooth muscle cells (Leskinen et al., 2001; Leskinen et al., 2003) and endothelial cells (Lätti et al., 2003) in vitro. These and abundant experimental data suggest a role for mast cells in the regulation of atherosclerotic plaque stability.

The specific aims of the study were:

1. To clarify if mast cells have an effect on lipid accumulation and the level of vascular inflammation, two major determinants of plaque instability, in vivo.

2. To further elucidate the mechanisms of mast cell-induced apoptosis of smooth muscle cells and endothelial cells.

3. To clarify if mast cells have an effect on extracellular matrix remodeling and apoptosis in vivo.

4. To study mast cell protease-induced endothelial erosion, and characterize its possible mechanisms.
MATERIALS AND METHODS

1. Methods used in publications I-IV

The main methods used in this study are listed in table 9, and detailed materials and methods are presented in the original publications. If a more detailed protocol has been described elsewhere, the reference is provided in table 9. Only methods for the unpublished data are described below.

Table 9. Methods used in this study.

<table>
<thead>
<tr>
<th>Method</th>
<th>Original publication</th>
<th>Reference/Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Animal protocols</td>
<td>I, II, III</td>
<td></td>
</tr>
<tr>
<td>Human coronary artery samples</td>
<td>IV</td>
<td>(Wang and Kovanen, 1999)</td>
</tr>
<tr>
<td>Isolation and culture of rat aortic smooth muscle cells</td>
<td>II</td>
<td>(Piper et al., 1990)</td>
</tr>
<tr>
<td>Isolation and culture of rat cardiac microvascular endothelial cells</td>
<td>III, IV</td>
<td>PromoCell, Germany</td>
</tr>
<tr>
<td>Isolation of serosal mast cells</td>
<td>III</td>
<td>(Kokkonen and Kovanen, 1989a)</td>
</tr>
<tr>
<td>Stimulation of serosal mast cells</td>
<td>III</td>
<td>(Lindstedt et al., 2001)</td>
</tr>
<tr>
<td>Isolation of mast cell chymase</td>
<td>III, IV</td>
<td>(Andrews and Faller, 1991)</td>
</tr>
<tr>
<td>Isolation of mitochondria</td>
<td>II</td>
<td></td>
</tr>
<tr>
<td>Preparation of nuclear extracts</td>
<td>II, III</td>
<td>(Andrews and Faller, 1991)</td>
</tr>
<tr>
<td>Western blotting</td>
<td>II, III, IV</td>
<td></td>
</tr>
<tr>
<td>Histochemical stainings</td>
<td>I</td>
<td></td>
</tr>
<tr>
<td>Sudans IV, oil-red-O, toluidine blue</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Immunochemical stainings</td>
<td>II, III</td>
<td>(Pentikäinen et al., 2002b)</td>
</tr>
<tr>
<td>Electrophoretic mobility shift assay (EMSA)</td>
<td>II, III</td>
<td></td>
</tr>
<tr>
<td>Competitive reverse transcriptase polymerase chain reaction (RT-PCR)</td>
<td>II</td>
<td>(Lätti et al., 2003)</td>
</tr>
<tr>
<td>Determination of apoptosis</td>
<td>II, III</td>
<td></td>
</tr>
<tr>
<td>Caspase activity (3, 8, 9)</td>
<td>II, III</td>
<td></td>
</tr>
<tr>
<td>DNA fragmentation (ELISA)</td>
<td>III</td>
<td></td>
</tr>
<tr>
<td>Flow cytometry</td>
<td>III</td>
<td></td>
</tr>
<tr>
<td>Normal and fluorescence microscopy</td>
<td>I, II, III, IV</td>
<td></td>
</tr>
<tr>
<td>Confocal microscopy</td>
<td>II</td>
<td></td>
</tr>
<tr>
<td>Scanning electron microscopy</td>
<td>IV</td>
<td></td>
</tr>
<tr>
<td>Computer-assisted morphometry/image quantification</td>
<td>I</td>
<td></td>
</tr>
<tr>
<td>Enzyme-linked immunosorbent assay (ELISA)</td>
<td>I, III</td>
<td></td>
</tr>
<tr>
<td>Lipid extraction</td>
<td>I</td>
<td>(Folch et al., 1957)</td>
</tr>
<tr>
<td>Lipid measurements</td>
<td>I</td>
<td></td>
</tr>
<tr>
<td>Size-exclusion chromatography</td>
<td>I</td>
<td></td>
</tr>
<tr>
<td>Determination of serum PLTP activity</td>
<td>I</td>
<td>(Damen et al., 1982; Jauhiainen and Ehnholm, 2005)</td>
</tr>
</tbody>
</table>

2. Brachiocephalic arteries

To induce atherosclerosis, 12-week-old male mast cell-deficient and -competent LDL receptor knockout mice, \( ldl^{-/-}\)Kitt\( W_{sh}/W_{sh} \) and \( ldl^{-/-}\)Kitt\( +/+ \), respectively, were fed a Western diet (21% fat,
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0.15% cholesterol, Harlan-Teklad 88137, Madison, WI) for 26 weeks (same mice as in Study I). The whole aorta was dissected out and fixed with 4% paraformaldehyde (Sigma, Germany) in PBS overnight. The brachiocephalic artery was dissected out under a preparation microscope, fixed in ~4% formalin (Sigma, Cat. Accustain HT50-1-128), and embedded in paraffin (HistoLab Products, Sweden, Cat. 00402-1). 3 μm sections were cut throughout the vessel, and stainings were performed on sections within the segment of the brachiocephalic artery 100-250 μm proximal from the point where the brachiocephalic artery branches into the carotid and subclavian arteries (Teupser et al., 2003). Every 10th section, corresponding to sections every 30 μm, was used for the the particular staining.

3. Histochemistry and immunohistochemistry

Sections were dewaxed and rehydrated, and elastin and collagen were visualized with ACCUSTain Elastic Stain (Sigma, Cat. HT25) and Trichrome Stain (Masson) (Sigma, Cat. HT15), respectively, according to the manufacturer’s instructions. Elastin staining is a modified Verhoeff van Gieson staining with a hematoxylin-iodine-ferric chloride solution and van Gieson solution detecting elastin and collagen, respectively. With Masson’s trichrome staining, collagen is stained by aniline blue, nuclei with Weigert’s iron hematoxylin, and cytoplasm and muscle with Beibrich scarlet-acid fuchsins. Mast cells were detected with toluidine blue staining: sections were stained with 0.25% toluidine blue (Fluka, Cat. 89640) in 1% NaCl in 70% ethanol for 15 min. and washed with water for 15 min. Apoptosis, macrophages and smooth muscle cells were detected by immunohistochemical stainings with cleaved caspase-3 (Cell Signaling Technology, Cat. 9661, 1:100), Mac-3 (BD Biosciences, Cat. 550292, 1:50) and α-smooth muscle actin (Sigma, Cat. A-5228, 1:200) antibodies, respectively. For cleaved caspase-3 staining, antigen retrieval was performed by incubation in 10 mM sodium citrate buffer pH 6.0 at sub-boiling temperature for 10 min. Endogenous peroxidase was blocked with 3% hydrogen peroxide for 5-10 min. For α-smooth muscle actin staining (Johnson et al., 2005), sections were blocked with 20% normal goat serum (Vector Laboratories, Cat. S-1000) for 30 min, and after overnight incubation with primary antibody Mouse on Mouse kit (Vector Laboratories, Cat. PK-2200) was used according to the protocol of the manufacturer. Other stainings were performed with VectaStain ABC Elite kits (Vector Laboratories, Cat. PK-6101 and PK-6104), according to the protocol of the manufacturer, except for the overnight incubation of primary antibodies. 3,3'-diaminobenzidine (DAB, Sigma, Cat. D5637) was used as a chromogen, and Mayer’s hematoxylin (Sigma, Cat. 51275) as a counterstain. For cleaved caspase-3 staining, incubation with blocking peptide (Cell Signaling Technology, Cat. 1050) and primary antibody, instead of primary antibody alone, served as negative control. For Mac-3 and α-smooth muscle actin stainings, incubation with an isotype control (BD Biosciences, Cat. 559072 and AbD Serotec, Cat. MCA929, respectively), instead of primary antibody, served as negative control. Samples were either mounted with Aqueous mounting media (Dako, Cat. S3025, for toluidine blue staining) or dehydrated and mounted with UltraKitt mounting media (J.T. Baker, Cat. 3905, for other stainings).

4. Image analysis

Tissue sections were viewed with a Nikon Eclipse E600 microscope (Nikon Co., Tokyo, Japan) and photographed with a digital camera (Spot RT color operated with Spot advanced software, version 4.1, Diagnostic Instruments, Sterling Heights, MI, USA). Cleaved caspase-3 positive cells and all nucleated cells were counted manually, and a total area with positive color was
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quantified from the Mac-3, α-smooth muscle actin and collagen images in a blinded fashion using computer-assisted image quantification (Image-Pro Plus software, Media Cybernetics).

5. Statistics
Data are expressed as medians±SD. To compare two groups, a Mann-Whitney U-test was used. A level of $P<0.05$ was considered statistically significant.
RESULTS AND DISCUSSION

1. Mast cells induce an atherogenic lipid profile and lipid accumulation in the arterial intima (I and unpublished data)

When beginning this work, in vivo studies on the effects of mast cells in lipid accumulation in atherosclerosis were scarce (Kaartinen et al., 1995; Uehara et al., 2002). Thus, since mice with spontaneous mutations in c-kit gene exhibit virtual mast cell deficiency (Grimbaldeston et al., 2005), and since the lipoprotein profile of LDL receptor knockout mice (ldlr⁻/⁻) fed a Western diet most closely resembles that of human (Ishibashi et al., 1994; Zadelaar et al., 2007), we crossbred mast cell-deficient Kit⁺⁻/⁻ mice with ldlr⁻/⁻ mice to generate mast cell-deficient and -competent ldlr⁻/⁻ mice, notably ldlr⁻/⁻/Kit⁺⁻/⁻ and ldlr⁻/⁻/Kit⁺/⁺, respectively. After feeding the mice a Western diet (21% total fat, 0.15% cholesterol) for 26 weeks, we estimated the effect of mast cell deficiency on the progression of atherosclerosis in vivo. Since a serum lipid and lipoprotein profile containing high levels of apoB-containing lipoproteins VLDL, IDL and LDL is a prerequisite for the development of atherosclerosis and critical in determining the rate of the progression of atherosclerosis (Ishibashi et al., 1994), we measured these factors in the mice. Mast cell-deficient ldlr⁻/⁻ mice had lower levels of cholesterol and triglycerides in serum compared to their mast cell-competent ldlr⁻/⁻ mice (Study I, Fig. 3), and the decrease of cholesterol and triglycerides was seen in apoB-containing lipoproteins VLDL, IDL and LDL (Study I, Fig. 6). Thus, the lipoprotein profile in mast cell-deficient ldlr⁻/⁻ mice is less atherogenic than in mast cell-competent littermates, suggesting a role for mast cells in the regulation of plasma lipoprotein profile, and subsequently possibly also in the accumulation of these lipoproteins in the arterial intima. The intake of lipids from diet is important in determining plasma lipoprotein profile both in humans and in mice (Brown et al., 2007; Sanders, 2009). Interestingly, the small intestine contain abundant numbers of mast cells (Bischoff, 2009) which might participate in the regulation of absorption of dietary lipids.

In a similar study by Sun and coworkers, in which mast cell-deficient ldlr⁻/⁻/Kit⁺⁻/⁻ mice and mast cell-competent ldlr⁻/⁻ littermates were fed a Western diet for 26 weeks, no significant changes in lipid profiles were reported (Sun et al., 2007). Interestingly, as compared to the levels reported by Sun and coworkers, the lipid profile of our ldlr⁻/⁻ control mice showed higher levels of total cholesterol (37.9±14.2 mmol/l versus 21.7±1.7 mmol/l, respectively) and triglycerides (5.6±2.5 mmol/l versus 1.3±0.2 mmol/l, respectively) in serum (Table 10). Since the background of the mice in both studies is similar, C57BL/6, the differences in lipid levels may result from differences in the diet even though the differences in diets seem minor. Compared to the diet we used, the diet used by Sun and coworkers (Western diet from Research Diets) contains 1% corn oil and 20% milk fat instead of 21% milk fat, and 10% maltodextrin and 5% corn starch instead of 15% corn starch. Corn oil contains more polyunsaturated fatty acids known to have atheroprotective properties whereas milk fat contains more saturated fatty acids promoting atherosclerosis (Temel and Rudel, 2007). Dextrins are a group of carbohydrates generated by the hydrolysis of starch and may have the ability to affect the lipid profile. For instance, when administered in the diet, α-cyclodextrin derived from corn starch is non-absorbable and induces a decrease in the plasma cholesterol level (Wagner et al., 2008). Corn starch and maltodextrin, however, are similarly digested in the intestine (Bauer et al., 2003) suggesting that the difference between the two Western diets concerning carbohydrates may be irrelevant. How these dietary
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differences then could explain the differences seen between mast cell-deficient and -competent mice remains to be elucidated. Furthermore, in mast cell-deficient Sl/Sl^d mice fed on a diet containing high levels of fat, cholesterol, and cholic acid for 17 weeks, no changes in plasma cholesterol were reported, but a decrease in triglycerides compared to normal mice was reported (Dileepan et al., 2004). Overall, the importance of the lipoprotein profile for the stability of the plaques in humans is highlighted by the facts that high total cholesterol, low HDL-cholesterol, and a high total cholesterol/HDL-cholesterol ratio is correlated with the incidence of plaque rupture (Burke et al., 1997; Burke et al., 1998). Whether this applies to ldlr^−/− or apoE^−/− mice on a Western diet as well is presently not known. Actually, it may be difficult to get such correlations since plaque rupture is only rarely seen in mice (Schwartz et al., 2007). In mice, however, simvastatin, when administered to apoE^−/− mice exhibiting advanced unstable plaques, has been reported to induce an increase in serum cholesterol but a decrease in intraplaque hemorrhage and calcification, signs of plaque vulnerability (Bea et al., 2002). These unexpected results are compatible with the idea that the statin exhibited lipid-independent plaque-stabilizing pleiotropic effects.

Table 10. Comparison of lipid accumulation and factors affecting it between the study by Sun et al 2007 and this study.

<table>
<thead>
<tr>
<th></th>
<th>Study by Sun et al 2007</th>
<th>This study</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Diet (for 26 weeks)</strong></td>
<td>Western diet (Research Diets, D12079B)</td>
<td>Western diet (Harlan-Teklad, TD.88137)</td>
</tr>
<tr>
<td>Fat</td>
<td>20% milk fat 21% milk fat</td>
<td>21% milk fat</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>5% corn starch 10% maltodextrin</td>
<td>15% corn starch</td>
</tr>
<tr>
<td><strong>Mice (male)</strong></td>
<td>ldlr^−/−/KitW-sh/W-sh</td>
<td>ldlr^−/−/KitW-sh/W-sh</td>
</tr>
<tr>
<td>Body weight gain (g)</td>
<td>not mentioned</td>
<td>9.1±6.7</td>
</tr>
<tr>
<td>Serum cholesterol (mmol/l)</td>
<td>23.5±2.8</td>
<td>21.7±1.7</td>
</tr>
<tr>
<td>Serum triglycerides (mmol/l)</td>
<td>1.23±0.2</td>
<td>1.26±0.2</td>
</tr>
<tr>
<td><strong>Level of atherosclerosis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aortic arch (longitudinal section, mm²)</td>
<td>0.13</td>
<td>0.26</td>
</tr>
<tr>
<td>Aortic sinus (cross section, mm²)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>En face aorta</td>
<td>Without arch 12%</td>
<td>32%</td>
</tr>
<tr>
<td></td>
<td>With arch</td>
<td></td>
</tr>
</tbody>
</table>

*only constituents differing are shown

As expected based on the differences in lipid profiles, our results showed that the accumulation of lipids in the arterial wall decreased in the mast cell-deficient ldlr^−/− mice. The areas of atherosclerotic plaques in the aorta were smaller in the mast cell-deficient ldlr^−/− mice compared to mast cell-competent littermates when measured both from Sudan IV-stained en face prepared whole aortas (in male mice, 18±7% versus 27±8%, respectively) (Study I, Fig. 1) and oil-red-O-stained aortic root sections at the level of the aortic sinus (in male mice, 190±110×10³ μm² versus 419±60×10³ μm², respectively) (Study I, Fig. 2). Plaque area in the brachiocephalic artery, however, as measured luminal to the internal elastic lamina from elastin-stained cross sections, did not show differences between mast cell-deficient and -competent ldlr^−/− mice (0.18±0.12 mm² versus 0.21±0.08 mm², respectively, P=1.00) (Figure 4A). Similarly to our findings in the aorta,
Sun and coworkers have reported decreased plaque size in the aorta of mast cell-deficient \(ldlr^{-/}\)/Kit\(^{W-sh/W-sh}\) mice compared to their mast cell-competent littermates (Sun et al., 2007) (Tables 10 and 11). In addition, Bot and coworkers have reported increased plaque size in the brachiocephalic artery after systemic activation of mast cells in \(apoE^{-/-}\) mice fed a Western-type diet (15% cacao butter and 0.25% cholesterol) for 8 weeks (Bot et al., 2007). Instead, after local adventitial mast cell activation in the carotid artery in the same study, no changes in the size of carotid plaques were seen (Bot et al., 2007). In another study of collar-induced carotid artery atherosclerosis in \(apoE^{-/-}\) mice fed a Western diet (15% lard and 0.25% cholesterol) for 5 weeks, plaque area increased upon mast cell activation (Tang et al., 2009). Thus, it seems that the aorta is the most susceptible artery to show increased accumulation of lipids induced by mast cells, and the effects in the brachiocephalic and carotid arteries are variable even though direct comparison between different mouse models and in different activation states of mast cells is difficult to carry out. The possible effects of mast cells on the size of the plaques depend on the presence of mast cells, an issue that will be discussed in the next section. The localization of the plaques in the arterial tree, and subsequently local shear stress conditions, may also have a role in determining the susceptibility of a plaque to further progression of atherosclerosis. In mice, the most susceptible sites may not be similar to those in humans. In mice, the aortic root shows a considerable amount of atherosclerosis whereas in humans, atherosclerosis has characteristically not been detected at this site (VanderLaan et al., 2004; Wouters et al., 2005). Thus, it would be important to know at what site of the mouse arterial tree the plaques most closely resemble those in humans. In terms of plaque rupture or disruption, this has been suggested to be the brachiocephalic artery (Schwartz et al., 2007). Regarding plaque size, differences have been reported in one location while no effects or even reversed effects were seen in the other location (Reardon et al., 2003; Teupser et al., 2004). Thus, the effect of mast cells on the size of the plaque may differ depending on the site of the plaque in the arterial tree as well as on the experimental setup. Interestingly, differences in the plaque area in the brachiocephalic artery of fractalkine (CX3CL1)-deficient and -competent \(ldlr^{-/-}\) mice have been reported at the location of 200 \(\mu\)m but not at 400 \(\mu\)m or 600 \(\mu\)m from the point where the brachiocephalic artery branches into the carotid and subclavian arteries (Teupser et al., 2004). In addition, the size of the plaque and its stability may be modulated independently (Bea et al., 2002; Jackson et al., 2007; Johnson et al., 2005).

2. Mast cells are located in the arterial adventitia in mouse (I and unpublished data)

In humans, abundant numbers of mast cells are located in the arterial intima (Kaartinen et al., 1994a; Kaartinen et al., 1994b; Kovanen et al., 1995). In mice, however, intimal mast cells have been reported only by one group, which found them in the aortic arch by mMCP-4 immunostaining together with toluidine blue staining (Sun et al., 2007). Our results from both the aortic root (Study I, Fig. 2) and the brachiocephalic artery (Figure 4B) of \(ldlr^{-/-}\) mice fed a Western diet for 26 weeks show, by toluidine blue staining, adventitial/perivascular mast cells whereas in the intima we could not see any mast cells. Interestingly, in the aortic root, we found a significant positive correlation between the extent of atherosclerosis and the number of adventitial/perivascular mast cells (Study I, Fig. 2). Since the arterial media in mouse is very thin, adventitial mast cells may have the capacity to regulate the progression of atherosclerosis in the intimal layer via diffusion of its mediators into the intima across the medial layer. Indeed, Bot and coworkers have reported considerable numbers of adventitial mast cells (on average, 5 mast
Figure 4. Characterization of atherosclerosis in the brachiocephalic arteries. A) Plaque areas were measured from elastin-stained sections. The data are presented as means of sections in the segment studied for each mouse (dots) and as medians for the groups of mice (horizontal lines) (left); and as curves showing plaque areas in individual sections in the region studied (right). B) Mast cells are localized in the adventitia as shown by toluidine blue staining. Macrophages (C) were detected by Mac-3 immunostaining, collagen (D) by Masson’s trichrome staining, smooth muscle cells (E) by α-smooth muscle actin immunostaining, and apoptosis (F) by cleaved caspase-3 immunostaining. Data represent means of sections in the segment of the brachiocephalic artery between 100-250 μm proximal from the point where the brachiocephalic artery branches to subclavian and cephalic arteries (dots), and medians of each group of mice (horizontal lines).

cells/mm², 45% of them activated) in the adventitia of collar-induced carotid lesions, and suggest a role for them in atherosclerotic plaque destabilization (Bot et al., 2007). Also human coronary adventitia contains an abundance of mast cells, the number of them being increased in the plaques (41 mast cells/mm²) and especially in ruptured plaques (98 mast cells/mm²) (Laine et al., 1999). Interestingly, decreased size of atherosclerotic plaques in mast cell-deficient Idlr⁻/⁻ mice compared to mast cell-competent littermates has been demonstrated in the aorta (Sun et al., 2007; Study I, Fig. 2), the artery that in humans has been reported to show very abundant amounts of mast cells even before initiation of atherosclerosis (Kaartinen et al., 1994b). Instead, the numbers of mast cells in normal human coronary and carotid intima are lower (Jeziorska et al., 1997; Kaartinen et al., 1994a; Lehtonen-Smeds et al., 2005) suggesting that only the largest arteries in humans contain mast cells in the intima (Pollak, 1957). Thus, the number of mast cells seems to
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reflect the thickness of the intima. If this applies also in mice with an extremely thin intima, it is understandable that mast cells are absent in normal mouse intima, and scarce or even absent in atherosclerotic intima. It could well be that the atherosclerotic lesions only in the aortic arch reach the required thickness for the infiltration of mast cells in the intima.

Table 11. Studies showing the effects of mast cell manipulation on atherosclerosis in the mouse.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Mast cell deficiency</th>
<th>Mast cell activation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sun et al 2007</td>
<td>This study</td>
</tr>
<tr>
<td><strong>Mouse</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>genetic background</td>
<td><strong>ldlr</strong>/-</td>
<td><strong>ldlr</strong>/-</td>
</tr>
<tr>
<td>age (weeks)</td>
<td>36</td>
<td>38</td>
</tr>
<tr>
<td>Diet (for weeks)</td>
<td>26</td>
<td>26</td>
</tr>
<tr>
<td>Western diet (fat, cholesterol)</td>
<td>21%, 0.15%</td>
<td>21%, 0.15%</td>
</tr>
<tr>
<td>Artery studied</td>
<td>aortic arch en face</td>
<td>aortic sinus en face</td>
</tr>
<tr>
<td><strong>Plaque area</strong></td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td><strong>Inflammation</strong></td>
<td>↓ ns ns (↓)</td>
<td>ns ns (↓)</td>
</tr>
<tr>
<td><strong>Collagen</strong></td>
<td>↓ ns ns (↓)</td>
<td>ns ns (↓)</td>
</tr>
<tr>
<td><strong>Smooth muscle cells</strong></td>
<td>± ns ns (↓)</td>
<td>ns ns (↓)</td>
</tr>
<tr>
<td><strong>Apoptosis</strong></td>
<td>↓ ns ns (↓)</td>
<td>ns ns (↓)</td>
</tr>
</tbody>
</table>

BCA, brachiocephalic artery; ↑ increased change as compared to control; ↓ decreased change as compared to control; ± no change as compared to control; ns, not studied; arrow in parenthesis, change not significant (only trend).

3. Mast cells induce vascular inflammation (I and unpublished data)

Since mast cells have proinflammatory properties such as the ability to recruit other inflammatory cells (Lindstedt et al., 2007), and infiltration of mast cells is associated with the infiltration of macrophages and T-lymphocytes in human atherosclerotic plaques (Kaartinen et al., 1994a; Laine et al., 1999), we measured the level of the other major regulator of plaque stability, inflammation, in mast cell-deficient **ldlr**/-/Kit**W-sh/W-sh** and -competent **ldlr**/-/Kit**+/+** mice fed a Western diet for 26 weeks. The serum levels of soluble ICAM-1 (sICAM-1), a marker of vascular inflammation (Lawson and Wolf, 2009), were markedly decreased in mast cell-deficient **ldlr**/- mice compared to mast cell-competent littermates (Study I, Fig. 8). In contrast, serum amyloid A, a marker of systemic inflammation (Ceciliani et al., 2002), did not differ between mast cell-deficient and -competent **ldlr**/- mice (Study I, Fig. 8). Macrophage content in the brachiocephalic arteries, as measured by immunohistochemical staining with the Mac-3 antibody, did not show significant changes between mast cell-deficient and -competent **ldlr**/- mice (5.6±6.3% and 12.1±6.8%, respectively, P=0.18, Figure 4C). In a similar study by Sun and coworkers, decreased levels of macrophages and T-lymphocytes were reported in the aortic arch of mast cell-deficient **ldlr**/-/Kit**W-sh/W-sh** mice fed a Western diet for 26 weeks compared to mast cell-competent littermates (Sun et al., 2007) (Table 11). On the contrary, in the study by Bot and coworkers, systemic activation of mast cells in **apoE**/- mice did not change the level of macrophages in the
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brachiocephalic artery (Bot et al., 2007). Still, the same study reported increased leukocyte adhesion in collar-induced carotid plaques that were locally stimulated to activate adventitial mast cells (Bot et al., 2007). In addition, another study of collar-induced carotid plaques with systemic (intraperitoneal) mast cell activation in apoE<sup>−/−</sup> mice reported increased levels of macrophages in stimulated carotid plaques as compared to nonstimulated ones (Tang et al., 2009). Interestingly, circulating levels of sICAM-1 have been reported to be predictive for future acute coronary syndromes (Haim et al., 2002). On the other hand, systemic inflammation may also increase the risk for atherothrombosis (van Leuven et al., 2008), even though our results, based on similar levels of serum amyloid A in both mouse groups, do not propose a role for mast cells in the regulation of systemic inflammation in ldlr<sup>−/−</sup> mice fed the specified Western diet for 26 weeks. Furthermore, characteristics of unstable plaques in humans include increased circulating levels of ICAM, VCAM, MMPs, TNF-α, IL-6, IL-10 and IL-18 (Sima et al., 2009). In addition, in patients with unstable carotid plaques, circulating levels of MMP-1, MMP-7, TIMP-1, TNF-α and IL-8 have been reported to be increased as compared to patients with stable plaques (Pelisek et al., 2009). Furthermore, in patients with coronary artery disease showing transmural narrowing of ≥50%, increased circulating levels of MMP-9 have been reported (Kalela et al., 2002). It remains for future studies to clarify whether others of these molecules besides sICAM-1 are also decreased in mast cell-deficient ldlr<sup>−/−</sup> mice compared to mast cell-competent littermates fed a Western diet. Furthermore, a similar study by Sun and coworkers suggested major roles for the proinflammatory cytokines IL-6 and IFN-γ in the mast cell-mediated promotion of atherosclerosis based on their results obtained by adoptive transfer of IL-6 and IFN-γ-deficient mast cells into mast cell-deficient ldlr<sup>−/−</sup> mice (Sun et al., 2007).

4. Mast cells induce extracellular matrix remodeling (III, IV and unpublished data)

Collagen is an abundant extracellular matrix protein in the fibrous cap, and important for the tensile strength of the plaque and the stability of the plaque (Burleigh et al., 1992; Falk et al., 1995). Collagen is produced by smooth muscle cells (Libby et al., 1995). We measured the level of collagen and smooth muscle cells in the brachiocephalic artery in mast cell-deficient ldlr<sup>−/−</sup>/Kit<sup>W-sh/W-sh</sup> and -competent ldlr<sup>−/−</sup>/Kit<sup>+/+</sup> mice fed a Western diet for 26 weeks. As shown in Figure 4D, the level of collagen in the plaques, as measured from Masson’s trichrome-stained sections, did not show significant changes between mast cell-deficient and -competent ldlr<sup>−/−</sup> mice (52.7±10.7% and 43.9±10.1%, respectively, P=0.09). As shown in Figure 4E, the content of smooth muscle cells in the plaques, as measured from α-smooth muscle actin-stained sections, were similar between mast cell-deficient and -competent ldlr<sup>−/−</sup> mice (8.7±3.5% and 14.0±6.9%, respectively, P=0.06). In contrast, Sun and coworkers have reported increased levels of collagen in the aortic arch of mast cell-deficient ldlr<sup>−/−</sup>/Kit<sup>W-sh/W-sh</sup> mice fed a Western diet for 26 weeks compared to mast cell-competent littermates (2.1% and 1.2%, as a % of positive area of picrosirius red birefringence, respectively) (Sun et al., 2007) (Table 11). In addition, increased content of smooth muscle cells have been reported in the study of collar-induced carotid plaques with systemic (intraperitoneal) mast cell activation in apoE<sup>−/−</sup> mice as compared to nonstimulated ones (Tang et al., 2009). Furthermore, Sun and coworkers have shown that mast cell-derived IL-6 and IFN-γ have capabilities in activating both MMPs and cathepsins in ldlr<sup>−/−</sup> mice (Sun et al., 2007), thus increasing the level of extracellular matrix degradation.
In addition, fibronectin and vitronectin are present in atherosclerotic lesions (Kakolyris et al., 1995; van Aken et al., 1997). Furthermore, in advanced human plaques, fibronectin has been reported as fragmented strands or as almost absent (Kakolyris et al., 1995), and a granular staining pattern has been reported for vitronectin in fibrous caps (van Aken et al., 1997). Thus, we studied the effect of mast cell proteases on the degradation of these glycoproteins. As suggested based on earlier reports (Banovac and De Forteza, 1992; Vartio et al., 1981) rat mast cell chymase (rMCP-1) degraded fibronectin and vitronectin both as purified proteins, and also in rat cardiac microvascular endothelial cells (Study III, Fig. 4). Furthermore, fibronectin degradation was seen in human coronary artery intima after incubation with chymase, tryptase, and a mixture of both in the lumen of the arteries ex vivo (Study IV, Fig. 6). In vitro, the degradation of fibronectin by chymase proceeds very rapidly. In the arterial wall, chymase inhibitors present in the intimal fluid such as α1-antitrypsin, α2-macroglobulin and α1-antichymotrypsin (Lindstedt et al., 2001) have the potential to decrease the rate of degradation. Nevertheless, in addition to heparin proteoglycans secreted together with chymase and tryptase in mast cell granules (Leskinen et al., 2003; Lindstedt et al., 2001; Pejler and Berg, 1995), proteoglycans isolated from human aorta have also been reported to stabilize tryptase (Lee et al., 2002b). Indeed, in coronary intima, fibronectin was almost completely lost after intraluminal incubation with chymase and/or tryptase (Study IV, Fig. 6). Pericellular fibronectin and vitronectin are critical for the survival of vascular smooth muscle and endothelial cells, and “outside-in” survival signaling is mediated through integrins α5β1 and αvβ3 that bind to fibronectin and vitronectin, respectively (Dejana et al., 1988; Matter and Ruoslahti, 2001; Wary et al., 1996). Mast cell chymase-mediated degradation of fibronectin with subsequent inhibition of survival signaling induces apoptosis of smooth muscle cells (Leskinen et al., 2003), and mast cell-mediated endothelial cell apoptosis includes degradation of both fibronectin and vitronectin as well as inhibition of survival signaling (Study III). Mechanisms of these apoptotic pathways will be discussed in the next section. In addition, chymase-induced epithelial cell apoptosis has been shown to be mediated by degradation of fibronectin (Ebihara et al., 2005b). Furthermore, various fragments of fibronectin have been reported to induce apoptosis of smooth muscle cells (Leskinen et al., 2003), endothelial cells (Fukai et al., 1998), epithelial cells (Schedin et al., 2000) and periodontal ligament cells (Dai et al., 2005). On the other hand, vitronectin has been reported to decrease apoptosis in endothelial cells (Isik et al., 1998).

5. Mast cells induce apoptosis (II, III and unpublished data)

5.1. Mast cell chymase-induced smooth muscle cell apoptosis is mediated by disruption of NF-κB-mediated survival signaling (II)

Smooth muscle cell apoptosis has been reported in the fibrous caps and the shoulder regions of atherosclerotic lesions (Björkerud and Björkerud, 1996; Geng and Libby, 1995), colocalized with the infiltration of inflammatory cells such as mast cells (Crisby et al., 1997; Kaartinen et al., 1994a; Kockx et al., 1998), and thought to contribute to the instability of the plaque (Bennett, 1999; Clarke et al., 2006). Mast cell chymase has been shown to induce apoptosis of rat aortic smooth muscle cells in vitro (Leskinen et al., 2001) by a mechanism involving degradation of pericellular fibronectin, disruption of focal adhesion complexes and inactivation of Akt-mediated survival signaling (Leskinen et al., 2003). Thus, we wanted to clarify the mechanisms downstream of Akt. Chymase was able to prevent the nuclear translocation of NF-κB (Study II, Fig. 1), which is required for the effects of NF-κB in inducing the activation, through binding to
DNA, of various effector genes (de Winther et al., 2005). Chymase also reduced the binding of p65 and p50 subunits of NF-κB to DNA (Study II, Fig. 2). Furthermore, chymase-induced nuclear p65 inhibition was mediated by caspases (Study II, Fig. 3). One of the genes activated by NF-κB is anti-apoptotic bcl-2 (de Winther et al., 2005), the expression of which was decreased by chymase both at the mRNA and protein level (Study II, Fig. 5). Instead, pro-apoptotic bax was only slightly decreased (Study II, Fig. 5). Also the localization of bcl-2 and bax changed upon chymase treatment: the mitochondrial staining of bcl-2 decreased whereas bax localization in the mitochondria increased with subsequent swelling of mitochondria (Study II, Fig. 4 and 6). Furthermore, chymase induced the release of cytochrome c from mitochondria (Study II, Fig. 7). Finally, both initiator caspase-8 and -9 as well as effector caspase-3 were activated (Study II, Fig. 8) (Figure 5). Thus, chymase seemed to activate both intrinsic and extrinsic pathways of apoptosis. As amplification of apoptosis may proceed through caspase-8-induced bid degradation with subsequent activation of the intrinsic pathway (Duprez et al., 2009; Youle and Strasser, 2008), the pathway described here may reflect a loop of amplification. The results of upstream pathways, leading from fibronectin degradation and disruption of focal adhesions to Akt and NF-κB inactivation (Leskinen et al., 2003) (Study II), suggest that the initiation of apoptosis proceeds via an extrinsic pathway. Indeed, chymase has extrinsic effects in the induction of smooth muscle cell apoptosis. Whether chymase can be internalized in smooth muscle cells and have direct intracellular effects as well remains to be clarified in future studies. In addition, chymase-induced mechanisms of smooth muscle cell apoptosis may involve other mechanisms and/or amplification inside the cell as well. Thus, the observed caspase-mediated nuclear p65 inhibition (Study II, Fig. 3) may derive from activation of apoptosis by various upstream pathways. The role for NF-κB in atherosclerosis and plaque stability, based on its ability to activate transcription of various inflammatory mediators such as MCP-1, ICAM-1, VCAM-1, selectins and cytokines (de Winther et al., 2005), may be considered as promoting the progression of the disease and plaque destabilization. In terms of smooth muscle cells, however, NF-κB is induced during their proliferation (Hoshi et al., 2000; Selzman et al., 1999) and is known to activate many antiapoptotic proteins such as bcl-2 (de Winther et al., 2005) thereby increasing the content of smooth muscle cells and the stability of the plaque. Thus, on the other hand, mast cells may induce plaque destabilization by inducing apoptosis of smooth muscle cells via inhibition of NF-κB.

5.2. Mast cell chymase and TNF-α-induced endothelial cell apoptosis is mediated by inactivation of FAK and Akt-dependent survival signaling (III)

Endothelial cell apoptosis is thought to be a contributor to plaque erosion (Durand et al., 2004), and is typically seen in the downstream areas of atherosclerotic plaques where turbulent blood flow prevails (Tricot et al., 2000). These areas prone to atherothrombotic events also contain infiltration of mast cells (Kaartinen et al., 1994a; Kovanen et al., 1995). Activated mast cells have been shown to induce apoptosis of rat cardiac microvascular endothelial cells by TNF-α-mediated downregulation of bcl-2 (Lätti et al., 2003). The study showed that TNF-α alone did not explain the level of apoptosis induced by activated mast cells. Thus, a combination of TNF-α neutralizing antibody and chymase inhibitor was necessary for full inhibition of the observed level of mast cell releasate-induced endothelial cell apoptosis (Study III, Fig. 1). Mast cell releasate, containing all the mediators released from activated mast cells upon stimulation by compound 48/80, induced FAK degradation and Akt dephosphorylation (Study III, Fig. 2), the effects of which were mediated by chymase, but not by TNF-α (Study III, Fig. 3). Furthermore,
Results and discussion

as already mentioned above, chymase induced degradation of fibronectin and vitronectin in the microvascular endothelial cells (Study III, Fig. 4). Similar to mast cell-induced apoptosis of smooth muscle cells, NF-κB nuclear translocation was inhibited by mast cell releasate in endothelial cells as well (Study III, Fig. I in the supplemental data). Furthermore, mast cell releasate induced activation of both initiator caspase-8 and -9 (Study III, Fig. 5). Finally, human chymase and TNF-α were also able to induce apoptosis of human coronary artery endothelial cells (Study III, Fig. 6). The described pathway of mast cell-mediated apoptosis in endothelial cells shows similarities to mast cell chymase-mediated apoptotic mechanisms in smooth muscle cells (see above, Figure 5). In smooth muscle cells, however, fibronectin fragments were shown to induce apoptosis to a comparable level as chymase (Leskinen et al., 2001; Leskinen et al., 2003). Here, chymase-generated fibronectin and vitronectin fragments had only a minor direct effect on endothelial apoptosis (Study III, data not shown). Thus the additional effects of chymase remain to be studied in future experiments. In addition, mast cell-induced endothelial cell apoptosis involves interplay between intrinsic and extrinsic pathways. Interestingly, the apoptotic potential of TNF-α has been reported to be significantly increased when protein or RNA synthesis is inhibited (Pohlman and Harlan, 1989; Polunovsky et al., 1994). Here, chymase may trigger the intrinsic pathway via inhibition of FAK- and Akt-mediated survival signaling thereby subsequently increasing the ability of TNF-α to induce apoptosis via extrinsic pathway. Thus, the observed interplay between chymase, inhibiting survival, and TNF-α, promoting apoptosis, disturbs the balance of survival and death and leads to apoptosis of endothelial cells. The experimental setup for this study, however, differs from the situation in the arterial wall where mast cells reside in the basolateral side of endothelial cells. Thus, demonstration of the pathways in more physiological models is needed.

Figure 5. Proposed mechanisms of mast cell-mediated smooth muscle cell and endothelial cell apoptosis and plaque erosion.

5.3. The effect of mast cells on the level of apoptosis in atherosclerotic plaque in vivo (unpublished data)

As mast cells have been shown to induce apoptosis of smooth muscle cells (Leskinen et al., 2001), endothelial cells (Lätti et al., 2003), and lately also of macrophages (Bot et al., 2007) in vitro, we wanted to find out if this happens also in vivo. We measured the level of apoptosis in
the brachiocephalic artery plaques in mast cell-deficient \( \text{ldlr}^{-/-}/\text{Kit}^{W-sh/W-sh} \) and -competent \( \text{ldlr}^{-/-}/\text{Kit}^{+/+} \) mice fed a Western diet for 26 weeks. By immunohistochemical staining with cleaved caspase-3 antibody, we could not see significant changes in the level of apoptosis in atherosclerotic plaques between mast cell-deficient and -competent \( \text{ldlr}^{-/-} \) mice (0.91±0.60 and 0.36±0.89, apoptotic cells as a % of all cells, respectively, \( P=0.57 \), Figure 4F). In a similar study by Sun and coworkers, decreased levels of both apoptotic and proliferating cells were reported in mast cell-deficient \( \text{ldlr}^{-/-}/\text{Kit}^{W-sh/W-sh} \) mice fed a Western diet for 26 weeks compared to mast cell-competent littermates (0.7% and 1.7%, as a % of all cells, respectively) (Sun et al., 2007) (Table 11). Local activation of adventitial mast cells in collar-induced carotid plaques increased the level of apoptosis especially in the core region of the intima, and mast cells were shown to induce macrophage apoptosis in vitro, an effect which was attributed to mast cell-derived histamine (Bot et al., 2007).

6. Mast cells may induce endothelial erosion (IV)

In human coronary atherosclerotic plaques, mast cells were detected in the subendothelial intima in association with endothelial erosion and parietal microthrombi (Study IV, Fig. 2 and 3). Based on the ability of chymase and tryptase to induce desquamation of the endothelium (Study IV, Fig. 5 and 6), and since neutrophil migration through the endothelial cell layer was known to involve degradation of vascular endothelial (VE)-cadherin (Hermant et al., 2003), we wanted to clarify whether mast cell proteases are capable of degrading VE-cadherin. Indeed, chymase, tryptase and cathepsin G were all able to degrade the endothelial cell-cell contact molecule VE-cadherin in protein extracts of human coronary artery endothelial cells (Study IV, Fig. 7). Instead, the levels of CD31 and CD146 remained intact after incubation of these mast cell proteases (Study IV, Fig. 7, data not shown). The degradation of VE-cadherin could be inhibited by chymostatin and leupeptin, which are specific inhibitors of mast cell proteases, but not by EDTA, a general inhibitor of MMPs (Study IV, data not shown). This suggests that the mast cell proteases themselves, and not via activation of MMPs, induced the degradation. Together with the ability to induce endothelial cell apoptosis and degradation of VE-cadherin, mast cell proteases may thus have the ability to induce endothelial erosion (Figure 5). Our studies concerning VE-cadherin, however, lack data from the plaques. The presence of VE-cadherin has been reported in atherosclerotic plaques and in endothelial cells at sites of neovascularization, and its expression to be increased during the progression of atherosclerosis (Bobryshev et al., 1999; Sigala et al., 2003). Furthermore, high expression of VE-cadherin has been reported in symptomatic patients as compared to asymptomatic ones and in complicated lesions as compared to fibrous lesions, suggesting that high expression of VE-cadherin is associated with plaque instability (Sigala et al., 2003). Interestingly, levels of the soluble form of VE-cadherin in plasma have been reported to be increased in patients with acute myocardial infarction, stable angina pectoris and old myocardial infarction (Soeki et al., 2004). Furthermore, in some neovessels in atherosclerotic plaques, loss of VE-cadherin protein expression was associated with increased infiltration of T-lymphocytes, macrophages and dendritic cells (Bobryshev et al., 1999). This suggests that atherosclerotic plaque VE-cadherin may be susceptible to degradation by inflammatory cells. Whether this is the case in association with mast cells in vivo remains to be clarified in future studies.
7. General discussion

In the brachiocephalic artery, we found only trends, but not significant changes in the levels of macrophages, collagen, smooth muscle cells, or apoptosis between mast cell-deficient ldlr<sup>−/−</sup>/Kit<sup>W−/−</sup> and -competent ldlr<sup>−/−</sup>/Kit<sup>+/+</sup> mice fed a Western diet for 26 weeks (Figure 4). The trends of changes as a composite, i.e. lower level of macrophages, lower level of apoptosis and higher level of collagen in mast cell-deficient mice, however, are consistent with the hypothesis that mast cells would have a role in inducing plaque destabilization (Table 1). Smooth muscle cells, instead, showed a trend of lower content in mast cell-deficient mice, suggesting mast cell-mediated increased plaque stability via increased production of collagen. The plaque, however, may also contain increased level of proteases capable of collagen degradation thus shifting the balance towards plaque destabilization. A similar study by Sun and coworkers has reported decreased levels of macrophages and apoptosis and increased levels of collagen in the aortic arch of mast cell-deficient ldlr<sup>−/−</sup>/Kit<sup>W−/−</sup> compared to mast cell-competent littermates fed a Western diet for 26 weeks (Sun et al., 2007). In addition, Bot and coworkers have reported increased levels of apoptosis and leukocyte adhesion in collar-induced carotid plaques that were locally stimulated to activate adventitial mast cells in apoE<sup>−/−</sup> mice (Bot et al., 2007). By systemic activation of mast cells in apoE<sup>−/−</sup> mice, however, they did not see changes in the level of macrophages in the brachiocephalic artery (Bot et al., 2007). Furthermore, another study of collar-induced carotid plaques with systemic (intraperitoneal) mast cell activation in apoE<sup>−/−</sup> mice reported increased levels of macrophages and smooth muscle cells in stimulated carotid plaques as compared to nonstimulated ones (Tang et al., 2009). Thus, without stimulation of mast cells, aortic arch seems to be the artery where the differences between mast cell-deficient and -competent mice are most clearly seen ((Sun et al., 2007), Study I). In the carotid artery, effects of mast cells were seen after stimulation of mast cells (Bot et al., 2007; Tang et al., 2009). In the brachiocephalic artery, differences were not seen between mast cell-deficient and -competent mice in vivo (Figure 4) and not even under systemic stimulation of mast cells (Bot et al., 2007). On the other hand, direct comparison is difficult to carry out due to differences in mouse models and diets. Still, the results of the studies mentioned above suggest that the aorta (aortic arch) may contain the highest numbers of mast cells and also intimal mast cells, whereas the aortic sinus, carotid and brachiocephalic arteries may contain only adventitial mast cells.

In conclusion, mast cells have the capability to induce atherosclerotic plaque destabilization, both by increasing the accumulation of cholesterol in the plaque and by affecting matrix remodeling and the cellular content of the fibrous cap. Besides macrophages and T-lymphocytes (Ross, 1999), mast cells are also able to mediate the events of atherosclerosis at every stage of the disease. The stability of an atherosclerotic plaque may be determined by its inflammatory cell infiltrate, in which mast cells present a third cell type in addition to macrophages and T-lymphocytes (Kovanen, 2007), whose recruitment at the same time may also partly be induced by mast cells. Thus, mast cells possess the potential to induce plaque erosion and rupture.
CONCLUSIONS

In this thesis study, the effects of mast cells on the regulation of the formation and stability of atherosclerotic plaques were studied. The following main results were obtained:

1. Mast cell-deficient \(ldlr^{-/-}/Kit^{W-sh/W-sh}\) mice fed a Western diet (21% fat, 0.15% cholesterol) for 26 weeks had less atherogenic lipid profiles, decreased levels of lipid accumulation in the aortic arterial wall and decreased levels of vascular inflammation as compared to mast cell-competent littermates.

2. Mast cells were localized in the mouse arterial wall only in the adventitial/perivascular tissue as studied in the aortic sinus and the brachiocephalic artery of \(ldlr^{-/-}\) mice fed a Western diet for 26 weeks.

3. Mast cell chymase-induced rat aortic smooth muscle cell apoptosis was mediated by inhibition of NF-\(\kappa B\) activity, downregulation of bcl-2 expression and release of mitochondrial cytochrome c with concomitant activation of caspase-8, -9 and -3.

4. Mast cell-induced rat cardiac microvascular endothelial cell apoptosis was mediated by chymase and TNF-\(\alpha\), and included chymase-mediated degradation of fibronectin and vitronectin, and inactivation of FAK and Akt-mediated survival signaling. In addition, mast cells induced inhibition of NF-\(\kappa B\) activity and activation of caspase-8 and -9. Furthermore, chymase and TNF-\(\alpha\) induced apoptosis of human coronary artery endothelial cells.

5. Mast cells were localized in the subendothelial intima in association with endothelial erosion and parietal microthrombi. In ex vivo studies with isolated human coronary arteries, chymase and tryptase induced desquamation of endothelium and degradation of fibronectin, and chymase, tryptase and cathepsin G induced degradation of VE-cadherin.

Our data suggest that mast cells that are present in the vulnerable regions of human atherosclerotic plaques, may have a role in the regulation of atherosclerotic plaque stability both by increasing the formation of plaques and by inducing matrix remodeling and apoptosis, and so may participate in the generation of atherothrombotic events.
ACKNOWLEDGEMENTS

This study was carried out at the Wihuri Research Institute during the years 2003-2010. I want to thank the Jenny and Antti Wihuri Foundation for providing the excellent facilities for the work.

I am grateful to Professor Petri Kovanen, the head of the Wihuri Research Institute and my second supervisor, for accepting me into Wihuri and for all his support and advice. It has been a pleasure to work with him especially during the final years of this study.

I wish to express my gratitude to my supervisor, docent Ken Lindstedt. His enthusiasm inspired me to join the Wihuri and his positive support has been important during the years of hard work. His knowledge and advice in experimental designing, scientific thinking and writing, as well as his knowledge of the world of wines, is greatly appreciated.

I am grateful to Professor Perttu Lindsberg and docent Vesa Olkkonen for carefully reviewing my thesis and for their constructive feedback.

I wish to express my gratitude to all my co-authors. Markus Leskinen is sincerely acknowledged for his enthusiasm, from the very beginning, and for introducing me to the Wihuri lab and to many practical things. The weekly critical discussions on specific research articles were a good starting point for scientific thinking and reading. This study would have been much easier to finalize if Markus had been at Wihuri for a longer time. Mikko Mäyränpää is especially acknowledged for his knowledge of immunohistochemistry, as well as his generosity in allowing me to use his article in this thesis. Julia Trosien is acknowledged for introducing me to the mice projects, Soili Lätti for the extensive work completed before I arrived at Wihuri, Mika Laine for his expertise in confocal microscopy, and Jukka Hakala, Markku Pentikäinen, Matti Jauhiainen, Jari Metso, Mei Speer and Andrew F. Walls for their valuable contributions.

I am especially grateful to the kind and skillful technicians. Jaana Tuomikangas is thanked for so many things, including cell culture, all the litres of blotting buffer, and for introducing me to a sandwich delicacy: bananas on rye bread. Mari Jokinen is acknowledged for her expertise in cell culture, as well as for making it easy for me to give fancy clothes to my daughter. Maija Atuegwu is thanked for Western blots and the Tupperwares. Leena “Lennu” Saikko from the Department of Pathology, University of Helsinki, and Monica “Monna” Schultz from the Transplantation Laboratory, University of Helsinki, are both acknowledged for the FACS analysis, and Lennu is also thanked for teaching me how to grow tomatoes. I am grateful to Elina Kaperi for her significant contributions to the project, including cutting the brachiocephalic arteries and her additional work with mice. Suvi Sokolnicki is thanked for staining the brachios and the artistic pictures, not only of the brachios, but also of my daughter. Jarmo Koponen is acknowledged for the staining, microscopy and analysis of the brachios. I would like to thank the staff at the Department of Forensic Medicine, University of Helsinki — Hilkka-Liisa Vuorikivi, Anna Pasanen, Kati Jyrkiäinen, Kirsu Huoviala, Pentti Korhonen and Johanna Virri — for their help in paraffin-embedding of the brachiocephalic arteries.

I am also grateful for the help of Kati, Jani, Minna, Mervi, Miriam, Inka, Laura, Suvi, Satu, Kari, Wolfgang and all the other people at Wihuri. Laura Fellman is acknowledged for her attention to numerous practical matters. Satu, my roommate during most of my years at Wihuri, is thanked...
for her nice companionship and her good advice. Riia and Kata are thanked for sharing the last moments of our PhD projects, as well as for their friendship. Everyone at the Wihuri, either past or present — Anna, Riina, Mia, Jaakko, Mikko, Marru, Artturi, Julio, Tuula, Marja, Päivi, Krisse, Katri, Kata, Ilona, Reija, Kati, Nick, Andrea, Steffi, Maria, Hanna, Terttu, and Pia — are acknowledged for the unique Wihuri atmosphere during the years, both during pleasant coffee breaks and at Wihuri parties.

My friends, Maarit, Laura, Johanna, Anu, Päivi, and Anne are thanked for sharing the moments that make up the journey of completing a PhD, and Laura, Hanna, Minna, Laura, Lotta, and Ulla are thanked for all the other things in life.

I wish to thank my mother and daddy, my siblings Ansku and Tumppi, as well as my parents-in-law, for all their support. Finally, I want to thank Antti, for his patience, support and love. Without you I would not be here today. I also want to thank our daughter, Anna, for reminding me what life is for.

This work has been financially supported by the Jenny and Antti Wihuri Foundation, the Orion-Farmos Research Foundation, the Aarne Koskelo Foundation and the other supporters specified in the studies I-IV.

Helsinki, August 2010

Hanna
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