Virchows Archiv

European Journal of Pathology

Volume 471 · Supplement 1 · September 2017

Springer

428 • 471(S1) S1-S352 (2017)
ISSN: 0945-6317 (print)
ISSN: 1432-2307 (electronic)

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which in turn may be able to reduce the invasiveness of a conventional autopsy.

OFP-03-006

Netosis in coronary plaque ruptures, plaque erosions and intraplaque hemorrhages of myocardial infarction patients at autopsy

K. R. Pertiwi*, A. C. van der Wal, D. R. Pabittei, R. J. de Winter, X. Li, O. J. de Boer
*Academisch Medisch Centrum, Biology Education Dept., Amsterdam, The Netherlands; Yogyakarta State University, Indonesia

Objective: Netosis is a form of cell death characterized by formation of neutrophil extracellular traps (NETs). To evaluate its participation in coronary atherothrombosis, we investigated the presence and distribution of NETs in coronary plaque ruptures, plaque erosions and intraplaque hemorrhages (IPHs).
Method: Forty-four coronary plaques were retrieved at autopsies from 36 myocardial infarction patients, of which, in HE-stains, were classified as 9 erosions, 18 ruptures and 17 IPHs. 20 intact plaques were selected as controls. Thrombus material in plaques was graded as either fresh, lytic or organized. Immunohistochemistry was performed to visualize neutrophils (MPO) and NETs (citrullinated histone-3/CitH3 and PAD4). Results of immunostaining were scored semi-quantitatively.
Results: Neutrophils (MPO+) and NETs (CitH3+ and PAD4+) were abundantly present in all types of complicated plaques, with no significant differences in extent between ruptures, erosions and IPHs. NETs were found in the thrombus, the underlying plaque tissue and adventitia, the latter with the highest amount in eroded plaques. Fresh and lytic thrombi contained significantly higher numbers of neutrophils and NETs than organized thrombi. In contrast, intact plaques contained no neutrophils and NETs.
Conclusion: Netosis takes part in all distinct types of atherothrombosis, with presumed role in thrombus progression towards vessel occlusion.

OFP-03-007

Balances of different types of cell death in coronary thrombus in relation to thrombus age and instability, after myocardial infarction

P. A. Gabriels*, K. R. Pertiwi, O. J. de Boer, R. J. Winter, A. C. van der Wal
*Academic Medical Center, Pathology, Amsterdam, The Netherlands

Objective: Fragile thrombus (instability) is associated with a higher mortality rate after acute myocardial infarction (AMI). We investigated which types of cell death are present and involved in thrombus stabilization, over time.
Method: Coronary thrombosuction aspirates of AMI patients were histologically classified in HE-stains as fresh (15), lytic (13) or early organizing (8). An immunohistochemical sequential triple staining was performed using anti-C-reactive protein (necrosis), anti-caspase-3 (apoptosis) and anti-citrullinated histone H3 (ETosis) as primary antibodies. For each specimen, the presence and most prominent type of cell death were semi-quantatively recorded and presented as a percentage of total observations.
Results: All 3 types of cell death were found to be present in all 3 age categories. The most prominent types of cell death observed in fresh and lytic thrombi were ETosis (44.9 and 40 % of specimens, respectively) and apoptosis (43.6 and 35.7 %, respectively), followed by necrosis (11.5 and 24.3 %, respectively). ETosis appeared the most prominent type of cell death found in organizing thrombi (40 % of specimens), but in these thrombi necrosis (37.5 %) was more dominant than apoptosis (22.2 %).
Conclusion: Cell death, along several pathways, is a prominent mechanism in thrombus tissue of AMI patients, and can lead to thrombus instability / fragility.

OFP-03-008

New formula for cardiothoracic ratio for the diagnostic of cardiomegaly on post-mortem CT

M. Jotterand*, M. Faouzi, F. Dedouit, K. Michaud
*CURML, UMF, Lausanne, Switzerland

Objective: The cardiothoracic ratio (CTR) is considered to be a reliable detector of cardiomegaly on the CT for livings. Our study aimed to establish an adjusted CTR based score to predict cardiomegaly at post-mortem computed tomography (PMCT).
Method: We selected adult's autopsy cases between 2009 and 2016. Two groups (normal heart weight and overweighed heart) were considered. The CTR was measured on axial images. Logistic regression analysis was performed to investigate the discriminating power of the CTR between groups when adjusting to the confounding factors.
Results: 120 cases with normal heart weight and 100 cases with overweighed heart were analyzed. The factors associated to the cardiomegaly are CTR (p-value = 0.003, OR = 3.57), BMI (p-value = 0.055, OR = 1.09), age (p-value <0.001, OR = 1.67) and gender (p-value 0.002, OR = 4.85). An integer-based point-scoring system was derived based on their β-Coefficients. The score ranged from 21 to 45 with highest values indicating a more likely cardiomegaly. For a threshold of 33, the sensitivity, specificity and the correctly classified were 0.84, 0.78 and 0.81 respectively.
Conclusion: CTR alone cannot be used to discriminate between normal heart weight and overweighed heart at PMCT. A new formula has been developed, including age, gender and BMI to diagnose the cardiomegaly at PMCT.

OFP-03-009

Extra-pulmonary tuberculosis in Nepal: A tip of an iceberg

R. Baral*
*Patan Academy of Health Science, Dept. of Pathology, Kathmandu, Nepal

Objective: Tuberculosis is a common condition in the underdeveloped countries like Nepal annual case notification rate (CNR) is 136 per 100,000 populations. Tuberculosis is the 6th leading cause of death in Nepal. Tuberculosis is a preventable disease if diagnosed in time. Extra Pulmonary Tuberculosis is 23.1 % out of total registered Tuberculosis cases in the country. Therefore, objective of this study is to determine the Extra Pulmonary Tuberculosis (EPTB) pattern in the specimen received in pathology lab that may help to understand the prevalence and disease identification.
Method: Pathology Lab Database analysis of Histo-cytology specimens for Extra Pulmonary Tuberculosis during 5 years period from 2011 to 2016 at PAHS, Kathmandu, Nepal.
Results: Out of approximately 20,000 specimens received in the Pathology Department in the 5 year period 1 % was of Extra Pulmonary Tuberculosis (EPTB). Lymph nodes comprised of 58 %, followed by Gastrointestinal and Skin in 10 each and 8 % cases were seen in the urogenital tract.
Conclusion: This study represents facility based data only; so it may reflect the tip of an iceberg of at risk population who dwell in the rural mountainous area where the diagnostic facility are not available. Merely the clinical judgement should not overlook the probability of Extra Pulmonary Tuberculosis.

OFP-03-010

Introducing MiniTEM for ultrastructural pathology

I.-M. Sintorn*, L. Haag, G. Kylberg, P. Malm, M. Ryner, K. Hultenby, A. Dragomir
*Vironova A/B, Stockholm, Sweden

Objective: MiniTEM is a desk-top transmission electron microscopy and analysis platform with a high degree of automation in the microscope

Balances of different types of cell death in coronary thrombus in relation to thrombus age and instability, after myocardial infarction

Pauline AM Gabriels[1,2], **Kartika R Pertiwi[1,3]**, Onno J de Boer[1], Robert J Winter[4], Allard C van der Wal[1]

[1]Cardiovascular Pathology, Academic Medical Centrum, Amsterdam, the Netherlands
[2]Faculty of Sciences, University of Amsterdam, Amsterdam, the Netherlands
[3]Biology Education Department, Yogyakarta State, Indonesia
[4]Heart Center, Academic Medical Centrum, Amsterdam, the Netherlands

Objective: Fragile thrombus (instability) is associated with a higher mortality rate after acute myocardial infarction (AMI). We investigated which types of cell death are present and involved in thrombus stabilization, over time.
Methods: Coronary thrombosuction aspirates of AMI patients were histologically classified in HE-stains as fresh (15), lytic (13) or early organizing (8). An immunohistochemical sequential triple staining was performed using anti-C-reactive protein (necrosis), anti-caspase-3 (apoptosis) and anti-citrullinated histone H3 (ETosis) as primary antibodies. For each specimen, the presence and most prominent type of cell death were semi-quantatively recorded and presented as a percentage of total observations.
Results: All 3 types of cell death were found to be present in all 3 age categories. The most prominent types of cell death observed in fresh and lytic thrombi were ETosis (44.9% and 40% of specimens, respectively) and apoptosis (43.6% and 35.7%, respectively), followed by necrosis (11.5% and 24.3%, respectively). ETosis appeared the most prominent type of cell death found in organizing thrombi (40% of specimens), but in these thrombi necrosis (37.5%) was more dominant than apoptosis (22.2%).
Conclusion: Cell death, along several pathways, is a prominent mechanism in thrombus tissue of AMI patients, and can lead to thrombus instability / fragility.

INTRODUCTION

The development of thrombosis towards the eventual clinical symptoms, caused by cardiovascular diseases (CVDs), is not fully understood up till now. CVDs are the leading causes of morbidity and mortality worldwide, with approximately 17.5 million deaths every year. This amount represents 31% of all deaths globally, of which 80% is in low- and middle income countries (1). Among these, atherosclerosis related diseases such as myocardial infarction, stroke and peripheral artery disease are most prevalent. This group of disorders can most often be prevented by addressing behavioral risk factors, such as poor diet, smoking, lack of physical activity and harmful use of alcohol. The effects of these risk factors present in patients as raised blood pressure, raised blood glucose and lipids, overweight and obesity (2, 3), which increase the risk of atherosclerosis related diseases. Pathologically, atherosclerotic plaques may become symptomatic due to disruption of plaque tissue, fibrous cap rupture or massive endothelial erosion, which evokes abrupt local activation of the coagulation system, followed by partial or even complete thrombotic occlusion of the coronary artery,

which critically lowers the blood flow to the heart (4, 5). This causes ischemia of the heart muscle, which eventually might lead to a life-threatening and time-sensitive acute myocardial infarction (AMI) (6-8).

The main mechanisms known of plaque disruption are plaque erosion and plaque rupture. Plaque erosion is typically characterized by the absence of endothelium, abundant activated SMCs and proteoglycans, absent or minuscule necrotic core and minimal inflammation (19). The uncovered SMCs express pro-coagulant transcription factors to the bloodstream, stimulating thrombosis. Recently, histopathological investigations of thrombectomy materials, derived from AMI patients, revealed that approximately 50% of the aspirated thrombi were older than 1 day, some even weeks old (25). This implies that plaque disruption and subsequent thrombosis do not always coincide directly with the onset of clinical symptoms. In these patients, the final coronary thrombotic occlusion often follows plaque instability with progressive growth of the thrombus mass. This suggests that after thrombus formation the development of the thrombus can encounter different pathways; directly causing an infarct, slow progression with an acute coronary occlusion as final stage manifesting clinical symptoms or even healing of the rupture, without any clinical outcomes (5, 25-27). Importantly, it is questioned what factors determine the (in)stability pathway the thrombus will encounter during development. Similar to wound healing the 'healing' of a thrombus is a tightly regulated process overtime. Thrombus organization is a process that converts an initially soft and fragile thrombus into strong and stable (scar)tissue that will be incorporated into the plaque mass (28). Inflammation, angiogenesis and fibrosis influence this process, during which not only cell proliferation is of importance, but also cell death plays a role in these three aspects of thrombus (in)stability (22).

OBJECTIVES

Coronary thrombus formation is a biologically heterogeneous process that changes over time. Consequently, the tissue composition of thrombi changes over time as well during organization, influencing the (in)stability of the thrombus. Identifying the major types of cell death and cell types inside the evolving coronary thrombus is required to investigate the ratios of different types of cell death in relation to the histologically determined age and stability of the coronary thrombus after myocardial infarction. Episodes of instability of the thrombus mass can have adverse effects on the clinical outcome of patients. We raise the hypothesize that different types of cell death are involved in destabilizing the tissue structure of a coronary thrombus over time and that different cell types will be specifically involved in each developmental step. We propose that tissue markers for cell death can serve as biomarkers for thrombus age and (in)stability.

MATERIALS AND METHODS

Thirty coronary thrombus specimens, retrieved from patients who presented with an AMI, were collected from the pathology archive of thrombectomy specimens in the Academic Medical Centre (AMC), in Amsterdam. These formalin-fixed paraffin embedded tissues were cut in 5 µm thin sections with a microtome (Thermo Scientific™ HM 340E Electronic Microtome; feed 5, trim 30) and mounted on adhesive coated glass slides (Klinipath, Duiven, the Netherlands). To confirm the thrombus age, an haematoxylin and eosin (H&E) staining was performed, following standard laboratory procedures. Histological classification was done according to previously published and accepted definitions: (1) fresh thrombus (<1 day old), characterized by layered patterns and intact granulocytes; (2) lytic thrombus (1 - 5 days old), composed of areas of necrosis and karyorrhexis of granulocytes; (3) organized thrombus (> 5 days old), showing ingrowth of SMCs, angiogenesis and/or deposition of connective tissue (27, 28, 38, 43).

Immunohistochemical staining

For immunohistochemistry, the following primary antibodies were used: anti-C-reactive protein (CRP) to identify necrosis, anti-caspase-3 (cleaved) for apoptosis, anti-citrullinated histone H3 (Citr. H3) for NETosis, anti-CD15 for granulocytes, anti-CD68 for macrophages and anti-smooth muscle α-actin (SMA) for SMCs. The 'triple sequential staining' technique was used to give a simultaneous overview of necrosis, apoptosis and NETosis within one section, by using three different chromogens. Briefly, paraffin sections were dewaxed in xylene and rehydrated in graded alcohols. This was succeeded by incubation of 20 minutes, with 0,3% hydrogen-peroxide in methanol, to block endogenous peroxidase activity. Subsequently, heat-induced antigen retrieval (Lab Vision™ PT Module; ThermoFisher Scientific, Fremont, CA, USA) was performed using Tris-EDTA (pH = 9.0; ThermoFisher Scientific) for 20 minutes, at 98 °C. After washing with Tris-HCl buffered saline (TBS), the sections were incubated with one droplet of Lab Vision™ Ultra-V Block (ThermoFisher Scientific) per slide, to block nonspecific binding (10 minutes, at room temperature (RT)). The fluid was blotted off and sections were incubated with 200 µl primary antibody, in optimized dilutions. Depending on the antibody, incubation was performed overnight at 4 °C or for 60 minutes at RT. After incubation with the primary antibody, the sections were incubated with the secondary antibody. For single staining, BrightVision polymer horseradish peroxidase (HRP) anti-rabbit (anti-Rb) IgG (1:1)(ImmunoLogic) or anti-mouse (anti-Ms) IgG (1:1)(ImmunoLogic,) was used, depending on the primary antibody, for 30 minutes at RT. Washing was performed prior to incubation with Vector NovaRED (Vector Laboratories), for 10 minutes at RT, for visualization. Finally, the slides were counterstained with undiluted haematoxyline (Klinipath), for 1 dip and dried on a hot plate (50 °C).

preceding mounting with Vectamount (Vector Laboratories,). During the sequential triple staining the first staining was performed the same way as above, with anti-CRP as primary antibody. However, HRP activity was visualized using DAB+ (3,3'Diaminobenzidine (DAB); ImmunoLogic) instead of Vector NovaRED. DAB+ is chosen as it forms a protective layer over the first antibody layer, preventing cross-reactions between antibodies from similar hosts. Following visualization of the first staining with anti-CRP, the sections were washed in TBS, prior to incubation with the second primary antibody, anti- Citr. H3, overnight at 4 °C. Secondary incubation was performed with BrightVision Alkaline Phosphatase (AP) anti-rabbit (anti-Rb) IgG (1:1)(ImmunoLogic,), for 30 minutes at RT. This antibody was visualized with Perma Blue (Diagnostic BioSystems, Pleasanton, CA, USA), for 10 minutes at RT. Subsequently, the slides were washed in tap water and a second antigen retrieval step was performed, using Tris-EDTA (pH = 9.0; ThermoFisher Scientific), however for 10 minutes at 98 °C. The sections were again incubated with Ultra V Block, for 10 minutes at RT. Thereupon, incubation with primary antibody anti-caspase-3 (cleaved) (1:500) took place for 60 minutes, at RT, followed by incubation with secondary AP anti-rabbit antibody (1:1), for 30 minutes at RT. AP activity was visualized with Vector RED (Vector Laboratories), for 10 minutes at RT and finally slides were placed on a hot plate (50 °C), prior to mounting with Vectamount.

Image analysis of immunohistochemical stained sections

Areas of the different age categories were selected from histological H&E stained thrombus sections, using a Olympus BX40 microscope (LabX, Midland, Canada). For the triple sequential staining, Multi Spectral Images were taken (wavelength; 420 till 720 nm, interval; 20 nm) of the selected areas, using a CRi Nuance camera (Nuance, Thermo Fisher Scientific, USA), attached to a Brightfield microscope (Leica CTR5500, Leica, Germany). Using the Nuance 3.0.2 imaging program, color spectra were made of DAB+, Perma Blue, NovaRED and background according to single stains. After determining the spectra, the three colors were separated from each other using the Unmix function. Subsequently these images were all saved, after which Image Pro Premier 9.2 (64 bit) software (MediaCybernetics, UK) was used to set a threshold for the three different types of cell death, which were treated separately (citr. H3 range 30, dark 230; caspase range 35, dark 185; CRP range 15, dark 175). This threshold was used to determine the area of true positive cells, which was then automatically counted (in pixels) and exported to an Excel sheet. With the use of IBM SPSS statistics 24, the percentage of the three different types of cell death was calculated.

Statistical analysis was performed using Two-Way Anova, followed by the multiple comparisons Bonferroni post-hoc test. This test was also used to determine the significance between the different age categories, for each individual type of cell death. $P < 0.05$ was considered to be statistically significant.

RESULTS

The selected archived specimens which were previously classified for the age of the thrombus, were graded according to the oldest part of the specimen. In the recent study, we found a heterogeneous tissue composition within aspirates, consisting of fresh, lytic and organized areas simultaneously. This resulted in a selection of a total of 46 areas (17 fresh; 15 lytic; 14 organized), within the 30 chosen specimens. Figure 1 illustrates representative examples of fresh (figure 1A), lytic (figure 1B) and organized (figure 1C) thrombi.

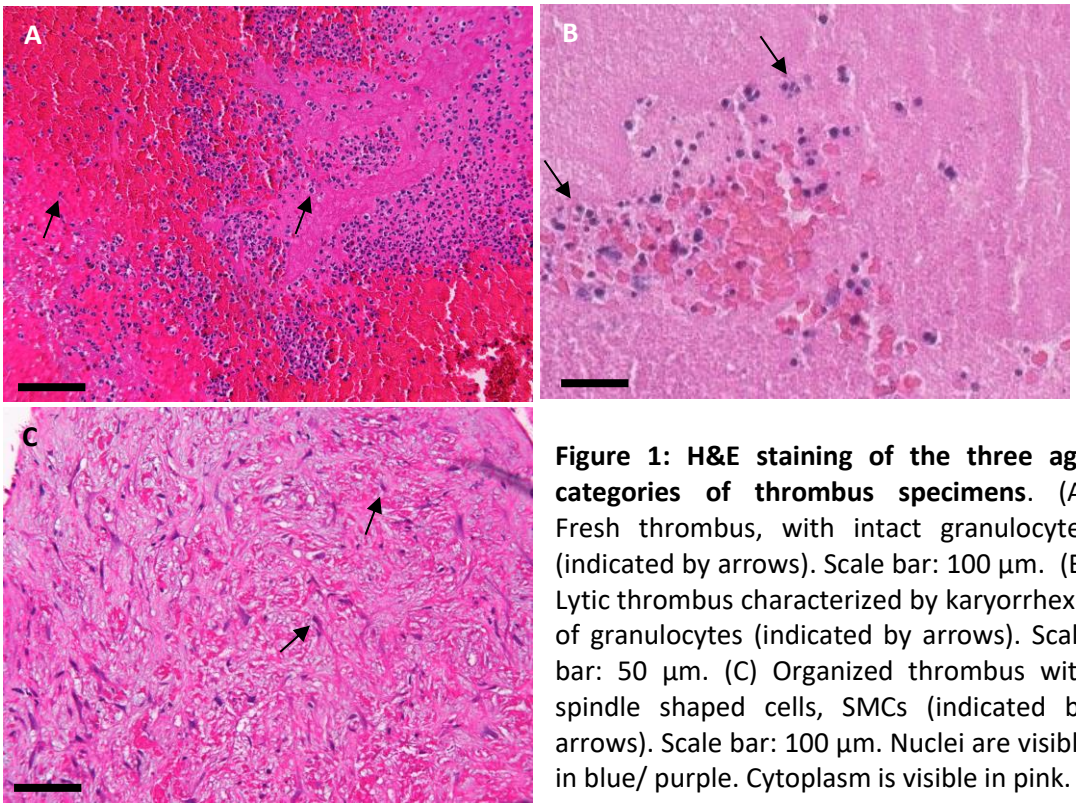

Figure 1: H&E staining of the three age categories of thrombus specimens. (A) Fresh thrombus, with intact granulocytes (indicated by arrows). Scale bar: 100 µm. (B) Lytic thrombus characterized by karyorrhexis of granulocytes (indicated by arrows). Scale bar: 50 µm. (C) Organized thrombus with spindle shaped cells, SMCs (indicated by arrows). Scale bar: 100 µm. Nuclei are visible in blue/ purple. Cytoplasm is visible in pink.

Immunohistochemical triple sequential stainings were performed to investigate the proportion of necrosis, apoptosis and NETosis within fresh, lytic and organized thrombi. Representative examples of the triple stains are illustrated in figure 2. It is clear that within every age category, a great variation in types of cell death occurs.

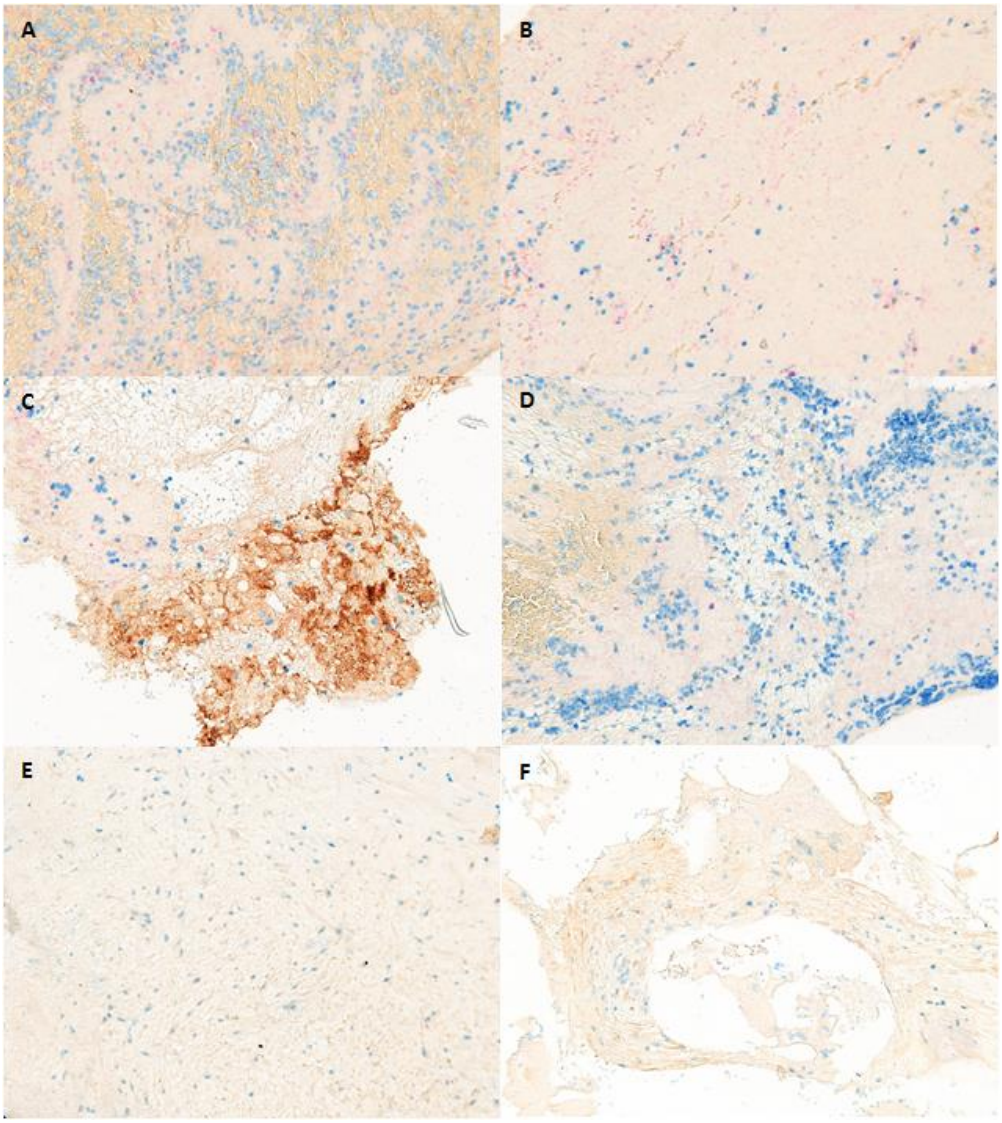

Figure 2: Representative examples of Fresh (A, B), Lytic (C, D) and Organized (E, F) thrombi. Necrosis is stained with DAB+ (1:4000), visible in brown. Apoptosis is stained with Vector RED (1:500), visible in red. NETosis is stained with Perma Blue (1:8000), visible in blue. In all images the original magnification was 20x.

To quantify the amount of expression of each individual antibody, individual staining patterns were separated and visualized using spectral imaging. The immunopositive surface area of each antibody was measured (see figure 2) and the amount of necrosis, apoptosis and NETosis was expressed as percentage, depicted in Figure 3. When investigating each age category individually, it can be appreciated that NETosis was present most, compared to apoptosis and necrosis. The difference between NETosis and either apoptosis or necrosis was significant ($P < 0.05$), in all three age categories. It can be esteemed that the percentages of apoptosis, necrosis and NETosis is relatively constant over time. Moreover, each individual type of cell death was investigated over time. NETosis was seen to be

least in lytic thrombi, while necrosis and apoptosis staining showed most positivity in lytic thrombi. No significant difference was found for either necrosis or apoptosis or NETosis during organization.

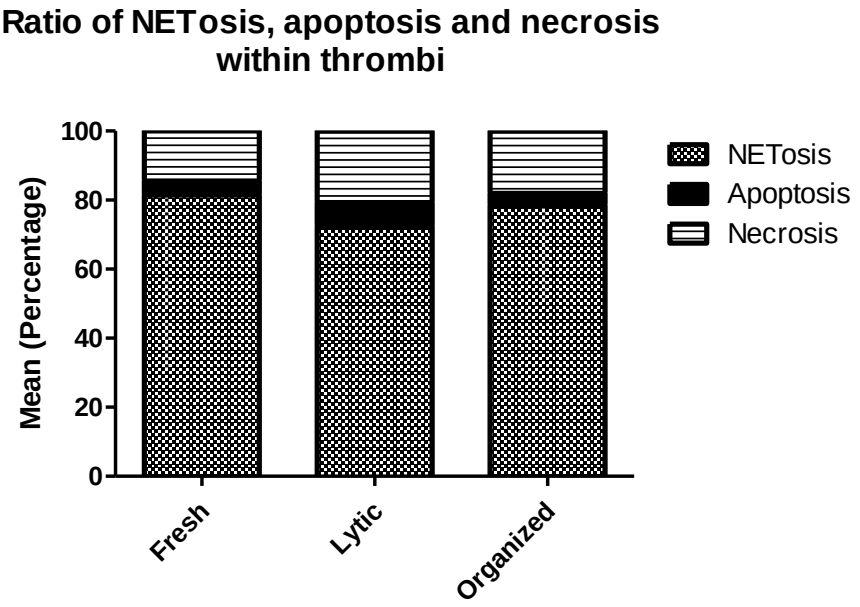

Figure 3. Graph representing the percentages of NETosis, apoptosis and necrosis relative to each other, within each age category.

DISCUSSION

In order to identify tissue destabilizing factors in the coronary thrombus of myocardial infarction patients, we investigated the occurrence of three different types of cell death, namely necrosis, apoptosis and NETosis. They were studied in the various stages of thrombus evolution, which occur after the onset of a thrombotic occlusion; fresh (early), lytic (older) and organized (oldest) thrombi. Moreover, different cell types involved in these cellular death mechanisms were studied. To the best of our knowledge, no information is known about the ratios of different types of cell death occurring in an organizing thrombus.

The three types of cell death under investigation were found to be present in all three age categories of a thrombus. In each age category, we found that NETosis, as measured by the relative immunopositive surface area, contributed significantly more to the overall rate of cell death than necrosis and apoptosis. The ratio between these types of cell death remained relatively constant over time, indicating an individual role for each type of cell death within stabilization of coronary thrombi.

In a previous study, apoptosis has been suggested to play an important role in thrombosis and thrombus destabilization (26). Maagdenberg et al. found a highly significant association between

thrombus age and the amount of apoptosis, with more apoptosis in lytic thrombi compared to fresh or organized specimens. Lytic thrombi are known to contain weak tissue, leading to thrombus instability. Lower apoptotic rates in organized thrombi indicate the role of apoptosis in organizing the evolving thrombus towards stability, as apoptosis provides a more stable and rigid tissue structure (26). However, in the present study, no significant differences between the different age categories were found for immunopositive apoptotic staining.

Apart from apoptosis, increasing evidence suggests that NETosis also plays a role during thrombosis. NETs appear to be decorated with TF and therefore promote fibrin deposition, erythrocyte recruitment and provide a stimulus for the formation of fibrin networks, which are of importance in platelet aggregation (29). De Boer et al. (2012) found NETs to be present focally most in lytic thrombi, followed by fresh and absent in organized specimens. Nevertheless, during the present study NETs were also observed in organized specimens and no significant difference in the presence of NETs was found between the age categories. An explanation for the different result could be that De Boer et al. identified NETs using only H&E, Feulgen and H1 stains and that they did not use the more sensitive and specific immunohistochemical technique with Citr. H3 antibody. Taken together, we showed that NETs may also contribute to thrombus growth and stabilization during organization.

Moreover, necrosis was present in all three age categories, and repeatedly with no significant difference during the process of thrombus organization. Theoretically, a significant higher amount of necrosis was expected to be found in lytic specimens, as this age category is by definition characterized by the presence of a colliquative type of necrosis in conventional H&E stains (28). These necrotic areas are a buildup of recently died cells, as in apoptosis and NETosis, but also include entirely dead tissue areas including matrix components, lipids and cell debris. The latter do no signal to be cleared away. This causes tissue weakness, a delay in organization and thus thrombus instability. We believe it is doubtful whether CRP is indeed a useful marker for presence of necrosis.

It was questioned whether immunohistochemistry is the correct method to compare the extent of each type of cell death. The percentages are based on the number of immunopositive pixels per section. However, each type of cell death has its own morphological appearance. Apoptotic cells shrink and are visible as small dots, undergoing nuclear fragmentation. Contrary, cells undergoing NETosis occupy a big surface area, visible as meshes. Consequently, immunopositive areas of NETosis cannot be simply compared with immunopositive areas for apoptosis, to find out which type of cell death predominates in a tissue. In other words, it remains elusive, whether apoptosis is more important than NETosis (or vice versa). On the other hand, the screening of immunopositive areas remains very useful to

estimate the extent of a type of cell death, be it NETosis or apoptosis, in the different stages of thrombus evolution, from fresh to organized.

CONCLUSION

It can be concluded that the occurrence of cell death is a prominent feature in thrombus tissue of AMI patients. This is appreciated in the finding that necrosis, apoptosis and NETosis occur in the evolving coronary thrombus. The percentages of these three types of cell death, as determined by the immunopositive stained areas, are constant during all stages of organization. It is speculated that the process of cell death, in which particularly neutrophils and macrophages take part, leads to induction of instability and fragility of the thrombus. We propose that these tissue markers for cell death in thrombi can serve as biomarkers for the onset of an AMI, caused by thrombus instability. Such biomarkers can be instrumental for clinical risk assessment, can be used as markers for in vivo imaging of the coronary thrombus during treatment or for personalized forms of (anti-thrombotic) therapy.

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