The vulnerable plaque: From plaque instability towards thrombus instability

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Microvascular endoglin (CD105) expression correlates with tissue markers for atherosclerotic plaque vulnerability in an ageing population with multivessel coronary artery disease

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Abstract

Aims
Vulnerable atherosclerotic plaques are lesions with high propensity to develop plaque disruption and superimposed thrombosis. No systematic studies have been carried out on tissue markers for plaque vulnerability throughout the entire coronary artery system in the end stages of coronary atherosclerosis.

Methods
Nine autopsied patients (mean age 77 years) with angiographically severe trivascular coronary atherosclerosis were selected for this study. All visible lesions in the post-mortem coronary angiograms (n=125) were histologically and immunohistochemically screened for the presence of intraplaque hemorrhages, (activated) microvessels and inflammatory infiltrates.

Results
Intraplaque hemorrhages were observed in 76/125 plaques (61%). Chronic inflammation was found superficially in 59/125 plaques (47%) and deeply inside the plaque tissue in 103/125 plaques (83%). Microvessels were found in 100/125 lesions (80%), of which 58% showed endothelial expression of the vascular activation marker CD105. Moreover, microvascular CD105 positivity correlated positively with plaque hemorrhage and deeply seated plaque inflammation.

Conclusions
Plaque inflammation and hemorrhages can be found in high frequency throughout the coronary artery system of elderly patients with multivessel coronary atherosclerosis. Microvascular expression of CD105, which correlates positively with both of these features of plaque vulnerability, can serve as a marker for the risk to develop coronary thrombotic complications.
Introduction

Coronary atherosclerosis is held responsible for the largest mortality in adults, at least in the Western world, but also rapidly upcoming in many developing countries.\(^1,2\) The pathophysiological mechanism underlying acute myocardial infarction (AMI) is in many cases a rupture or erosion of the plaque surface followed by thrombotic occlusion of the involved artery. Over the years, several pathology studies on autopsy derived atherosclerotic vessels and materials retrieved through coronary artery interventions such as directional atherectomy have forwarded distinct inflammatory activity inside atherosclerotic plaques as a factor that increases the vulnerability of a plaque to develop such acute plaque complications.\(^3-6\)

More recently, also intraplaque hemorrhages (IPH) came to the attention as an additional mechanism of plaque tissue destabilization that may lead to progression towards critical stenosis of the vessel lumen.\(^7-9\) Leaky or ruptured microvessels inside the plaque are held responsible for the onset of plaque hemorrhages, and indeed, recent studies have shown that clinically unstable plaques contain significantly higher numbers of microvessels when than asymptomatic plaques.\(^10, 11\) Microvessels arise in plaques through a process of angiogenesis, likely driven by tissue hypoxia.\(^12\) This is further endorsed by the endothelial expression of endoglin (CD105) on a subfraction of plaque microvessels,\(^13\) which is considered as a marker of active angiogenesis, not only in plaques but also in chronic inflammatory processes\(^14\) and in solid tumors such as mamma carcinoma.\(^15\)

Risk factors for atherosclerosis related cardiovascular disease such as smoking, hypercholesterolemia and diabetes mellitus appear to influence the vulnerable components of atherosclerotic plaques at least to some extent,\(^16\) which could implicate that plaque vulnerability is a widespread phenomenon in the end stage of atherosclerotic coronary artery disease. This is of importance, since the population of patients with advanced coronary artery disease is growing as a result of progressive aging of the population.\(^17\) However, up to date such an association has not been defined clearly. The aim of the present study was to investigate systematically several histopathological markers of plaque vulnerability, including inflammation, microvessels and plaque hemorrhage throughout the entire coronary artery system of autopsied patients with angiographically established severe end stage coronary atherosclerosis.

Materials and methods

Material selection and tissue processing

Hearts were obtained at autopsy from nine patients. Patients were selected based on
post-mortem angiograms for the presence of severe trivascular stenosis in all 3 major epicardial coronary artery branches (Fig. 1). This study was prepared in accordance with the Helsinki Declaration. Age, sex, and cause of death were recorded of each patient. Written permission to obtain the hearts for research purposes was granted by the family of the patients. After postmortem angiography, the entire hearts were fixed in buffered formalin for at least 24 hours. Myocardial ischemia was assessed by means of Nitroblue Tetrazolium (NBT) staining of a fresh transversal biventricular slice at the midpapillary level of the heart. Decolorization of grossly normal appearing myocardium was interpreted as recent onset ischemia. Coronary arteries were carefully dissected en bloc from the epicardial surface of the heart, and decalcified in ethylene diamine tetra acetic acid (EDTA) for 4 days. All segments containing visible lesions at corresponding post-mortem angiograms (in total n=128) were cut in 3 mm segments, and after routine processing embedded in paraffin.

**Classification of atherosclerotic lesions**

For histomorphological classification of plaque composition, five-μm-thickness sections of all 128 lesions were stained with hematoxylin and eosin and Elastic van Gieson
staining respectively. For detection of iron a Perls stain was used. Lesions with a fibrous cap overlying a lipid core were classified as fibrolipid plaques; lesions that were for the largest part calcified, were classified as calcified plaques; and lesions that consisted mainly of fibrous tissue were classified as fibrotic plaques. Plaque disruption was defined as the presence of intraluminal thrombus superimposed on erosion of the superficial endothelial layer (plaque erosion) or rupture of the fibrous cap (plaque rupture)\textsuperscript{19}. The compositions of the disrupted plaques were also classified as stated above.

**Immunohistochemistry**

For immunohistochemistry the following monoclonal antibodies were used: anti-CD45 (Leukocyte common antigen, dilution 1:200, DAKO), anti-CD68 (pan-macrophage, dilution 1: 200, DAKO), anti-CD31 (PECAM, dilution 1:20, DAKO), anti-vWF (von Willebrand factor, dilution 1:50, DAKO), anti-CD105 (endoglin, dilution 1:1000, Thermo) and anti-glycophorin A (1:200, DAKO) antibodies.

Paraffin tissue sections were dewaxed in xylene and rehydrated in graded alcohols. Endogenous peroxidase activity was blocked with 0.3% \( \text{H}_2\text{O}_2 \) in methanol for 20 min. Sections were subsequently digested with 0.25% pepsin dissolved in 10mM HCl for 10 min at 37°C prior to CD31 and vWF antibody labeling. Heat-induced epitope retrieval was performed for 20 min at 98°C in a Pretreatment Module (Thermo Fisher/Labvision, Fremont, CA, USA) with 10mM citrate (pH6.0) prior to labeling with CD105 and CD68 antibodies. Subsequently, sections were loaded in a Thermo Fisher/Labvision 360 immunostainer using a 2-step polymer detection protocol. In short: serum-free protein-block (Dako) for 10 min; primary antibodies for 1hr at room temperature (LCA, CD31, vWF, glycophorin A, CD68) or overnight at 4°C (CD105); appropriate anti-mouse or anti-rabbit HRP-labeled polymers (Immunologic, Duiven, The Netherlands) for 30 min; HRP-activity was visualized using DAB+ (Dako) for 8 min.

**Histological grading of markers for plaque vulnerability**

*Microvascular leakage and CD105 expression.*

Numbers of CD31+ staining microvessels in each plaque was graded as: *absent:* <10 vessels, *moderate:* 10-50 vessels and *high:* >50 vessels. The activated microvessels were recognized as CD105+ luminal structures, which were confirmed by CD31 positivity of these structures in adjacent sections. Microvessel leakage was defined as diffuse immune staining of von Willebrand factor (vWF) around microvessels in plaque as previously described\textsuperscript{20, 21}. 31
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**Plaque inflammation.**
Severity and topographic location of plaque inflammation was semi-quantitatively scored as either superficially adjacent to the lumen endothelium, deep inside the plaque around the atheroma or both: The severity of inflammation was graded as *absent*: no inflammatory cells; *moderate*: inflammation involving <50% of the plaque area, and *severe*: inflammation involving >50% of the plaque area.

**Intraplaque hemorrhage (IPH).**
Glycophorin A is expressed on the membranes of red blood cells (RBC), including membrane fragments that remain present in the tissue long after the onset of a bleeding.\textsuperscript{22} IPH were classified as either grossly or microscopically recent onset IPH (intact RBC in glycophorin A stain), old IPH (depositions of glycophorin A positive RBC fragments and/or iron), and ongoing IPH: simultaneous presence of both recent and old hemorrhage in one plaque.

**Statistics**
Statistical analysis was performed with SPSS 17. Results are expressed as percentages and evaluated with $\chi^2$ test. A P-value of $\leq 0.05$ was considered statistically significant.

**Results**
In this study, 128 coronary atherosclerotic plaques were collected that corresponded with lesional sites on the postmortem angiograms. Clinical data and numbers of plaques obtained from each patient are described in table 1. Three lesions showed a chronic total occlusion histologically, and were excluded from the series. The remaining 125 plaques were classified as fibrolipid (36/125), calcified (60/125) and fibrotic (29/125) (table 1). In total, eight disrupted plaques with superimposed lumen thrombosis were found in 4 patients, of which 2 were plaques ruptures (no.2) and the remaining 6 were eroded plaques. The 2 patients who died from AMI (no. 2 and 9) had 2 and 4 disrupted plaques respectively. Earlier plaque stages such as fatty streaks were not encountered among the angiographically visible lesions.
IPH were found in 76 out of 125 (61%) of lesions (Fig 2 A-D). Of these, 17 out of 125 (14%) showed recent microscopic hemorrhages, 14 out of 125 (11%) had macroscopically visible fresh hemorrhages, 24 out of 125 (19%) plaques had old hemorrhages, and 21 out of 125 plaques (17%) showed features of both recent and old hemorrhages (interpreted as ongoing).
CD45 immunopositive inflammatory infiltrates were found in 110 (88%) plaques (Fig. 3). Deep infiltrates located around the lipid core were found in 103/125 (82%) of plaques, and superficially located infiltrates continuity with the surface endothelium were found in 59/125 (47%) of plaques. Overall, CD31+ microvessels were present in 100/125 (80%) of plaques (Fig. 2E and 2F). Of these, diffuse perivascular anti-vWF staining, as a marker for microvascular leakage, was found in 58% of plaques, particularly lesions containing dilated thin walled vessels. In addition, numbers of microvessels were positively associated with presence of recent hemorrhages (P<0.001), and also with presence of deep plaque inflammation (P<0.0001).

Although plaque inflammation, microvascularization, vascular leakiness and plaque hemorrhage were found in all 9 patients, their presence varied in each individual case from only few lesions to nearly all lesions (Fig. 4). For example, the percentage of plaques of single patients containing superficial plaque inflammation ranged from 5% to 87%.

**CD105 expression of microvascular endothelium**

CD105+ microvessels were found in 58/125 (46%) of all plaques (Fig. 5). Again, huge variation among patients was observed, since the presence of intraplaque CD105+ vessels ranged from 8% up to 90% of all the plaques of each individual patient. As shown in table 2, there was a positive association between the presence of CD105+ microvessels and the presence of IPH (p=0.044), irrespective of the age of the hemorrhage (recent, old or ongoing). Moreover, plaques with CD105+ microvessels showed more

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**Table 1. Clinical data and histomorphological classification of 125 plaques in the coronary arteries of 9 patients with severe trivascular atherosclerosis.**

<table>
<thead>
<tr>
<th>pt</th>
<th>age</th>
<th>sex</th>
<th>no. of plaques</th>
<th>classification</th>
<th>Disease state</th>
<th>Recent myocardial ischemia*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>82</td>
<td>M</td>
<td>11</td>
<td>3</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>2</td>
<td>90</td>
<td>M</td>
<td>10</td>
<td>4</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>82</td>
<td>M</td>
<td>21</td>
<td>10</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>4</td>
<td>61</td>
<td>M</td>
<td>12</td>
<td>8</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>75</td>
<td>M</td>
<td>12</td>
<td>0</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>63</td>
<td>M</td>
<td>20</td>
<td>0</td>
<td>13</td>
<td>7</td>
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<td>V</td>
<td>10</td>
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<td>M</td>
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<td>9</td>
<td>86</td>
<td>M</td>
<td>15</td>
<td>8</td>
<td>6</td>
<td>1</td>
</tr>
<tr>
<td>tot</td>
<td>125</td>
<td></td>
<td>36</td>
<td>60</td>
<td>29</td>
<td></td>
</tr>
</tbody>
</table>

COPD: chronic obstructive pulmonary disease; MI: myocardial infarction; CVA: cerebral vascular accident.

*Recent onset myocardial ischemia: assessed by regional or circumferent pale areas in Nitro blue tetrazolium (NBT) stain of myocardial slice at autopsy.
Figure 2. Overview of intraplaque hemorrhage and leakage of microvessels
Fig 2A – H&E stain of a cross-section of a coronary atherosclerotic plaque (x20 magnification), inserts show intraplaque hemorrhage
Fig 2B – details of the black insert of 2A (x200 magnification), immunostained for Glycophorin A, showing extravasation of intact erythrocytes, which indicates fresh hemorrhage
Fig 2C – details of the red insert of 2A (x200 magnification), immunostained for Glycophorin A, showing erythrocyte fragments, which indicates old hemorrhage
Fig 2D – details of the black insert of 2A (x200 magnification), Perls stain for detection of iron (blue deposits), indicating old hemorrhage
Fig 2E – intraplaque microvessels identified with immunostain for CD31 (x40 magnification), microvessels are indicated with arrows; macrophages also stained positively with CD31
Fig 2F – adjacent tissue section of 2E immunostained for von Willebrand factor (vWF) (x40 magnification), leakage of intraplaque microvessels is identified by presence of diffuse perivascular vWF deposits
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Figure 3. Intraplaque inflammation visualized with immunostains against CD68 and CD45

**Fig 3A** – immunostain against CD68 (x40 magnification), showing superficially (white arrow) and deeply seated infiltration of macrophages (arrow)

**Fig 3B** – immunostain against pan-leucocyte marker CD45 (x200 magnification), showing inflammatory cells localized around microvessels (arrows)

Figure 4. Dot-plot figures showing the percentages of plaques of each of the 9 patients included in the study, containing microvessels (A), hemorrhages (B) and inflammation (C) respectively. The figures illustrate high overall incidence but huge variation for all 3 parameters among the individual patients. NB: % of plaques (Y-axis) represents the % of the total amount of plaques of each patient.
frequently inflammatory infiltrates deep in the atherosclerotic lesion (p=0.007). Finally, the total number of microvessels was larger in plaques with CD105+ vessels compared with those without CD105+ vessels (p=0.012).

Markers of plaque vulnerability in disrupted plaques
The vulnerable markers of the 8 disrupted plaques were compared with the non-disrupted (intact) plaques. No statistical analysis was performed due to the limited numbers of the disrupted plaques, but all the markers of plaque vulnerability were observed more frequently present in disrupted plaques than in non-disrupted plaques: presence of CD105+ microvessels (63% vs 45%), high density of microvessels (63% vs 40%), IPH (100% vs 58%), superficial inflammation (75% vs 45%) and deep inflammation (100% vs 81%).

Figure 5. Intraplaque microvessels identified with immunostains against CD31 and Endoglin (CD105) (x100 magnification)
Fig 5A – microvessels immunostained for CD31 (arrow)
Fig 5B – adjacent tissue section of 4A, immunostained for CD105, showing positive CD105 stain (arrow), which indicates a subgroup of intraplaque microvessels undergoes active angiogenesis
Fig 5C – vessels in de adventitia positively stained for CD31 (arrow)
Fig 5D – adjacent tissue section of 4C, immunostained for CD105 (arrow), showing no CD105 + microvessels
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Discussion

Plaque inflammation, growth of microvessels, microvascular leakage and IPH have all been forwarded as factors playing a pathophysiological role in the onset of acute coronary artery disease, and as such are considered as tissue markers for plaques vulnerability.\(^4,5,23\)

In this study, we systematically investigated these histological markers throughout the coronary arteries of 9 patients who had angiographically proven severe trivascular atherosclerosis at autopsy, but were not primarily hospitalized for coronary artery disease.

Intraplaque inflammation and angiogenesis

By far, most plaques (88%) contained inflammatory infiltrates, although there was variation in severity and location of the infiltrates among the patients. Superficial infiltrates closely apposed to the arterial endothelium or throughout the entire fibrous cap of lesions were found less frequent (47%) than deep infiltrates (82%), but are likely more relevant for the process of plaque erosion or fibrous cap rupture resulting in arterial thrombus than the deeply located infiltrates.\(^5\) Still, inflammatory activity deep inside the plaque is thought to be of relevance for inducing plaque instability through the cytotoxic or tissue degrading activities of reactive oxygen products or inflammatory proteins such as matrix metalloproteinases.\(^3\) These findings, which visualize plaque inflammation throughout the coronary arterial tree, support the view that plaque inflammation is a diffuse process of the arterial system rather than being a feature of one culprit lesion,\(^4\) at least in this specific population under investigation, i.e. elderly patients with multivessel coronary artery disease. Still, the outcome of our study confirms the importance of evaluating inflammation as a marker for plaque vulnerability, since the 8 disrupted and thrombosed lesions contained more frequently inflammatory infiltration than the 117 intact lesions, although the huge difference in sample sizes did not allow statistical interpretation.

Table 2. Contingency table shows the association between CD105 expression on microvessels and parameters of plaque vulnerability.

<table>
<thead>
<tr>
<th>Hemorrhage</th>
<th>Superficial inflammation</th>
<th>Deep inflammation</th>
<th>Microvessels</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absent</td>
<td>Recent</td>
<td>Old</td>
<td>Absent</td>
</tr>
<tr>
<td>CD105 - (total = 67), n (%)</td>
<td>33 (49)</td>
<td>24 (36)</td>
<td>10 (15)</td>
</tr>
<tr>
<td>CD105 + (total = 56), n (%)</td>
<td>16 (28)</td>
<td>28 (48)</td>
<td>14 (24)</td>
</tr>
</tbody>
</table>

P: 0.044, 0.28, 0.007, 0.012
Several studies have indicated a possible mutual relationship between these inflammatory infiltrates and the growth of microvessels in plaques. Microvessels can serve as a gateway for the recruitment of inflammatory cells into atherosclerotic plaques, and at the same time certain cytokines such as VEGF and TGF-β that are released by these cells can stimulate the angiogenic process inside plaques. Our study, which showed the number of microvessels was positively associated with deep plaque inflammation, further confirms this concept. In addition, activated microvessels identified by positive Endoglin (CD105) immunostaining were observed in about half of all the plaques (46%). Interestingly, these microvessels are significantly associated with both IPH and plaque inflammation. These findings further support the paradigm supposed by Li et al, since plaque inflammation could create a hypoxic environment within the atherosclerotic plaques; and the hypoxia is considered as a strong stimulus for upregulation of endoglin (CD105) gene expression on endothelial cells, which in turn prevents these cells from apoptosis and subsequently contributes to growth of the microvessels (angiogenesis). In disrupted plaques, not only microvessel density but also microvascular CD105 positivity appeared indeed higher than in the intact plaques. The soluble form of endoglin in circulating blood has also been evaluated as a potential prognostic marker for coronary artery disease. Ikemoto et al demonstrated that plasma soluble endoglin is positively associated with adverse clinical outcomes for patients with stable angina. However, Cruz et al found a decrease of soluble endoglin in AMI patients within the first two days after onset of symptoms, which appeared significantly associated with higher cardiovascular mortality. Blood samples of the patients included in our study were not available, so we were not able further evaluate the issue of soluble endoglin as a prognostic marker.

**Fresh and old intraplaque hemorrhages**

Signs of either fresh or old IPH were found frequently inside the plaques (altogether 61% of lesions). Most studies on IPH have been carried out in symptomatic (thrombosed) plaques which are derived from coronary- or carotid artery surgical specimens, or from occluded vessels that are found at autopsy. Recently, Gao et al showed, in a review of 31 studies on carotid artery IPH, that the percentage of such complications is indeed much higher in symptomatic than in asymptomatic lesions in most of these reports. However, the rate of IPH differed substantially among the included studies, from 9% up to 78%, reflecting large differences in patient selection and/or in methodology. Similar to our study, Kolodgie et al investigated also IPHs in coronary plaques that were not related to a clinical event, and found 53% of fibroatheromatous plaques, and 77% of thin cap fibroatheromatous plaques positive for this complication, which is in the same range as we found in all visible lesions on the postmortem angiograms.
However, Kolodgie et al evaluated the coronary arteries of sudden coronary cardiac death victims, whereas in our study the patients were selected for the presence of extensive trivascular atherosclerotic disease. In fact, in our series 7 out of 9 patients showed signs of recent myocardial ischemia at autopsy (as a final manifestation of their severe coronary artery disease), but 7 of these 9 patients were hospitalized for a non-cardiac disease. Another interesting observation was the simultaneous presence of both fresh and old haemorrhages in one and the same plaque, indicating an ongoing process of plaque hemorrhage. Together with the subsequent organization and accumulation of free cholesterol derived from erythrocyte membranes, the ongoing IPH could play an important role in increase of plaque volume (plaque growth) in a relatively short period of time. 21, 32

The age of the patients in our study ranged from 60 to 90 years (mean 77 yrs). This implies that both inflammation and angiogenesis can be prominent atherosclerotic plaque features also in old individuals, despite the impairment of immune function inherent to ageing. 33, 34 Similarly, also a recent study on carotid artery plaques of 383 patients could not find a correlation between age and macrophage contents in the plaque tissue. 35 Apparently, the deteriorating effects of aging on systemic inflammation and angiogenesis do not influence atherogenesis in situ, probably as it is overcome by the lipid rich pro-inflammatory- and pro-angiogenic milieu of the atherosclerotic plaque.

**Conclusion**

Our findings illustrate that several markers of plaque vulnerability coincide frequently in the lesions of patients with severe end stage coronary artery disease. Plaque hemorrhages of recent or older date are present frequently and correlate with the presence of activated CD105+ microvessels. These markers can be identified in plaques throughout the coronary arteries, signifying prominent inflammation and angiogenesis as a feature of end stage atherosclerosis also in older patients.
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References


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