Dynamics of intracoronary thrombosis in STEMI and sudden death patients
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Early onset of endothelial cell proliferation in coronary thrombi of patients with acute myocardial infarction - implications for plaque healing

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Abstract

Aims: Coronary thrombotic occlusion in ST-segment elevation myocardial infarction (STEMI) patients is often preceded by episodes of progressive growth of the thrombus mass. Similar to wound healing, the organization of thrombus could depend on ingrowth of microvessels in order to stabilize its structure. We investigated the patterns of neovascularization in different stages of coronary thrombus evolution.

Material and methods: Thrombectomy materials obtained from STEMI patients were histologically classified according to thrombus age in three groups: fresh (<1 day), lytic (1-5 days) or organized (>5 days) thrombi. Forty thrombi of each group were randomly collected. Neovascularization in the thrombi was evaluated histomorphologically and with immunodouble stains to visualize various differentiation antigens of endothelial cells (ECs) and primitive cells.

Results: Morphologically, ECs in the coronary thrombi manifested as: single cells, cell clusters or microvessels. CD31+/CD34+ ECs were present in 98% of all the thrombi. In addition, endothelial clusters were found in 63% of the fresh thrombi (<1 day). CD105+, Ki67+, or C-kit+ ECs (active, proliferating cells) were observed in all the stages, but significantly more in organized thrombi (>5 days) compared with fresh and lytic ones (<5 days), and mainly as cell clusters (p<0.05 for all). CD133+ primitive cells were found only sporadically in 11% of all the samples.

Conclusion: EC proliferation is initiated very early, and gradually progresses during the organization process of thrombus after coronary plaque disruption, with only limited contribution of primitive cells in this process.
Introduction

The acute coronary syndromes (unstable angina, acute myocardial infarction and sudden coronary death) usually result from coronary atherosclerotic plaque disruption with superimposed thrombus formation. Large plaque lacerations followed by massive local activation of the coagulation system will readily lead to vessel occlusion, and hence the onset of ischemic symptoms. However, plaque disruption and thrombosis do not always coincide directly with the onset of clinical symptoms. Postmortem investigations on young adults who witnessed sudden cardiac death due to coronary thrombus have revealed ingrowth of vessels and smooth muscle cells into the occlusive thrombus mass, which indicates a discrepancy in the initiation of thrombus and the onset of death. And more recently, we revealed in histological studies of in vivo derived thrombectomy specimens of ST-segment elevation type of myocardial infarction (STEMI) patients that approximately 50% of the aspirated thrombi were in fact days to even weeks old, which further suggest that thrombus formation starts at a variable time before the onset of symptoms. Thrombus organization through ingrowth of fibrovascular tissue plays an important role in sealing and stabilizing of disrupted plaques, a notion endorsed by postmortem observations on coronary arteries which showed that progression of many atherosclerotic plaques occurs through healing of (often repeated) silent plaque ruptures. In fact, such histological “footprints” of healed plaque ruptures are a very common finding throughout the coronary arteries at autopsy. Similar to wound healing, angiogenesis is an imperative component of thrombus organization, since the newly formed vessels provide oxygen and nutrients during the healing process, thus assuring proper functioning of the cells involved, such as inflammatory cells and fibroblasts. Initially, the formation of neovessels in healing tissues was considered to be achieved exclusively by sprouting from preexisting blood vessels, but recently some evidence came up for participation of bone marrow derived primitive cells that are recruited into the thrombus. Detailed insight in these processes is of great importance, since they apparently orchestrate the stabilization of plaques after plaque rupture. However, current knowledge on mechanisms of neovascularization of thrombi is mainly derived from observations on venous thrombus in animal models, pulmonary thromboembolectomy specimens and postmortem studies on the recanalization process in coronary thrombus leading to chronic total occlusions. The current study focuses on in vivo derived coronary thrombectomy specimens from STEMI patients which provide a unique opportunity to investigate angiogenesis in coronary thrombi in vivo. With the use of immunodouble staining methods we identified the consecutive steps of neovessel formation in thrombus and correlated these findings with the histologically assessed age of the thrombi. In addition, we investigated the potential participation of primitive cells herein.
Materials and methods

Selection of materials
Paraffin embedded tissue blocks containing thrombus aspiration materials derived from STEMI patients were retrieved from the archives of the Department of Pathology of the Academic Medical Center, Amsterdam. In our hospital, thrombus aspiration is part of the routine clinical care for patients with an indication for acute percutaneous coronary intervention treatment. All patients received aspirin 300 mg and unfractionated heparin 5,000 to 10,000 IU before the procedure. Additional use of glycoprotein IIb/IIIa inhibitors was decided by the operator. Clopidogrel was administered in a loading dose of 300 or 600 mg directly before or immediately after the procedure. Since 2001, the retrieved thrombi are routinely processed following standardized methods and histomorphologically graded on H&E stained sections according to the age of thrombus as previously described. In detail, thrombus age is described in 3 categories: 1) fresh thrombus (<1 day old) with completely intact blood cells (platelets, erythrocytes and/or granulocytes); 2) lytic thrombus (1-5 days): colliquation type of tissue necrosis and nuclear fragmentation of granulocytes; 3) Organized thrombus (>5 days): presence of (myo)fibroblasts and depositions of extracellular matrix in the thrombus mass (see also Figure 1). Thrombus materials with mixed composition of different ages were graded according to the oldest part. From the total file of archived specimens we randomly selected 120 specimens, 40 of each age category, and blinded to the clinical data of the corresponding patients. Prospectively collected, baseline demographic and angiographic variables of the patients were also available.

Immunohistochemistry
Visualization of the essential steps of angiogenesis: survival, migration, activation, and proliferation of ECs with capillary tube formation, was carried out with the use five different immunodouble staining combinations. Since CD34 is a sensitive pan-endothelial marker for the detection of microvascular ECs, anti-CD34 antibody was used as the first antibody in all double staining combinations with antibodies against CD31, CD105, Ki67, C-kit and CD133 respectively. Detailed description of these antibodies and their antigenic specificity on ECs is shown in Table 1.
Figure 1: Thrombus ages (Haematoxylin and Eosin stain).
1A – fresh thrombus, characterized by completely intact blood cells. 1B – lytic change of thrombus, showing homogenization of structural elements, inset demonstrates. karyorrhexis of inflammatory cells. 1C – organized thrombus, characterized by infiltration of fibroblasts.

Table 1. Overview of antibodies used for the immunodouble stains.

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Clone number</th>
<th>Dilution</th>
<th>Time/temp</th>
<th>Source</th>
<th>Specification*</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD34</td>
<td>QBEnd-10</td>
<td>1:1000</td>
<td>60 min, RT</td>
<td>Neomarkers'</td>
<td>All ECs and primitive cells [19]</td>
</tr>
<tr>
<td>CD31</td>
<td>JC40A</td>
<td>1:1000</td>
<td>60 min, RT</td>
<td>DAKO²</td>
<td>Mature ECs [20]</td>
</tr>
<tr>
<td>CD105</td>
<td>RB-9291-P1</td>
<td>1:1000</td>
<td>Overnight, 4°C</td>
<td>Neomarkers' (Hypoxia) activated ECs [21, 22]</td>
<td></td>
</tr>
<tr>
<td>Ki67</td>
<td>SP6</td>
<td>1:1000</td>
<td>60 min, RT</td>
<td>Neomarkers'</td>
<td>Proliferating ECs [23]</td>
</tr>
<tr>
<td>C-kit</td>
<td>A4502</td>
<td>1:500</td>
<td>60 min, RT</td>
<td>DAKO²</td>
<td>Primitive cells, and cells in transition towards fully matured ECs [24, 25]</td>
</tr>
<tr>
<td>CD133</td>
<td>HPA 4922</td>
<td>1:2000</td>
<td>Overnight, 4°C</td>
<td>Gift ³</td>
<td>Primitive cells with potential to differentiate into ECs [26]</td>
</tr>
</tbody>
</table>

* Specification of antibody reactivity is mentioned only as far it relates to endothelial cells and their precursor cells. 1 = ThermoScientific, Fremont, CA. 2 = Dako, Glostrup, Denmark. 3 = Gift from Kenneth Wester, HPA group, Uppsala, Sweden. EC = Endothelial cells.
Methods of the immunohistochemical double stains were used as previously described. Briefly, after dewaxing and rehydration, a heat-induced antigen retrieval procedure using Tris-EDTA at pH 9.0 for 20 minutes was performed on all tissue sections. Subsequently, the sections were incubated with a primary antibody for 1 hour at room temperature (CD34, CD31, Ki67, and C-kit) or overnight at 4 °C (CD105 and CD133) in a two-step polymer/alkaline phosphatase assay. Visualization of antibody reactivity was performed with the Vector Red (for CD34) and Vector Blue (for CD31, CD105, Ki67, C-kit and CD133) substrate kits (Vector Laboratories) as chromogens. Negative controls were performed on thrombus sections using the same methodologies but with omission of the primary antibodies. Paraffin embedded tonsil and bone marrow tissue were used as positive controls for the specific antibodies.

Quantitative analysis of the immunohistochemical double stains
Immunostained tissue sections were screened light microscopically (Zeiss Oxiostar) at scanning view (x40) to identify the areas of highest density of double-positive immunostaining (hotspot areas). Within these areas, the numbers of immunodouble stained ECs were counted in 5 non-overlapping high power fields (x400). All numbers of immunopositive cells were expressed per mm² thrombus area.

Statistical analysis
Continuous data were expressed as mean ± SD or median with range. The means of normally distributed data among the groups were compared with the one-way Anova test. For analysis of density of ECs, groups were compared with the Wilcoxon/Kruskal-Wallis (rank sums) test. Categorical data were expressed as percentages and evaluated with chi-square test. A p value of ≤0.05 was considered statistically significant. Statistical analysis was performed with the Statistical Package for Social Sciences software (SPSS 17.0 for Windows, SPSS Inc, Chicago, IL).

Results
Patient characteristics
Patient clinical characteristics are summarized in Table 2. There were no differences in major risk factors for cardiovascular disease among the patients in the three groups classified according to thrombus age. However, thrombi retrieved from the left anterior descending coronary artery were more frequently fresh than lytic or organized (p=0.02).
Table 2. Patient clinical characteristics.

<table>
<thead>
<tr>
<th></th>
<th>Fresh (41)</th>
<th>Lytic (37)</th>
<th>Organized (44)</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male, n (%)</td>
<td>34 (83)</td>
<td>27 (73)</td>
<td>30 (68)</td>
<td>0.29</td>
</tr>
<tr>
<td>Age (yr, mean ± SD)</td>
<td>61.8±13.8</td>
<td>62.0±14.0</td>
<td>62.0±12.5</td>
<td>0.97</td>
</tr>
<tr>
<td>Hypertension, n (%)</td>
<td>15 (37)</td>
<td>16 (43)</td>
<td>16 (36)</td>
<td>0.78</td>
</tr>
<tr>
<td>Dyslipidemia, n (%)</td>
<td>12 (29)</td>
<td>9 (24)</td>
<td>7 (16)</td>
<td>0.33</td>
</tr>
<tr>
<td>Diabetes mellitus, n (%)</td>
<td>4 (10)</td>
<td>5 (14)</td>
<td>7 (16)</td>
<td>0.70</td>
</tr>
<tr>
<td>Smoke (current), n (%)</td>
<td>19 (46)</td>
<td>15 (41)</td>
<td>11 (25)</td>
<td>0.11</td>
</tr>
<tr>
<td>Statin</td>
<td>11 (27)</td>
<td>6 (16)</td>
<td>11 (25)</td>
<td>0.50</td>
</tr>
<tr>
<td>BMI (mean ± SD)</td>
<td>27.7 ± 3.9</td>
<td>26.2 ± 4.2</td>
<td>26.3 ± 4.7</td>
<td>0.18</td>
</tr>
<tr>
<td>Prior MI, n (%)</td>
<td>8 (20)</td>
<td>7 (19)</td>
<td>7 (16)</td>
<td>0.90</td>
</tr>
<tr>
<td>Prior PCI, n (%)</td>
<td>3 (7)</td>
<td>3 (8)</td>
<td>2 (5)</td>
<td>0.79</td>
</tr>
<tr>
<td>Prior CABG</td>
<td>2 (5)</td>
<td>4 (11)</td>
<td>2 (5)</td>
<td>0.46</td>
</tr>
<tr>
<td>Infarct-related artery</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>RCA, n (%)</td>
<td>11 (27)</td>
<td>24 (65)</td>
<td>22 (50)</td>
<td>0.02</td>
</tr>
<tr>
<td>LAD (including LMCA), n (%)</td>
<td>21 (51)</td>
<td>9 (24)</td>
<td>15 (34)</td>
<td></td>
</tr>
<tr>
<td>LCx, n (%)</td>
<td>9 (22)</td>
<td>4 (11)</td>
<td>7 (16)</td>
<td></td>
</tr>
</tbody>
</table>

BMI: body mass index; MI: myocardial infarction; PCI: percutaneous coronary intervention; CABG: coronary artery bypasses graft; RCA: right coronary artery; LAD: left anterior descendent artery; LMCA: left main coronary artery; LCx: left circumflex branch.

Evaluation of EC and microvessels in immunodouble stains

ECs were identified with use of the two endothelial markers CD31 and CD34 (Figure 2). Morphologically, immunopositive ECs were observed in 3 distinct patterns: first, single isolated ECs embedded in the thrombus materials; second, EC clusters of more than 3 ECs; and third, small lumen forming structures lined with ECs (microvessels). In general, ECs were found in nearly all the coronary thrombi (98%). There was no difference in numbers of single immunostained ECs among the coronary thrombi of different ages (p=0.06, Figure 3A). EC clusters could be already observed in 63% of the fresh thrombi (age <1 day) and were more frequently present in organized thrombi (93%, p<0.001). Furthermore, the number of the cell clusters was significantly higher in organized thrombi than in fresh or in lytic thrombi (p<0.001, Figure 3B). By contrast, only low numbers of distinct microvessels were observed in either fresh, lytic, or organized thrombi (Figure 3C).
Figure 2: Immunodouble stain against CD31/CD34 on an organized thrombus visualized with the use of spectral imaging.

2A – overview of immunodouble stained thrombus, CD31 stained in blue and CD34 in red. 2B – details of black frame of 2A. Green arrow: single cell; red arrow: cell cluster; black arrow: microvessel. 2C-E – spectral analysis of 2B showing CD31 stain (C) in green and CD34 in red (D), colocalization of CD31 and CD34 visualized in yellow (E). Bar=0.1mm.
Figure 3: Quantitative results of different immunodouble stains.
Three distinct patterns of immunostained endothelial cells were found: single cells, cell clusters and microvessels. Data are expressed by: numbers of positively stained endothelium per mm². 3A-C: immunodouble stain with CD31/CD34; 3D-F: immunodouble stain with CD105/CD34; 3G-I: immunodouble stain with ki67/CD34; 3J-L: immunodouble stain with c-kit/CD34. * P≤0.05. ** P <0.001.
CD105+ endothelial cells
With use of immunodouble stains combining CD105 with the pan-endothelial marker CD34, we identified the presence of activated ECs in coronary thrombi (Figure 4A and 4B). CD105+/CD34+ ECs were found in 77% of fresh, 81% of lytic, and 95% of organized thrombi (p=0.06). Most CD105+ EC presented in thrombi as single cells. However, organized thrombi still contained more EC clusters than fresh and lytic thrombus (p=0.001, Figure 3E). Only few microvessels showed CD105 positivity.

Proliferating (Ki67+) endothelial cells
Ki67/CD34 immunodouble stain was used to identify proliferating ECs (Figure 4C and 4D), since nuclear expression of Ki-67 occurs during all active phases of the cell cycle (G1, S, G2, and mitosis) but is absent in resting cells (G0). Ki67+ EC were found in 43% of the fresh thrombi, in 44% of the lytic thrombi, and in 81% of the organized thrombi (p<0.001). Most Ki67+ ECs were arranged as cell clusters and significantly more Ki67+ endothelial cluster were observed in the organized thrombi than in fresh or lytic thrombi (p<0.001, Figure 3H).

C-kit expressing cells
C-kit, a receptor tyrosine kinase for stem cell factor, is expressed on a subpopulation of maturing ECs and promotes survival, migration and capillary tube formation of the cells. The immunodouble stain for C-kit/CD34 was used to identify this subgroup of ECs (Figure 4E and 4F). C-kit+ cells were present in 27% of the coronary thrombi, albeit in low numbers and significantly more frequently in organized thrombus than in fresh and in lytic thrombi, and most of them arranged in clusters (p=0.002, Figure 3K).

Primitive cells
Primitive cells can be differentiated from mature ECs with the use of CD133 antibody (Figure 4G and 4H). CD133+/CD34+ cells were found only in very low numbers in 11% of coronary thrombi and there was no significant difference noticed between their occurrence of in fresh, lytic, or organized thrombi (p=0.69). Most CD133+ primitive cells were present as single cells and were localized in the erythrocytes-rich regions, indicating that they were entrapped in the thrombi during thrombus formation. Due to the very low numbers, this was not further quantified.
Figure 4: Immunodouble stains for endothelial cells (ECs) and primitive cells, visualized with the use of spectral imaging.

4A/B – (A) light microscopic image of an organized thrombus, immunodouble stained against CD105 (blue) and CD34 (red); (B) spectral analysis of 4A, showing colocalization of CD105 and CD34 in yellow, identifying activated ECs. 4C/D – (C) light microscopic image of an organized thrombus, immunodouble stained against Ki67 (blue) and CD34 (red); (D) spectral analysis of 4C, showing Ki67 positivity (green) restrict to cell nuclei of the ECs, identifying proliferating ECs. 4E/F – (E) light microscopic image of an organized thrombus, immunodouble stained against c-kit (blue) and CD34 (red); (F) spectral analysis of 4E, showing colocalization of c-kit and CD34 in yellow, identifying a special subgroup of ECs. 4G/H – (G) light microscopic image of a fresh thrombus immunodouble stained against CD133 (blue) and CD34 (red); (H) spectral analysis of 4G, showing colocalization of CD133 and CD34 in yellow, identifying primitive cells. Bar=0.1mm.
Discussion

With use of the thrombus age classification as a time-scale, we investigated the process of angiogenesis in coronary thrombi which were aspirated in vivo from acute myocardial infarction patients. The earliest steps in the process of angiogenesis, i.e. cellular activation (CD105 expression) and proliferation (Ki67 expression, formation of EC clusters) could be observed already in the morphologically fresh appearing thrombi of not older than approximately one day. The density of the EC clusters differed not significantly between in fresh and in lytic thrombi, which suggests a time-span of slow progression of angiogenesis in the early days (up to 4-5 days) after thrombus initiation. In addition, only sporadic primitive cells were present in 11% of the coronary thrombi, which suggests that they seem to play a limited role in the formation of neovessels during thrombus organization.

Angiogenesis in coronary thrombus

Thrombus organization implies conversion of the initially soft and friable thrombus into strong repair tissue, which is considered pivotal for sealing and stabilization of disrupted plaques. Angiogenesis appears to be a crucial element in tissue repair, which includes thrombus organization, since the newly formed vessels provide oxygen and nutrients to the healing tissue. We found ECs in arterial (coronary) thrombi already in the earliest days after thrombus initiation, while most microvessels appeared in concert with detection of sprouts of myofibroblasts in the thrombus mass, which occurs after approximately 5 days. Activation and proliferation of ECs are essential steps for initiation of angiogenesis. We found immunohistochemical evidence for both steps (CD105+ ECs in 77% and Ki67+ ECs in 43%, many of them as clustered cells) already in morphologically fresh thrombi. However, the progression of this angiogenic response seems to be initially slow, since a significant increase of such cell clusters was found only in the organized stages of thrombus of at least 5 days old. A low angiogenic activity of ECs within the first 5 days after thrombus formation could be explained by temporal changes in local concentrations of angiogenic factors such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF). Waltham et al. showed in experimental intravenous thrombi that VEGF concentrations in thrombus are significantly higher at day 7 than at day 1, and reach peak levels not earlier than 14 to 21 days after induction of the thrombus, with basically similar results for bFGF in the same experimental setting. It could be that the concentrations of these important stimuli of angiogenesis are positively associated with progression of intrathrombus angiogenesis. Therefore, we suppose that during the first week after thrombus formation, the concentrations of intrathrombus VEGF and bFGF are progressively built up to levels that significantly stimulate angiogenesis. Regrettably, the small size of thrombectomy specimens did not allow us to verify this concept.
C-kit expressing cells
Recently, several studies reported that a subgroup of mature ECs expressing c-kit may play an important role in angiogenesis. Matsui et al reported that interaction between stem cell factor and c-kit receptor promoted the survival, migration and capillary tube formation of human umbilical vein endothelial cells. In our study, we found c-kit+/CD34+ cells in 11% of fresh thrombi compared with 45% of organized thrombi and they were mainly arranged in cell clusters. This finding indicates a significant expansion of the c-kit expressing cell population during progression of thrombus organization. The distinct expression of c-kit on cells in the microvessels in the coronary thrombi may indicate that these cells are not fully differentiated ECs, because c-kit on primitive cells decreases rapidly when they begin to transform into mature cells.

Role of the primitive cells
Bone marrow-derived primitive cells have been studied extensively and appear to play an important role in physiological and pathological vascularization processes. Recently, Modarai et al. demonstrated that large numbers of primitive cells are recruited into resolving venous thrombi of recipient mice, 7 days after bone marrow transplantation, but the migration pattern of these cells closely resembled that of monocytes. Interestingly, these cells did not integrate into newly formed vessels, suggesting that the majority of neovessels in thrombi under these circumstances arose from local endothelium. In line with these findings, we found only small numbers of primitive cells in 11% of the aspired coronary thrombi, and if present, they were mainly localized in erythrocyte-rich regions, indicating their entrapment during the process of thrombus propagation. Therefore, the role of these primitive cells seems to be limited in arterial thrombus organization at least at this specific site.

Clinical perspective
Although, EC proliferation, interpreted as the first step of angiogenesis, in coronary thrombi following plaque rupture starts as early as within the first day after thrombus formation, its progression was found to be slow during the first week. This could be due to low levels of intrathrombus angiogenic stimuli. Because treatment of ischemic heart disease and peripheral vascular disease with angiogenic growth factors has produced promising results, and an early angiogenic response is considered pivotal for proper tissue repair, similar treatments could be used to evoke rapid stabilization of disrupted plaques in patients with unstable coronary artery diseases.
References


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