The vulnerable plaque: From plaque instability towards thrombus instability
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General introduction and outline of this thesis
General introduction

Coronary artery disease (CAD) is the leading cause of death in the world. Each year, 3.8 million men and 3.4 million women worldwide die from CAD\(^1\). A particular challenge for prevention of CAD is the sudden and often unpredictable nature. For many patients, the first manifestation is acute myocardial infarction (AMI) or sudden cardiac death\(^2,3\). In addition, among patients who have survived a cardiovascular event, the risk of subsequent events remains relatively high, approaching 1 in 4 despite aggressive treatment\(^4\). These features highlight the need for further understanding the pathophysiology of CAD and to elucidate and evaluate tissue markers that may identify not only the plaques prone to be disrupted (so called vulnerable plaques)\(^5\) but also those that have already been ruptured recently and are complicated by superimposed thrombosis (so called complicated plaques)\(^6,7\).

Pathogenesis of atherosclerosis

Atherosclerosis is a chronic disease that affects innermost layer (intima) of medium and large-size arteries. The build-up of an atherosclerotic plaque is a slow process, developed over a period of several decades through a complex series of cellular events occurring within the arterial wall. The exact pathogenesis of atherosclerosis is still unknown, but is suggested to be an inflammatory and vascular healing response, driven by endothelial cells injury, lipid accumulation and oxidation, and thrombosis\(^8-11\). This process is considered to be initiated by endothelial dysfunction. Among a long list of factors, hemodynamic disturbance and hypercholesterolemia are considered the two most important causes that initiate endothelial dysfunction\(^12,13\). The injured endothelium shows increased vascular permeability and leukocyte adhesion, which facilitates accumulation of lipoproteins (mainly low density lipoprotein - LDL), inflammatory cells (mainly macrophages and T lymphocytes) in the vessel wall. Once inside the vessel wall, LDL are oxidized through the action of oxygen radicals locally generalized by macrophages and endothelial cells, and become toxic to the cells in the artery wall\(^14,15\). Subsequently, the body’s immune system responds to the damage by sending macrophages to absorb the oxidized LDL. But these cells are not able to process the oxidized LDL, resulting in lipid accumulation in the cells and forming specialized foam cells. The foam cells grow progressively and ultimately become ruptured, releasing a greater amount of oxidized cholesterol into extracellular space, which in turn augments macrophage activation and cytokine production. This further increase inflammatory cell adhesion and production of chemokines, creating a stimulus for recruitment of additional inflammatory cells and resulting in a vicious circle in the vessel wall\(^16\). In response to stimulation of growth factors produced by activated
inflammatory cells, vascular smooth muscle cells (SMC) begin to proliferate and produce extracellular matrix (ECM). The intimal SMC proliferation and ECM deposition covers over affected area and forms a hard fibrous cap, which separate the central necrotic core from blood stream, consequently transforming an early lesion into a mature atheroma.

**Histomorphology of atherosclerosis**

The development of atherosclerotic lesions is best illustrated by American Heart Association’s Classification, which divides the development of atherosclerosis in several stages based on their histological characteristics. The earliest recognizable lesion is so called the “intimal xanthomata” (also known as “fatty streak”) characterized by focal aggregation of lipid-laden macrophages or SMCs (foams cells) in the intima. This initial pathological change could already be observed in adolescents and most of these lesions are believed to be regressive. The intermediate lesion is a poorly defined entity, known as pathological intimal thickening (PIT). Histologically, PIT shows some disperse extracellular lipid present deep in the lesion, but there is no well-formed necrotic core and no evidence of cellular debris. Some scattered inflammatory cells may also be present, but these are usually sparse. Advanced atherosclerotic lesions are characterized by well-formed necrotic core and distinct fibrous cap. The advanced plaques are described histologically as fibroatheroma, thin cap fibroatheroma and fibrocalcified plaques, based on variation in their composition, size of necrotic core, thickness of fibrous caps, number of inflammatory cells and SMCs, state of endothelial function and degree of calcification.

**Acute plaque complications and the vulnerable plaque concept**

Typically, acute myocardial infarction (AMI) is caused by acute thrombotic occlusion superimposed on a disrupted plaque in coronary artery. It has been recognized that plaque composition rather than plaque size or stenosis severity is important for plaque rupture and subsequent thrombosis. Over the past 20 year, the concept of “vulnerable plaque” has been widely used to describe an atherosclerotic plaque which is prone to be disrupted and develop thrombotic complications. From observation on patients witnessed sudden cardiac death, about 60% of acute thrombotic occlusion resulted from rupture of so-called thin cap fibroatheroma (TCFA). TCFA shows several histological characteristics: a thin fibrous cap of ≤ 65 μm, extensive inflammatory infiltration by macrophages and T lymphocytes (>25 cells per 0.3mm diameter field), and a large lipid core of >40% plaque area. Other relevant histological features include small numbers of SMCs, the outward remodelling, dense intraplaque microvessels, or hemorrhage.
Of these plaque components, inflammation is the most important part of progression and destabilization of an atherosclerotic plaque. Accumulation of inflammatory cells, particularly macrophage and T lymphocytes, results in the release of proinflammatory cytokines and matrix metalloproteinases that contribute to degradation of collagenous components in the fibrous cap of the atheroma. Furthermore, inflammation may also play a key role in apoptosis of collagen synthesizing SMCs which further leads to loss of fibrous cap strength. When the structural integrity of the cap is overwhelmed by the extrinsic mechanical stresses, it will be disrupted, leading to a direct contact between the prothrombotic necrotic core and the circulating blood in the affected vessel. Consequently, coronary thrombosis will be formed through the elaboration of the pro-coagulant protein, tissue factor, produced by intraplaque inflammatory cells.

Another important stimulus of plaque vulnerability is intraplaque hemorrhage (IPH). Bleeding in the atherosclerotic plaque is a common finding in advanced lesions. IPH leads to sudden growth of the plaque necrotic core by accumulation of cholesterol derived mainly from extravasated red cell membranes. In addition, IPH further contributes to plaque instability by facilitating the recruitment and activation of inflammatory cells as well as produce of oxygen radical into the advanced lesions. The origin of plaque bleeding is still uncertain. It has been suggested that hemorrhage into a plaque occurs from fissures originating from the luminal surface, thereby allowing the entry of blood into the necrotic core. Alternatively, IPH has been considered to be secondary to the rupture of microvessels (vasa vasorum) inside the plaques. Histologically, these microvessels show several immature characteristics, such as paucity of pericytes and poorly formed endothelial junction. These fragile microvessels are leaky and prone to be disrupted leading to an intramural hemorrhage; and in addition, the microvessel density is parallel with severity of IPH and macrophage infiltration.

An improved understanding of the pathophysiology of plaque vulnerability and subsequent atherothrombosis should provide new insights into acute thrombotic cardiovascular events. It has been shown that the plaque rupture underlying AMI reflects instability of a single plaque, as result from interactions between factors intrinsic to the plaque and extrinsic forces. However, the risk factors precipitating in atherogenesis and plaque vulnerability, such as hyperlipidemia, hypertension, diabetes mellitus, inflammation etc., might exert their adverse effects in a widespread pattern throughout the whole coronary vasculature, rather than a single plaque. Indeed,
many studies have shown that plaque vulnerability is usually not a feature of a single (culprit) lesion, but may involve several plaques in the coronary system of a patient (pan-coronary vulnerability)\textsuperscript{46-50}. Therefore, the early identification of plaques prone to disruption (‘vulnerable plaques’) is desirable, as this may allow the implementation of preventative strategies and, possibly, effective therapeutic intervention. Such insights have boosted the search for in vivo markers of plaque vulnerability by means of high resolution coronary imaging techniques, to identify vulnerable plaques. In the recent decades, many clinical studies have demonstrated that histological characteristics of a vulnerable plaque, such as thin fibrous cap and necrotic core, could be identified by means of several invasive or noninvasive image modalities\textsuperscript{51-56}. However, other tissue markers of plaque vulnerability, such as plaque inflammation, IPH, are still not detectable with current diagnostic methods. Therefore, further investigation on in vivo detection of intraplaque inflammation and hemorrhage will be of great value for the patient tailored prevention or treatment strategy.

The atherosclerotic plaque after a rupture event: thrombus evolution and plaque healing

Large plaque disruption followed by massive local activation of the coagulation system will directly lead to coronary thrombotic occlusion, and hence the onset of clinical symptoms\textsuperscript{21-24}. However, in our lifespan, plaque disruption and thrombosis are not uncommon features and most of them remain clinically silent\textsuperscript{23, 24}. Post-mortem studies demonstrated that, healed plaque disruption could be observed in either culprit or non-culprit lesions in the coronary arteries of patients witnessed acute cardiac death; instead of leading to acute thrombotic occlusion, this plaque disruption with organization/evolution of the mural thrombus contributes to a phase of rapid plaque growth\textsuperscript{23, 48, 49, 57}.

Thrombus evolution/organization is a process converting a friable thrombus into stable fibrovascular-, and later fibrosclerotic (scar) tissue and incorporating the thrombus into plaque, which contributes significantly to repair process after plaque disruption. This healing process is tightly regulated multistep events overtime. Similar to skin wound healing, inflammation, angiogenesis and ingrowth of fibroblasts are imperative components of thrombus organization\textsuperscript{58}. Histopathologically, the thrombus organization can be divided into 3 phases: 1) fresh thrombus (up to 1 day old) composed of layered patterns of platelets and fibrin with intact blood cells; 2) lytic thrombus (1-5 days) characterized by homogenization of thrombus structure, necrosis and fragmented nuclei (karyorrhexis); and 3) organized thrombus (older than 5 days) featured by
ingrowth of (myo) fibroblasts, with or without collagen deposition and angiogenesis. This distinct pattern of temporal changes of thrombus morphology can be therefore used as a time-scale for investigation on pathophysiology of the healing process at the site of thrombus-plaque interface.

**Figure 1.** (A) Macroscopic image of a thrombectomy sample. (B) Fresh thrombus (< 1 day): layered patterns of platelets, fibrin, erythrocytes and intact granulocytes. (C) Lytic thrombus (1-5 days): areas of colliquation necrosis and karyorrhexis of granulocytes. (D) Organization of thrombus (> 5 days): characterized by ingrowth of smooth muscle cells, with or without depositions of young connective tissue and ingrowth of capillary vessels.

**Factors that may involve the process of thrombus evolution and plaque healing**

It is widely known that critical coronary stenosis (as in unstable angina) and total coronary occlusion (as in transmural myocardial infarction) results by far the most cases from plaque disruption with superimposed thrombus formation. And, although thrombi can be found in the infarct related coronary arteries of patients who died of AMI (at autopsy) and more recently in thrombectomy studies of patients who survive the acute coronary event, the knowledge on mechanisms of thrombus initiation,
progression and in later stages organization that underlie the final onset of acute events is far from complete. For example, several studies have now shown that process of plaque disruption and thrombus formation is often not immediately followed by the onset of clinical symptoms. First, thrombus formation and intra-plaque hemorrhage are well recognized elements in the progression of subclinical atherosclerosis, implying that thrombus formation does not always result in symptoms\textsuperscript{34, 48, 49}. Secondly, studies from autopsy, angiography and intravascular ultrasound (IVUS) show that in patients with AMI, plaque instability is not a feature of a single plaque, but is frequently present in multiple coronary plaques\textsuperscript{46, 47, 50, 59-61}, indicating that thrombosis occurs also in non culprit lesions. Thirdly, earlier histopathological study in our lab on thrombectomy specimens, retrieved from patients with ST-segment elevation myocardial infarction (STEMI), has shown that thrombus formation was initiated in about 50\% of cases already days or even weeks before the acute event\textsuperscript{62}. This feature was also described by Henriques et al. in autopsy study showing presence of organized thrombus in the coronary arteries of young individuals who died instantly or within 6 hours after onset of cardiac symptoms\textsuperscript{57}. Such observations suggest coronary thrombosis appears to be often a “wax and wane” process of the thrombus mass or a process

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{pathway.png}
\caption{Schematic description of the development after plaque disruption: healing processes do occur; but failure in complete healing in many cases may lead to acute total occlusion at a later time point.}
\end{figure}

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of repeating thrombosis (with probably repeated ruptures) rather than being a single hit event as was thought for a long time.

Based on these observations, one could speculate that the occurrence of acute thrombotic occlusion could be in part due to a failure of the ruptured plaque-thrombus interface to organize and repair, as it occurs in many patients with subclinical (symptomless) thrombosed plaques as alluded to above. This hypothesis forms the basis for the studies described in this thesis.

**Objectives of this thesis**

The general objective of this thesis was to get more insight in the mechanisms contributing to plaque disruption and luminal thrombus progression, which finally lead to critical stenosis or occlusion of coronary arteries, or otherwise may promote tissue healing after plaque disruption.

For these purposes, coronary atherosclerotic plaques identified on postmortem angiograms were collected from patients died from heterogeneous reasons, and were classified into early lesion, fibrolipid plaque, fibrocalcified plaque, fibrotic plaque and complicated lesion (plaque rupture or erosion). In this approach, we followed a modification of the classifications by Virmani et al. and American Heart Association. A large numbers of plaque sections were evaluated (immuno-) histologically to visualize in situ the parameters of plaque instability. In addition, thrombectomy specimens were also collected from patients treated with primary percutaneous coronary intervention (PCI) for STEMI with symptom shorter than 24 hours. Histologically, thrombus age can be classified as fresh (<1 day), lytic (1-5 days), or organized (>5 day), which was used as a time scale for evaluation of healing process. In addition, the thrombectomy materials contain also plaque components extruded into the coronary lumen from disrupted plaque, which may provide a unique opportunity to study in detail the various pathophysiological aspects of atherothrombosis at the site of culprit lesions.

**Outline of this thesis**

The first part of this thesis describes special histological features correlated with progression and disruption of atherosclerotic plaques, such as inflammation, angiogenesis and intraplaque hemorrhage. Chapter 2 explores the tissue markers of plaque vulnerability in end-stage coronary plaques in elder patients. Chapter 3 deals with
Chapter 1

the relationship between (micro-) calcifications and intraplaque inflammation in the culprit lesions of patients with ST-segment elevation myocardial infarction (STEMI). The influence of anti-coagulants and anti-platelet medications on intraplaque bleeding is evaluated in chapter 4.

The healing process after plaque rupture appears to be of pivotal importance for onset of acute cardiovascular events. Second part of this thesis concerns the several pathophysiological processes involving in the tissue healing after plaque disruption, such as angiogenesis, fibrogenesis, intra-thrombotic inflammatory cells and their secretory products, and the impact of thrombus organization on clinical outcomes. For this purpose, we used thrombectomy samples obtained from STEMI patients over a period of 2001 and 2008; and in this period a total of >1300 samples were included. Chapter 5 shortly reviews the histological studies to date on thrombectomy samples, highlighting the usefulness of these valuable materials for investigations on pathophysiology of atherothrombosis. Chapter 6 describes the association between thrombus morphology and clinical outcome of patients with AMI. Chapter 7 addresses the process of angiogenesis and the role of endothelial progenitor cells during the process of thrombus organization. Chapter 8 focuses on presence of neutrophil extracellular traps (NETs), IL-17A and - F in different stages of thrombus organization. In chapter 9, with use of autopsy myocardium samples and thrombectomy materials, we evaluate whether or not the foreign materials detached from the guidewires during primary percutaneous coronary intervention (PCI) embolize to the distal microvasculature. Chapter 10 gives a short review of studies, which focus on specific histomorphological and biophysical properties of coronary plaques in determination of the risk of PCI related vascular complications.

Finally, chapter 11 summarizes the findings of this thesis and gives a general discussion of these findings in the context of understanding the pathophysiology of plaque instability, coronary thrombus formation and subsequent healing process.
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General introduction


Chapter 1


