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Development of immunohistochemical triple staining method for the evaluation of different types of cell death in coronary thrombus

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Abstract. Immunohistochemistry is a powerful technique to identify the presence, distribution and extent of certain antigens at protein level in tissue specimen. Multiple immunostainings enable the detection, colocalization and comparison of several markers within one specimen. However, an optimization study is firstly needed to ensure the expression of multiple markers is clear and distinguishable. We formulated an optimized sequential triple immunostaining protocol which could identify the simultaneous presence of three types of cell death namely necrosis, apoptosis and etosis. These following antibodies were used: C-reactive protein (CRP, necrosis), (cleaved) Caspase-3 (Casp3, apoptosis) and citrullinated-Histone3 (CitH3, etosis). Several antigen retrieval methods, various concentration and order combinations of those antibodies as well as different combination color of chromogens were tested on coronary thrombus materials obtained from patients with acute myocardial infarction (AMI). The results showed that CRP (1:4000) visualized with 3,3'-diaminobenzidine (DAB) in brown, is better performed as the first staining, followed by CitH3 (1:8000) visualized with Perma Blue as the second staining and Casp3 (1:500) visualized with Vector Red as the last staining. In conclusion, we presented an immunohistochemical triple staining protocol to identify the comparative presence of different types of cell deaths: necrosis, apoptosis and etosis in coronary thrombus specimens.

Keywords: immunohistochemistry, multiple staining, sequential staining, cell death, thrombosis

1. Introduction

Immunohistochemistry (IHC) which enables the detection of tissue antigens (markers) on various specimens using the principle of a specific antigen-antibody interaction, is still considered as a valuable tool not only for diagnostics but also for research purposes in histopathological field [1]. In principle, IHC is based on a monoclonal or polyclonal primary antibody that binds the antigen on the tissue, forming an immune complex, to be bound by an enzyme-conjugated secondary antibody. When a specific chromogen is added, this enzyme is able to convert the chromogen into a specific insoluble color we could identify under the microscope [2].

Alongside the introduction of IHC in histopathological field, there had been a growing interest in how to investigate more than one antigens in the same tissue specimen using IHC. Since the use of



individual immunostaining on serial sections is quite problematic and requires laborious work, researchers had developed “multiplex IHC” [3]. Multiplex IHC generally combines multiple individual immunostainings on the same tissue section, allowing the simultaneous identification of multiple markers [4]. Using different colored chromogens, multiplex IHC could distinguish localization pattern of different antigens or detect colocalization of those antigens [1, 3].

In order to perform a successful multiple enzyme immunostaining, there are two important matters that need to be aware of, namely the cross-reactivity between individual staining and the best color combination of the different chromogens [1, 5]. We performed an optimization study to develop a triple immunohistochemical staining for the identification of different types of cell death: apoptosis, necrosis and etosis in coronary thrombosis specimens. Investigation on different types of cell death in coronary thrombosis is of importance as cell death leads to thrombus fragility and further associates with a higher morbidity and mortality rate in patients with coronary syndromes. The development of optimal immunostaining protocol to identify cell deaths may facilitate subsequent studies on the chronological pattern of different types of cell deaths and the potential roles of those markers as biomarkers for coronary thrombus (in)stability.

2. Methods

2.1. Specimens

Thrombus aspiration materials were obtained from patients with acute myocardial infarction (AMI) who underwent percutaneous coronary intervention (PCI) as a part of their treatment. The materials were placed in formalin and fixated at least for 24 hours. They were then embedded in paraffin and stored as tissue blocks in the pathology archives of the Academic Medical Center, Amsterdam, the Netherlands. For diagnostics purposes, they were cut in 5 μ m thin sections and stained with hematoxylin and eosin (H&E) to classify the specimens according to the histologically features of thrombus evolution as fresh (<1 day, red blood cells, intact granulocytes, platelets and fibrin), lytic (1-5 days, lytic areas, necrotic granulocytes) and organized (>5 days, ingrowth of smooth muscle cells/SMCs, angiogenesis and fibromyxoid matrix deposition) as previously described [6]. We randomly selected available tissue blocks of each thrombus age for this study (approx. 3 thrombi per category). For control sections, we used liver, tonsil and skin tissues. The study conformed to the ethical guidelines of the Declaration of Helsinki and the medical ethical review board of the hospital has granted informed consent waiver for the proper secondary use of human tissue in this study.

2.2. Antibodies

We employed antibodies as follow: anti- C-reactive-protein (CRP for necrosis), anti- (cleaved) Caspase-3 (Casp3) for apoptosis and anti- Citrullinated-Histone-3 (CitH3) for Etosis. For detailed specification of these antibodies and their respective protocol see table 1.

Table 1. Overview of the antibodies specification tested in this study

Antibodies (Anti-)	Host	HIER ^a buffer	Dilution tested	Incubation time	Source	Company
CRP	Rb	Tris-EDTA, Citrate	1: 4000 1: 2000 1: 500	overnight 4°C	Abcam (ab32412)	Cambridge, UK
Casp3	Rb	Tris-EDTA, Citrate	1:2000 1:1000 1: 500	60 mins at RT	Cell Signaling (9661S)	Beverly, USA
CitH3	Rb	Tris-EDTA, Citrate	1: 20000 1: 10000 1: 5000	overnight 4°C	Abcam (ab5103)	Cambridge, UK

^aHeat Induced Antigen Retrieval in Lab Vision™ PT Module (ThermoFisher Scientific, Fremont, CA, USA)

2.3. Immunohistochemistry

2.3.1. Single immunostaining

Prior to the development of the triple immunostaining, we first tested the presence of each type of cell death in our specimens. We employed a 2-step polymer antigen detection technique as previously described [7]. In brief, paraffin sections were dewaxed in xylene, rehydrated in graded alcohols and incubated with 0,3% hydrogen peroxide in methanol to block endogenous peroxidase activity for 20 minutes (mins). Subsequently, heat-induced antigen retrieval (HIER) was performed using Tris-EDTA or Citrate buffer (pH = 9 or 6, respectively) for 20 mins at 98°C. Following antigen retrieval step, the sections were washed in running tap water and Tris-HCl buffered saline (TBS), then were incubated in Ultra-V Block (ThermoFisher Scientific) to block unspecific binding for 10 mins at room temperature (RT). We then put the primary antibody at appropriate dilution (see table 1) either for overnight at 4°C or for 60 mins at RT. For antibody dilution, we used normal antibody diluent (ImmunoLogic). Next, the sections were incubated with the secondary antibody: anti-rabbit (anti-Rb) polymer horseradish peroxidase (HRP) IgG (ImmunoLogic) in 1:1 dilution for 30 mins at RT. The formed immune-complex was then visualized with Vector Nova Red (Vector Laboratories) chromogen, for 10 mins at RT. In between primary, secondary and chromogen step, the sections were washed with TBS 3 times for 2 mins each. Finally, the slides were counterstained with undiluted hematoxylin (Klinipath), dried on a hot plate (50°C) and cover-slipped with Vectamount (Vector Laboratories). Positive and negative controls were always included, i.e. controls for CRP, Casp3 and CitH3 were performed on liver, tonsil and skin samples, respectively.

2.3.2. Triple sequential immunoenzyme staining

For the optimization of the triple sequential staining, we tested either anti- CRP, Casp3 or CitH3 as first, second or third staining respectively with adjusted dilutions. Following the individual optimization, a test trial was performed to see if the three different colors (brown, blue and red) could be clearly distinguished from each other. Accordingly, we were able to perform the sequential triple staining. The first staining was performed similarly as single staining above, only HRP activity was visualized using DAB (Diaminobenzidine, ImmunoLogic) instead of Vector Nova Red. Following the first immunostaining, in the second immunostaining, the primary antibody was bounded with Alkaline Phosphatase (AP) anti-rabbit (anti-Rb) IgG (ImmunoLogic) and visualized with Perma Blue (Diagnostic BioSystems). Subsequently, a second antigen retrieval step was performed only for 10 mins at 98 °C prior to the third individual staining which was performed similarly with that of the second staining. Only the chromogen step would be different since we should use colors other than brown and blue. Thus, the last AP activity was visualized with Vector Red (Vector Laboratories). Finally, the slides were dried, mounted and cover-slipped with Vectamount.

2.4. Image Analysis

For single staining, immunostained sections were digitized using a slide scanner (Philips IntelliSite UFS, Philips Digital Pathology Solutions) and assessed by two observers (KRP and PG) using the Fiji Image J software. For sequential triple immunostaining, 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), attached to a Brightfield microscope (Leica CTR5500, Leica). All of these images would be saved for future measurement.

3. Results and Discussion

3.1. Different types of coronary thrombus specimens

Representative examples of coronary thrombus classification: fresh, lytic and organized were illustrated in Figure 1.

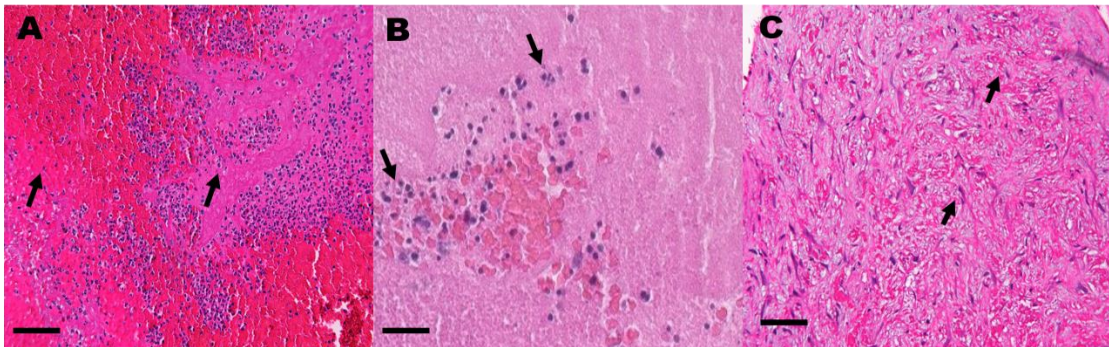


Figure 1. Classification of coronary thrombus specimens. These images were taken from different specimens stained with H&E staining to show fresh (A), lytic (B) and organized (C) thrombus. Fresh thrombus (A) was characterized by intact granulocytes (black arrows), platelets, fibrin and erythrocytes; lytic thrombus (B) with the presence of karyorrhectic granulocytes (black arrows) and organized thrombus (C) with distinct spindle-shaped smooth muscle cells/SMCs (black arrows). Bar scale in A and C: 100 μ m and in B: 50 μ m.

3.2. Necrosis, apoptosis and etosis were identified in coronary thrombi

Single immunohistochemical staining was first performed to detect the presence of three different types of cell deaths: necrosis, apoptosis and etosis in coronary thrombosis specimens. Representative images showing immunostained section with positive results for necrosis, apoptosis and etosis were presented in figure 2. We observed that positive staining with anti-CRP was found in necrotic areas such as the plaque materials (necrotic lipid core) and in cellular components such as SMCs (figure 2A), while both Casp3 (figure 2B) and CitH3 markers stained nuclear fragments (figure 2C). In addition, CitH3 also stained “threads-like” extracellular chromatin DNA. These three types of cell death were observed in all three thrombus categories.

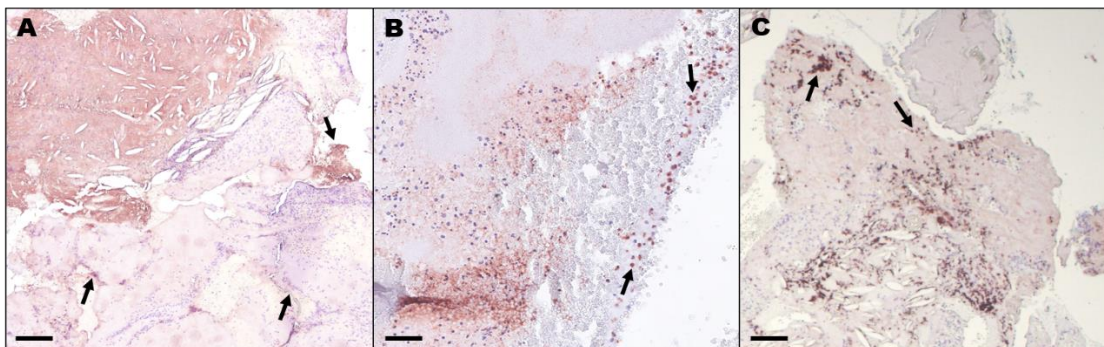


Figure 2. Identification of different types of cell deaths in coronary thrombus specimens. Single immunohistochemical staining for CRP(A) at a dilution of 1:4000, Casp-3(B) at 1:2000 and CitH3(C) at 1:10000 to visualize necrosis, apoptosis and etosis, respectively. Immunopositive cells and/ or areas are shown in brown/dark red (black arrows). Scale bar in A: 200 μ m, in B and C: 100 μ m.

3.3. Suitable combinations of antibodies with appropriate dilutions and antigen retrieval method for triple immunohistochemical staining

We tested the three antibodies to distinguish the simultaneous presence of three types of cell deaths in the same section. Table 2 summarizes the results of optimized immunostaining protocol for necrosis, apoptosis and etosis markers under different antigen retrieval buffer, the order of staining and antibody

dilution. Accordingly, based on our finding, we established the triple immunoenzyme staining method. The antibody order for the immunostaining was determined as follow: CRP (DAB), CitH3 (Perma Blue) and Casp-3 (Vector Red). For antigen retrieval method, as Casp3 could only be performed with Tris-EDTA, we eventually chose the first and second HIER with Tris-EDTA.

Table 2. Results of antibodies testing for immunodetection of cell deaths

Antibodies (Anti-)	Host	HIER ^a buffer	Rank/order	Dilution recommendation	Incubation time	Chromogens
CRP	Rb	Tris-EDTA	Only first	1: 4000	o.n. 4°C	should use DAB
Casp3	Rb	Tris-EDTA	Could be first, second or third	1:2000 (1st) 1:1000 (2nd) 1: 500 (3rd)	60 mins at RT	Optimal with Vector/Perma Red
CitH3	Rb	Tris-EDTA	Could be first, second or third	1: 20000 (1st) 1: 8000 (2nd) 1: 5000 (3rd)	o.n. 4°C	Optimal with Vector/Perma Blue

^aHeat Induced Antigen Retrieval in Lab Vision™ PT Module (ThermoFisher Scientific, Fremont, CA, USA)

Following our testing results, we performed triple immunohistochemical stainings to identify necrosis, apoptosis and etosis simultaneously on the same section of fresh, lytic and organized thrombi. Representative examples of the result of this triple immunoenzyme staining protocol that we developed were illustrated in Figure 3.

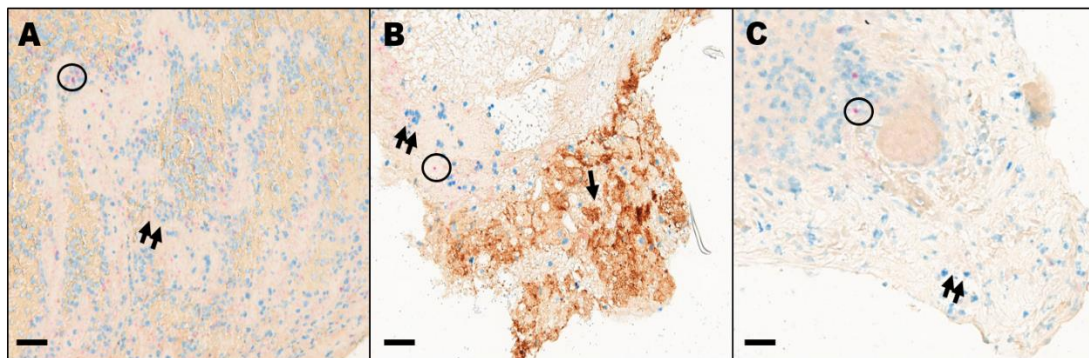


Figure 3. Representative examples of immunohistochemical triple staining to simultaneously identify necrosis, apoptosis and etosis on the same section in different thrombus categories: Fresh (A), Lytic (B) and Organized (C). Necrosis was stained with anti- CRP (1:4000) in DAB showing immunopositivity in brown (one black arrow), apoptosis was stained with anti- Casp3 in Vector Red (1:500) showing immunopositivity in red (black circle) and etosis was stained with Perma Blue (1:8000) showing immunopositivity in blue (two black arrows). Scale bar in A: 100 μ m, in B and C: 50 μ m.

3.4. Discussion

Coronary atherothrombosis, following atherosclerotic plaque disruption, is biologically dynamic and heterogeneous [6]. The changes of thrombus tissue composition and its biological activity influence thrombus (in)stability [8]. Thus, episodes of thrombus instability possess a hidden threat to patient life. One of biological mechanisms that may play roles in destabilizing tissue structure of a coronary thrombus over time is cell death [9]. Accordingly, identifying the major types of cell death inside the

evolving coronary thrombus is required to propose the possibility that cell death markers can serve as biomarkers for coronary thrombus (in)stability after myocardial infarction.

There are several types of cell death, of which the three types of our interest are necrosis, apoptosis and etosis. A necrotic cell is characterized by swelling organelles, a gain in cell volume, and/ or by ruptured plasma membrane and subsequent loss of intracellular contents [10, 11]. Apoptosis, a physiological programmed cell death, is the major cell death pathway during the progression of inflammatory diseases [9, 12, 13]. Etosis, firstly identified in neutrophils as netosis, is a distinct form of cell death, of which the dying cells release “thread-like” extracellular chromatin DNA[14].

We presented an immunohistochemical technique study to develop a sequential triple immunoenzyme staining which allows the simultaneous visualization of three types of cell death on the same tissue section of a coronary thrombus. First, we revealed that necrosis, apoptosis and etosis were indeed present in thrombus specimens (see figure 2). Second, we performed an optimization study to determine the order of immunostaining, the antigen retrieval method, the dilution of antibodies and the best color combinations. The results (see table 2) were used to establish the sequential triple immunoenzyme staining protocol of anti- CRP, Casp3 and CitH3. To evaluate the quality of the sequential triple staining, the staining pattern of each individual antibody was carefully compared among the triple staining (see figure 3), to the control (figures are not shown) and to the single staining (see figure 2). It appeared that the same cells and/ or area shown similar patterns of immunopositivity, thus, our sequential triple immunoenzyme staining protocol is genuinely reliable.

We determined the order of staining and the dilution of the antibodies (table 2) based on the observation that CRP did not only stain cells but also areas (see figure 2A) so that it would be better performed as the first staining. CitH3 could appear as fuzzy threads like fibers (see figure 2C) so that we ranked it as the second one. Eventually, Casp3 was performed as the last staining. Alongside the decision on the order of the staining, adjusted antibodies dilution was also determined as the high dilution of the early antibodies could exhibit cross-reaction with the next detection system [1].

In addition, we also have addressed some concerns about this technique which are cross-reactivity and color combination. The three antibodies we used are all rabbit origin, of which cross-reaction between the three anti-rabbit detection systems (similar hosts) are likely to occur. We succeeded in tackling this issue by using DAB chromogen when performing the first immunostaining with anti-CRP (see figure 3B), as suggested by previous studies of our lab [1, 5]. The cross-reaction between the antibodies of first and second staining was anticipated as the brown DAB reaction formed a protective layer covering the first immune-complex layer effectively [1]. Moreover, we also performed the second antigen retrieval with HIER step in between the second and third staining as previously recommended [15]. This heating step showed effective results either in preserving the first CRP-DAB-immunostaining or in protecting the second CitH3-PermaBlue-immunostaining (see double black arrows in figure 2A-C). Eventually, the combination of three colors: brown, blue and red, has shown the best contrast between the three markers (see figure 2A-C).

Overall, sequential triple staining is a useful technique that enables simultaneous visualization of different antigens on the same section [4]. On one hand, it provides a direct overview of the presence, localization and extent of three types of cell death individually, as such we are able to compare the immunopositivity pattern. On the other hand, sequential multiple staining is not recommended for the evaluation of colocalization by mixed-colors (the presence of co-expression of multiple antigens in one cell) since the mixed-colors could be difficult to be analyzed [1].

Furthermore, we also experienced some limitation and difficulties. First, triple immunostaining is designed to compare the extent of each type of cell death using the percentages of immunopositivity (based on the number of immunopositive pixels per section). However, each type of cell death has its own morphological appearance, for example apoptotic cells shrink and may be visible as small dots or nuclear fragments [9, 13] while cells undergoing etosis may not always appear as nuclear staining but they may occupy a big surface area, visible as fuzzy “cloud-like” appearance [16, 17]. Consequently, to find out which type of cell death is more dominant, immunopositive areas of etosis could not be simply compared with immunopositive areas for apoptosis. Still, the screening of immunopositive

areas remains very useful to estimate the extent of a type of cell death, whether necrosis, etosis or apoptosis, in the different stages of thrombus evolution, from fresh to organized. Second, we observed that the use of CRP as a marker for necrosis is arguable. Although another study have also employed CRP as necrosis marker [18] we observed that fresh and organized thrombi also show positive staining for CRP although they are not particularly marked by necrosis [6, 19]. Yasojima *et al.* [20] demonstrated that vascular SMCs and also macrophages inside atherosclerotic plaques are able to locally release CRP. Thus, it may also be the case in our study that the identified CRP positive staining in coronary thrombus could be due to the local CRP generation. On the other hand, lytic thrombi which by definition should contain a great amount of necrosis [6], did not show large immunopositive CRP areas in our study although the corresponding H&E stained showed pronounced morphology of necrosis. Indeed, many other studies previously used CRP as a marker for inflammation [21, 22]. Thus, we suggested that CRP appears inadequate as a marker for necrosis in coronary thrombus, nevertheless CRP may be more suitable as a marker for inflammation. Furthermore, since immunohistochemical detection of necrosis is hampered by the lack of a specific marker in paraffin-embedded tissue specimens, H&E stains are still considered as the best method for the identification of necrosis.

4. Conclusion

This study presented a reliable sequential immunohistochemical triple staining to identify simultaneous three types of cell deaths, namely necrosis, apoptosis and etosis. We tackled the issues regarding cross-reactivity, the order of staining, antibodies dilution and color combination successfully. However, we found that CRP may not be the best immunostaining marker candidate to visualize necrosis in paraffin-embedded tissue specimens. Future studies are needed to reinvestigate the more prosperous candidate marker for immunodetection of necrosis. Still, in general, a sequential multiple immunostaining remains important in histopathological diagnostics and research field.

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