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Full Length Article

Time dependent apoptotic rates in the evolving coronary thrombus mass of myocardial infarction patients

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ABSTRACT

Aim: To study the rate of apoptotic cell death in the process of thrombus evolution after plaque rupture in myocardial infarction.
Methods: Paraffin embedded thrombosuction aspirates of 63 patients were stained with haematoxylin & eosin (H&E) to assess histologically the age of the thrombi: fresh (intact blood cells; <1 day old), lytic (necrosis; 1–5 days old) or organized (ingrowth of cells; >5 days old). Presence of plaque constituents (atheroma including foam cells, cholesterol crystals calcifications and fibrous cap tissue) was also recorded. Immunohistochemical (double) stains with anti-caspase-3-antibody were used to visualize apoptosis and the cells involved. For the latter caspase-3 antibody was combined with cell-specific markers MPO (granulocytes), CD68 (macrophages), CD34 (endothelial cells), SMA-1 (smooth muscle cells) and a Feulgen stain (DNA). Second, the rate of apoptosis was evaluated in relation to the age of the thrombi. Platelet apoptosis was further evaluated with the use of TEM.
Results: From a total of 63 aspirates, plaque constituents were found in 33 of the aspirates, and in 15 of them lipid rich plaque tissue was the sole component. Age classification of all thrombus containing aspirates ($n = 48$) resulted in 12 fresh (25%), 18 lytic (37.5%) and 18 organized (37.5%) thrombi. Apoptosis was more extensive in lytic thrombi than in fresh or organized thrombi ($P < 0.0001$). Plaque-containing aspirates showed more apoptosis than aspirates without plaque ($P < 0.05$). Immuno staining with caspase-3 antibody in combination with cell-specific markers showed that apoptosis was most extensive in MPO + granulocytes. Caspase-3-positive platelets (CD61 + anucleate particles) were most abundant in lytic thrombi. Apoptosis in platelets was confirmed by ultrastructure.
Conclusion: This study demonstrated a significant association between thrombus age and occurrence of apoptosis of granulocytes and also platelets, with highest rates in (fragile) lytic thrombi. We propose that apoptotic cell death in athero thrombosis could potentially serve as a biomarker for thrombus instability.
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1. Introduction

Most cases of acute myocardial infarction (AMI) are caused by an acute thrombotic occlusion of a coronary artery(1). Large plaque ruptures leading to massive local coagulation, will readily lead to acute instantaneous occlusion [1–3].

However, recent histopathological investigations of thrombectomy materials derived from patients treated for acute myocardial infarction may have coronary thrombi older than 1 day in approximately 50% of the patients [4,5]. This implies that in those cases acute coronary occlusion can be the final stage in a series of consecutive thrombotic events dating back days or sometimes weeks before clinical symptoms become manifest [4–6]. The cellular mechanisms that underlie the process of thrombus progression after a coronary plaque rupture or erosion are still not fully understood, but clearly of importance since such knowledge could provide useful new (bio)markers for progression of thrombosis in atherosclerotic arteries. Apoptosis, a physiological, programmed and energy-dependent cell death cascade, has extensively been studied in atherosclerotic plaques, and shown to be abundant in mature advanced atherosclerosis but scarce in early lesions [7–9]. It has been suggested that apoptosis plays an important role in atherogenesis and plaque destabilization [7–10]. Also thrombus aspirates show a distinctive age-related pattern of morphological changes resembling apoptotic cell death, *e.g.* nuclear condensation and fragmentation [4,5]. Potentially, all cell types inside a thrombus can be involved: inflammatory cells (granulocytes and mononuclear cells), smooth muscle

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cells, and also platelets, as has been reported recently [13–15].Several atherosclerotic factors can induce apoptosis such as oxidative stress, hypoxia, interferon-γ and, in the case of macrophages, cholesterol overload [2,3].

The pathogenesis and mechanisms of development and destabilization of the atherosclerotic plaque have been well documented [3,11,12,16–18]. However, the role of apoptosis in the coronary thrombus in relation to thrombus evolution after its initiation has never been studied. To gain more insight herein, this study investigated the time related changes in the rate of apoptosis in coronary thrombus with the use of histological grading of the age of thrombi as previously described. For this purpose we used coronary thrombectomy samples obtained from AMI patients during primary percutaneous coronary intervention by means of thrombosuction followed by balloon dilatation and/or stent implantation.

2. Material and methods

Coronary thrombosuction specimens were obtained from patients who presented with an acute (≤12 h) ST elevation type of myocardial infarction (STEMI, ≥2 mm ST-elevation in ≥2 contiguous leads on EKG),and who were treated with thrombosuction as part of the therapeutic primary percutaneous coronary intervention (PCI) procedure [4]. Directly after thrombosuction the aspirated materials were fixed in buffered formalin for 24 h and paraffin embedded. Only specimens of >2 mm and suitable for histological grading of the age of the thrombus were enrolled, which resulted in a total of 63 aspirates for this study. Study design met the Declaration of Helsinki. The medical ethical review board of the hospital applies a waiver for informed consent when only leftover tissue of normal clinical procedures is used. All data were analyzed anonymously.

2.1. Histology

Paraffin sections (5 μm) were cut, mounted on glass slides, and haematoxylin and eosin (H&E) stained for histological grading of the age of the thrombus using a previously described and accepted classification [4,19]: first: fresh thrombus (<1 day old), characterized by intact red blood cells, platelets and granulocytes in a layered structure; second: Lytic thrombus (1–5 days old), characterized by areas of necrosis and karyorrhexis of cells; and third: organized thrombus (>5 days old), characterized by ingrowth of (myo-)fibroblasts, and/or deposition of connective tissue and angiogenesis. In addition, the presence of plaque constituents such as foam cells, inflammatory cells, collagen tissue and lipids, including cholesterol crystals and calcifications was recorded. Aspirates with a heterogeneous thrombus age were graded according to the age of the oldest component.

2.2. Immunohistochemistry

Serial sections adjacent to H&E stained section were used for immunostaining. After deparaffinization and rehydration in xylol and ethanol endogenous peroxidase was inhibited in methanol and H_2O_2 and heat induced antigen retrieval (HIER) in Tris EDTA buffer (pH = 9) for 20 min at 98 °C. After washing, sections were subsequently incubated with serum free protein block (Dako, Glostrup, Denmark) and incubated with cleaved caspase-3 antibody (rabbit anti-caspase-3, clone ASP175, Cell signalling Europe BV, Leiden, the Netherlands), as apoptosis marker, followed by Horse radish peroxidase conjugated polymers as the secondary antibody. Enzymatic activity detection was performed with 3,3′-Diaminobenzidine (bright-DAB, Immunologic BV, Duiven, the Netherlands). Finally, all sections were counterstained with haematoxylin.

Immunodouble stains were applied to identify the cellular origin of apoptosis in the thrombus material. Methods were essentially as previously described [20]. The first step differed only from the single staining by the use of Alkaline Phosphatase (AP) AP conjugated polymers as secondary antibody and the use of Vector Red phosphate substrate (Vector labs, Peterborough, United Kingdom) for detection of enzymatic activity. In the second staining step, HIER was performed for 10 min in Tris EDTA, followed by rinsing in TBS and a second protein block. Then the sections were incubated with one of the following primary cell specific antibodies: mouse anti-SMA (smooth muscle actin, smooth muscle cells, clone 1A4, Dako, Glostrup, Denmark), monoclonal rabbit anti-MPO (myeloperoxidase, neutrophils, Dako, Glostrup, Denmark), mouse anti-CD68 (macrophages, clone PG-M1, Dako, Glostrup, Denmark), mouse anti-CD34 (endothelial cells, clone QBend1, Immunologic BV, Duiven, the Netherlands) and mouse anti-CD61 (glycoprotein-IIIa, platelets, clone Y2/51, Dako, Glostrup, Denmark). After rinsing with TBS the sections were incubated with appropriate AP conjugated polymers and Vector Red substrate.

To enhance visualization of nuclear material also a Feulgen stain (Scytek Laboratories, Utah, USA) was applied as secondary staining step. After the first staining step (see above) sections were hydrolysed in HCl 1 N at 60 °C. After washing the section they were subsequently rinsed with a Blue Feulgen stain mixed with the decolourizer vial and a rinse solution (400 ml 0.005 N HCl and one rinse solution vial) and finally with distilled water.

Co-expression of epitopes by cells in immunodouble stained sections was analyzed with the use of Nuance spectral image analysis software (Perkin Elmer, Inc., Hopkinton, USA). Negative controls were performed on thrombus sections using the same methodologies but with omission of the primary antibodies. Tonsils were used as positive controls.

2.3. Evaluation of apoptosis

Immuno-stained sections were screened under a light microscope at scanning view (×40) to identify the area with the highest density of positive cells. Within this area the positively stained cells were counted in three adjacent high power fields (200×). From thrombosuction aspirates with a heterogeneous thrombus age composition the degree of apoptosis was measured in the area with oldest age.

To assess the presence and extent of apoptosis we developed a three-point scale labelling index of anti-caspase-3 immunostaining: 1. low (<10 positive cells and/or cell fragments per 200× field), 2. medium (at least one field with >10 but <20 positive cells and/or cell fragments) or 3. high (at least one field with >20 positive cells and/or cell fragments). thrombosuctions were scored by two observers (intra- and inter-observer variation < 10%).

2.4. Transmission electron microscopy (TEM)

To further evaluate the presence of apoptosis in platelets during thrombus evolution, additional thrombus aspirates were captured in Karnovsky fixative for ultrastructural analysis by means of TEM. Ultrathin sections were cut and screened for ultrastructural characteristics of apoptosis in platelets as have been described in the literature, such as cell membrane shrinkage, active membrane blebbing, filopod extrusion, nuclear and cytoplasmic condensation, cellular fragmentation and engulfment of apoptotic bodies by nearby cells [13,14].

2.5. Statistical analyses

All statistical analyses were performed with the SPSS software PASW Statistics 18, (IBM, Hong Kong). Categorical data were expressed as percentages and the associations of the different variables with apoptosis were evaluated using the Chi-square test. P values < 0.05 were considered to indicate statistical significance.

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