Micron 114 (2018) 1–7

Contents lists available at ScienceDirect

Micron

journal homepage: www.elsevier.com/locate/micron

NETs detection and quantification in paraffin embedded samples using confocal microscopy

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ARTICLE INFO

Keywords:
Microscopy
Inmunofluorescence
Image analysis
Neutrophil Extracellular Traps

ABSTRACT

Detection and quantification of Neutrophil Extracellular Traps (NETs) in tissue samples has become a topic of great interest to understand their pathological role in various diseases. We describe a semi-automatic method of visualization and quantification of NETs in paraffin-embedded intracoronary thrombus aspirate samples. This study is based on colocalization of myeloperoxidase (MPO) and citrullinated histone 3 (H3Cit) as hallmark of the presence of NETs. For the analysis we used the confocal immunofluorescence microscopy technology to quantify the number of fields and the total area (in μm^2) containing NETs in each thrombus sample. This observer-independent quantification method could be a useful tool to standardize the study of NETs in paraffin-embedded tissues, enabling comparison of results among different laboratories.

1. Introduction

Cardiovascular diseases (CVD) are the leading cause of mortality and morbidity in developed countries (Anwaier et al., 2017; Gaziano et al., 2016). Specifically, thrombosis is the main cause of death in the Western world (Shahidi, 2017). Although in the last 40 years its mortality rate has been reduced (Mensah et al., 2017), it continues to be a serious health problem worldwide. In thrombosis and other vascular ischemic diseases, the presence of neutrophils is associated with increased mortality and morbidity (Nagareddy and Smyth, 2013). In fact, recent studies have showed the involvement of Neutrophil Extracellular Traps (NETs) in atherosclerosis, atherothrombosis and venous thrombosis (Qi et al., 2017).

NETs were described by Brinkmann et al (Brinkmann et al., 2004) for the first time in 2004. They are defined as web-like structures formed by decondensed chromatin (histones and DNA) and antimicrobial components such as neutrophil elastase and myeloperoxidase (MPO) (Delgado-Rizo et al., 2017; Papayannopoulos and Zychlinsky, 2009; Yang et al., 2016). The release of NETs leads to a type of cell death known as NETosis (Fuchs et al., 2007; Hemmers et al., 2011; Khan and Palaniyar, 2017; Steinberg and Grinstein, 2007). NETosis is a form of defense against bacteria, fungi, parasites and viruses, that causes the trapping of pathogens and their destruction due to the concentration of antimicrobial agents within the network of extracellular DNA (Brinkmann and Zychlinsky, 2007; Della Coletta et al., 2015; Liu et al., 2017; Pieterse et al., 2016; Selders et al., 2017).

Quantification of NETs has been performed by means of immunofluorescence microscopy, multispectral imaging flow cytometry, or by plate assays in several *in vitro* studies (Carmona-Rivera and Kaplan, 2016; Pieterse et al., 2016). The main method for NETs analysis in tissue samples is microscopic observation, but this could be prone to observer-dependent variations (Brinkmann et al., 2013; Brinkmann and Zychlinsky, 2012; Coelho et al., 2015; Masuda et al., 2016), and until now there is no established protocol for their detection and quantification. As there is a need for standardization, we propose a semi-automatic system for detection and quantification of NETs in paraffin-embedded samples. Our method is based on the use of immunofluorescence, confocal microscopy technology and image analysis for

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https://doi.org/10.1016/j.micron.2018.07.002
Received 7 March 2018; Received in revised form 5 July 2018; Accepted 6 July 2018

the colocalization of MPO and citrullinated histone 3 (H3Cit), which is a consolidated indicator of the presence of NETs (Thålin et al., 2016; Zenaro et al., 2015).

Thrombus aspiration during primary percutaneous coronary intervention (PCI) in patients who suffer a ST-elevation myocardial infarction (STEMI) provides a valuable material for the analysis of the composition of thrombi (Blasco et al., 2017), and we have used this aspirates to implement our method.

2. Materials and methods

2.1. Samples

A total of 50 intracoronary thrombus aspirates extracted from as many STEMI-patients during primary PCI were included in the study. Clinical, angiographic, and procedure-related variables were prospectively collected in a database specifically designed for this purpose. The information recorded included epidemiological and clinical parameters (cardiovascular risk factors, presence of prodromal angina, Killip class, ST resolution, analytical parameters, left ventricular ejection fraction (LVEF) at discharge *etc.*), procedural times (eg, symptom onset-to-balloon, door-to-balloon), angioplasty information, and patient outcomes. The study was carried out in accordance with Good Clinical Practice guidelines and applicable regulations, as well as the ethical principles originating in the Declaration of Helsinki. The protocol was reviewed and approved by the Ethics Committee of Hospital Universitario Puerta de Hierro-Majadahonda (Acta nº 07.18).

2.2. Fixation and paraffin embedding

The aspirated samples were rinsed with phosphate buffer saline immediately after extraction and fixed in 10% neutral buffered formalin in the catheterization laboratory. Then, they were sent to the Pathology Department to be processed. Samples were dehydrated and embedded in paraffin using an automatic tissue processor (Shandon Excelsior ES, ThermoFisher Scientific). Paraffin blocks were sectioned in 4-μm slices, stained with Hematoxylin and Eosin and mounted for light microscopy observation, following an automatic standard laboratory procedure (Dako CoverStainer for H&E, Agilent).

2.3. Immunofluorescence of NETs

For immunofluorescence, the sections were deparaffinized and underwent antigen retrieval with High Buffer pH 9 (Dako DM 848), being washed with PBS and treated with NH_4Cl 50 mM for 10 min. After permeabilization with 1%Triton X-100, the samples were blocked with 5% BSA (Bovine Serum Albumin) during 30 min and incubated with a 1/50 diluted rabbit anti-H3Cit antibody (Abcam ab 5103) at 4 °C overnight. The next day the specimens were washed with PBS and incubated with a goat anti rabbit secondary antibody conjugated to ALEXA 488 (Invitrogen Life Technologies), applied at a dilution of 1/500 for 45 min at room temperature (r.t.). For a second immunodetection, the samples were blocked again with BSA 5%, incubated with a 1/25 diluted goat anti-MPO antibody (R&D Systems BAF3174) at 4 °C overnight, washed with PBS and incubated with a donkey anti goat secondary antibody conjugated to ALEXA 546 (Invitrogen Life Technologies) applied 1/500 for 45 min at r. t. The nuclei were stained with TO-PRO 3 (Invitrogen Life Technologies) diluted 1/500 for 30 min. Finally, the sections were mounted with a 50/50 PBS:glycerol mixture. Negative controls were processed in identical way but without primary antibody.

2.4. Visualization of neutrophil extracellular traps

Images were collected with a TCS SP5 confocal microscope (Leica Microsystems, Wetzlar, Germany) using a 63 × HCX PL APO (1.25–1.52 numerical aperture) oil-immersion objective. The three channels were acquired sequentially to avoid cross-talk between them. The following excitation and emission parameters used were: (488 nm, 500–540 nm) for anti H3Cit signal, (546 nm, 557–572 nm) for MPO and (633 nm, 645–750 nm) for TO-PRO 3. Three different lasers were used independently for each fluorophore (Alexa 546, Alexa 488 and TO-PRO). The gains were adjusted for each channel to avoid saturation in pixels intensity. Z-stacks were obtained from confocal sections at 1 μm intervals. Laser intensity and detector sensitivity settings remained constant for all image acquisitions for experimental replicas. Image processing was performed with the ASF Leica software.

2.5. Quantification

The NETs were identified as the colocalization area of both antibodies (H3Cit and MPO) and quantified using the ASF SOFTWARE VERSION 2.6.07266 (LEICA) to determine the colocalization ratio of both antibodies in each sample, by applying the Pearson correlation coefficient (PCC). An intensity threshold was uniformly applied to represent a positive signal for two channels, and also background was set for each channel. We used the “quantification” tool from ASF Software in which three categories are available: intensity, motion and colocalization. Colocalization implement was chosen, which carries out the analysis of the whole image. We also calculated the average of colocalization area in μm^2 per sample, and the total area of colocalization (μm^2) obtained by the sum of the areas contained in all the images taken by each sample.

3. Results

3.1. Detection of NETs in coronary aspirate samples

Based on the fact that not all thrombi have NETs and that NETs are not distributed equally in the thrombi, a scan of the entire sample was performed. In the positive regions for H3Cit and MPO, Z-series images were taken and a maximum projection was subsequently made. Figs. 1A to D are a representative example of a region of a coronary aspirate with NETs. H3Cit distribution is shown in Fig. 1A (green), MPO in Fig. 1B (red) and the colocalization of both proteins is illustrated in Fig. 1D (yellow). The colocalization of these two proteins and their conformation as fibrillary networks is an unequivocally indicative of NETosis. In Fig. 1C and D it is also to observe that DNA (blue) is spread throughout some regions of the thrombus and forms extracellular traps, which is another important feature of NET release. On the other hand, there were coronary aspirates where NETs were not found, as shown in Fig. 1E–H. In Fig. 1F and 1H an intracellular and extracellular labeling of MPO can also be seen, which is not colocalizing with H3Cit. Presumably, this MPO is located inside primary granules of neutrophils or it has been degranulated. Therefore, in regions with NETs we differentiate between the degranulated MPO from NETs and the MPO from granules, as it colocalizes with the H3Cit, which is specific to netting neutrophils (Fig. 1D).

After mapping each sample under the microscope, the number of fields with NETs was determined. Among the 50 coronary aspirates analyzed, almost one third (32%) did not contain NETs. Near half of the samples (46%) had between 1 and 6 fields with NETs; 14% of the samples had among 7–12 NETs fields and samples with more than 12 NETs fields represented only 8% of cases (n = 4). The maximum number of NETs fields detected in a single sample was 16 (Fig. 2).

We also tried to identify NETs in the same samples using the immunohistochemical technique. For this purpose, H&E and MPO stains were performed. Chromatin rich regions, stained with MPO, were photographed with a bright field microscope (Fig. 3A and B). Thereafter these regions were analyzed by fluorescence confocal microscopy for H3Cit and MPO labeling. The results indicate that many regions with haematoxylin smears and stained for MPO do not correspond to regions