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Research article

Comparison of different microscopy approaches to quantification of inhibitory effect on thrombus formation under flow conditions by the example of adenosine receptor agonist HE-NECA

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ABSTRACT

Introduction: Thrombus formation *in vitro* in flow conditions and its visualization and quantification with the use of microscopy are widely utilized to evaluate activity of compounds with a potential antithrombotic activity. Visualization and quantification of thrombi can be performed with the use of wide-field or confocal microscopy. Acquiring reliable numerical data from wide-field microscopy images of objects which have a complex three-dimensional structure is strongly influenced by the methods used for image analysis. This can be a possible source of inaccuracy in assessment of antithrombotic activity of a tested substance. We aimed to verify how different approaches to the quantification of wide-field images can affect the evaluation of an antiplatelet effect of a tested substance.

Methods: We compared three algorithms of image analysis to evaluate an effect of 2-hexynyl-5′-ethylcarboxamidoadenosine (HE-NECA), a compound of a moderate antiplatelet activity on thrombus formation, and of abciximab - a potent antiplatelet compound. Also, we studied how the results obtained in a wide-field imaging correspond to those obtained by means of confocal imaging.

Results: Three algorithms for analysis of wide-field images showed antiplatelet effect of HE-NECA or abciximab. Absolute values of thrombus area and outcomes of the evaluation of inhibition efficacy of HE-NECA were significantly different between the algorithms. Analysis of volumes and heights of thrombi obtained by confocal imaging confirmed inhibitory effect of HE-NECA, but the evaluated levels of inhibition were significantly different from that obtained by wide-field imaging.

Discussion: We conclude that wide-field imaging provides reliable qualitative data on an inhibitory effect on thrombus formation, despite differences which can emerge from various approaches to image analysis. However, quantitative evaluation and comparison of the efficacy of inhibitors on the basis of total area occupied by thrombi obtained by wide-field microscopy should be made with caution. To obtain a reliable quantitative assessment of the effect of a tested compound on thrombus structure the use of confocal microscopy is inevitable.

1. Introduction

In vitro testing of thrombus formation in flow conditions is widely used to evaluate activity of haemostatic system and specifically to test compounds with a potential antithrombotic activity (Fuentes, Caballero, Alarcon, Rojas, & Palomo, 2014; Hosokawa et al., 2011; Van Kruchten, Cosemans, & Heemskerk, 2012). These methods are based on passing blood at a controlled shear rate through channels which are coated with specific proteins, *e.g.* collagen, fibrinogen, tissue factor *etc.* These conditions reflect a pathological event of rupture of atherosclerotic plaque where subendothelial matrix proteins are exposed to a lumen of blood vessel and blood components come in contact with these proteins under high shear forces. Proof of an antithrombotic effect of a potential drug in such *in vitro* conditions is considered a prerequisite of its efficacy in a clinical situation. Quantification of thrombi which are generated in the channels varies along with the equipment.

Abbreviations: HE-NECA, 2-hexynyl-5′-ethylcarboxamidoadenosine

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In some of the methods quantification is based on the change of parameters of flow such as time to occlusion or an increase in pressure, as a result of the formation of occlusive thrombi. The latter approach is utilized in Total Thrombus-Formation Analysis System (Li, Hotaling, Ku, & Forest, 2014; Sugihara et al., 2016). These methods also allow to evaluate stability of thrombi exposed to high shear forces. In the other group of methods, quantification is based on the measurement of size of the aggregates visualized by means of microscopy.

In numerous studies published to date, thrombi were visualized with the use of wide-field microscopy and their sizes were evaluated by quantification of the areas covered by thrombi (Fuentes et al., 2014; Yoshida, Okamura, Watanabe, Ikeda, & Handa, 2011; Zafar et al., 2007). In this approach, a decrease in thrombus area caused by a tested compound is interpreted as a decreased size of thrombi. It has to be kept in mind however, that thrombi are in fact three-dimensional structures, and area of their horizontal projection, which is acquired by wide-field microscopy, is only an appropriate approximation of their actual size. The detrimental effect of intravascular thrombus formation stems from its ability to close lumen of the vessel, thus disabling the blood flow. This is, in turn, dependent on structure and stability of thrombus, which determines its ability to resist high shear stress and to build up (Kamada, Imai, Nakamura, Ishikawa, & Yamaguchi, 2013). Thus, it is rather the volume and not the area of thrombus, which actually determines its occlusive properties. To obtain data on the volume of thrombi, the use of confocal microscopy is inevitable. However, imaging with the use of confocal microscopy and analysis of data obtained with this technique are relatively time-consuming. Thus, although imaging of thrombi with confocal microscopy (Swieringa et al., 2016) and quantification of the volumes of images of such visualized thrombi has been performed (Kuwahara et al., 1998; Van Kruchten et al., 2012), wide-field is still a technique often used for imaging and quantification of thrombi. For this reason a proper quantification of wide-field images of thrombi is of particular importance.

In the present work we aimed to analyze to which extent different approaches to the quantification of wide-field images can affect the evaluation of inhibitory activity in a model of thrombus formation under flow conditions. Since the detection of the effects of moderate inhibitors is more challenging than that of the potent inhibitors, we have chosen a compound, which (on the basis of our preliminary research) did not have an ability to completely block formation of thrombus through a range of concentrations. 2-hexynyl-5′-ethylcarboxamidoadenosine (HE-NECA) has been previously shown to possess antiplatelet activity in classical aggregation tests (Cristalli et al., 1994). Its action is mediated by adenosine receptors (A2A and A2B), which are coupled to G_s protein. Activation of these receptors leads to an increased cAMP content in platelets, which translates into a decreased platelet activation (Cooper, Hill, Alexander, Rubin, & Horn, 1995). Antithrombotic action of HE-NECA under flow conditions has not been tested yet. To validate the algorithms of image analysis we also used the reference compound, abciximab, monoclonal antibodies against platelet GPIIbIIIa receptor, a potent antiplatelet inhibitor of fibrinogen-dependent aggregation (Coller & Scudder, 1985).

Finally, we compared the inhibitory effect of HE-NECA evaluated by means of wide-field imaging with data obtained with the use of confocal imaging.

2. Materials and methods

2.1. Blood collection

Experiments were approved by the committee on the Ethics of Research in Human Experimentation at the Medical University of Lodz, approval number (RNN/43/17/KE). After having received written consents from volunteers, blood was collected from five healthy donors into a vacuum tube containing 0.105 mol/l buffered sodium citrate (the final citrate:blood ratio 1:9 v/v).

2.2. Thrombus formation in flow conditions

Effect of HE-NECA or abciximab on thrombus formation was assayed with the use of the Venaflux platform (Cellix, Dublin, Ireland) according to protocol based on studies published elsewhere (Van Kruchten et al., 2012). The channels of Vena8 Fluoro+ biochip were coated with type I collagen (20 μg/ml) overnight at 4 °C and blocked with 0.1% BSA for 1 h at 4 °C. Biochip was mounted on a stage of an inverted AxioVert microscope thermostatically controlled throughout the experiment at 37 °C (Carl Zeiss, Oberkochen, Germany). Whole blood was incubated with 10 μmol/l HE-NECA (Sigma Aldrich, Saint Louis, USA) while control samples were incubated with 0.1% DMSO. In the other experiments abciximab (ReoPro) (Jenssen Biologics B.V., Leiden, the Netherlands) was used in the concentration of 20 μg/ml (control samples were incubated with equal volume of saline) for 3 min (37 °C) prior to measurements. Samples were recalcified with $CaCl_2$ (1 mM) shortly before measurement. Prior to measurements each channel was washed with PBS for 1 min at 5 dynes/cm^2. The samples were then perfused using a shear force of 60 dynes/cm^2 for 4 min. The thrombi were stained in channels by passing through them 10 μg/ml fluorescein dissolved in PBS for 2 min at 5 dynes/cm^2. Thereafter the samples were flushed with CellFix for 4 min at 5 dynes/cm^2. Such prepared channels were imaged by wide-field and confocal microscopy.

2.3. Wide-field imaging and quantification of thrombus areas

Wide-field imaging was performed with the use of AxioVert inverted epifluorescence microscope (Zeiss, Jena, Germany) equipped with 40× objective and an AxioCam702 monochromatic camera using a green filter. Images were acquired from 5 distinct fields of view along the channel with a resolution of 1920 × 1216 pixels and 16-bit depth.

Image analysis was performed with the use of FIJI software (Schindelin et al., 2012). Three different approaches were undertaken to quantify thrombus sizes. As a first step in all used algorithms the raw 16-bit images acquired in Zeiss '.czi' format were converted to an 8-bit greyscale.

Algorithm A - For better separation of the objects for quantification from background, a thresholding procedure was performed with the use of 'Auto Local Threshold' function (Bernsen method) with the radius value set at 15. To acquire areas of the objects an 'Analyze Particle' function was used with a lower limit for the cutoff size set at 20 μm^2 and an option 'include holes' enabled. The latter option includes into a calculation also these areas which were classified by the thresholding function as background, but since they were surrounded by a solid part of thrombi, they could be assumed to be in fact integral parts of thrombi. The cutoff size value of 20 μm^2 was chosen arbitrarily to exclude background areas which could be erroneously thresholded as thrombi. Since 20 μm^2 corresponds to roughly 4–5 non-activated platelets, which is too small to be considered as a thrombus, the setting of such a cutoff value does not generate a risk of losing meaningful information. 'Analyze Particle' function generated a set of data describing separately each thrombus identified on an image. This set of data includes *inter alia*: area of an object, its circumference, intensity *etc.*

Two another approaches were conducted with utilizing semi-automated calculations based on ImageJ macro language. The designated scripts were written for this purpose. The codes are attached in supplementary materials. The main differences with respect to the first algorithm are in the steps of image preprocessing and main processing stage.

Algorithm B - as a first step, contrast was improved by utilizing histogram equalization procedure in the 'Enhance contrast' function. This function employs a monotonic, non-linear mapping, which re-assigns the intensity values of pixels in the input image in such a way that the output image contains a uniform distribution of intensities. As a next step a 'Subtract Background' feature was used with "rolling ball radius" parameter set at 100 μm to cut off background. To eliminate