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Ambiguous role of F11Receptor/Junctional Adhesion Molecule-A in human blood platelet adhesion and thrombus formation

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Background

F11 receptor Junctional / Adhesion Molecule A (F11R/JAM-A) was primarily discovered in blood platelets. Its primary function is to form tight junctions in epithelial and endothelial cells. In these structures, F11R/JAM-A forms trans-homophilic complexes in which dimers of this protein located on one cell interact with their counterparts on the adjacent cells. In blood platelets, the JAM-A protein is connected both physically and functionally to the αIIbβ3 integrin one of the most prominent adhesion molecules on blood platelets. This protein, also known as glycoprotein IIb/IIIa is a well-known transmembrane receptor for fibrinogen and von Willebrand factor, and aids platelet activation, therefore it is a prime target of study in adhesion, migration, and clot formation. Given the nature of fibrinogen as a ligand, the interaction might be defined by both platelet-platelet interactions and those that involve fibrin.
Our previous studies guided us toward exploring both of those possibilities.

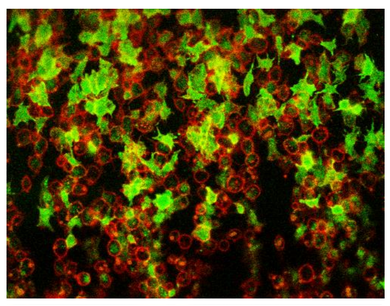

Figure 1 Confocal images of blood platelets adhered under flow conditions to fibrinogen-coated surface. Red staining – CD-41, green staining – F11R/JAM-A
Rich staining for JAM-A in the filopoidal population suggested a role of JAM-A in the transitioning of platelets between distinct morphological variants.

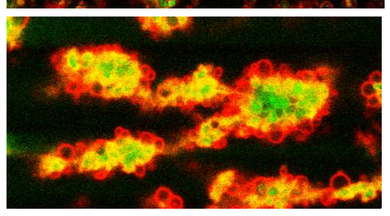

Figure 2 Confocal images of blood platelets adhered under flow conditions to a collagen-coated surface. Red staining – CD-41, green staining – F11R/JAM-A.
The central location in clots pointed toward a specific subpopulation of platelets involved in the initiation of the clotting process.

Methods

Blood donors: Blood was collected from healthy volunteers (age 25 – 50 years).
Confocal imaging: Blood platelets adhered to the channel surface were stained with platelet-specific anti-CD41 PE-conjugated antibodies and antiF11R/JAM-A FITC-conjugated antibodies and visualized using a confocal microscope **(Fig. 1),** platelet aggregates stained with the same technique **(Fig. 2)**.
Thrombus formation and blood platelet adhesion under flow conditions: The experiments were performed using the VenaFlux platform. The channels were coated with type I collagen and blocked with BSA. Blood was perfused with a shear force of 40 dynes/cm^2 (~890 s^{-1}) which is considered to mimic flow conditions in the stenosed artery. Adherent platelets were stained with anti-annexin V1 PE-conjugated antibodies and anti-JAM-A FITC antibodies. Imaging was performed using an epifluorescence microscope.
Blood platelet static adhesion: The experiments were performed using µ-Slide Angiogenesis chambers. The wells were coated with fibrinogen *(100ug/ml or 37.5ug/ml)* or fibrin *(37.5ug/ml)*, with or without recombinant human Fc chimera F11R/JAM-A *(100ug/ml)* and blocked with 0,1% BSA. Adherent platelets were stained with platelet-specific anti-CD41 PE-conjugated antibodies, phosphatidylserine specific annexin V protein, or PAC-1 specific for the activated form of the αIIbβ3 integrin. Imaging was performed using an epifluorescence microscope.
Machine learning: Quantification of thrombus formation and platelet adhesion was performed with the use of Ilastik and ImageJ software. Firstly, the collected microphotographs were preprocessed to file type accessible for Illastic software, then the initial human input was used to teach the software how to classify every pixel as belonging to the object (platelet) or not (background). This process of segmentation resulted in a probability map with a threshold value that can be adjusted by the analyst. When the program discerns objects from non-objects to the analyst's satisfaction, the results are forwarded to further analysis i.e. object classification. During this process the analyst once again inputs data that serve as a basis for establishing distinct classes of objects, in our case subpopulations of platelets based on their morphology **(Fig 4)**.
T-TAS® Total Thrombus formation Analysis System was used to analyse in vitro thrombus formation. This apparatus measured time to thrombosis onset and to full occlusion based on the changes in pressure in the system. The AR chip consisting of a single capillary channel coated with collagen and thromboplastin to assess the function of primary and secondary haemostasis.
Experimental thrombosis in mice: Male and female mice of C57/Bl strain were used in a $FeCl_3$-induced carotid thrombosis model. We have placed a patch of filter paper soaked with 10 % $FeCl_3$ directly contacting the exposed common carotid artery. Using i.v. route via the retroorbital plexus, animals were given injections of tested substances. The study measures changes in blood flow over time **(Fig 6).**

Results

*We were unable to detect any correlation between procoagulant platelets and increased JAM-A expression. There were no changes in annexin V staining coinciding with increased JAM-A staining **(Fig 3)**. Compared to fibrinogen, static adhesion to fibrin showed increased αIIbβ3 activation, changes in platelet morphology and changes in the localisation of PAC-1 staining (focal activation instead of diffuse across the platelet surface)(not shown). The addition of Fab fragments of J10.4 antibodies did not alter the degree or nature of expression of the activated form of αIIbβ3 on the platelet surface neither on fibrinogen nor fibrin. Experimental inhibition of the interaction by Fab fragments of J10.4 antibodies showed that antibodies that inhibit dimerisation reduce platelet adhesion.*
*A change in platelet morphology was observed in the presence of the JAM-A. A classifier trained in the Ilastik programme was used for quantitative evaluation. With the applied division of adherent platelets into 3 distinct morphological categories, the platelet proportions were distributed as follows: on fibrin (37.5ug/ml) inactive platelets(median with IQR) 30.64 (22.04 ; 43.31), filopodial 31.40 (22.59 ; 55.23) and lamellopodial 27.2 (4.488 ; 44.94). The addition of JAM-A (100ug/ml) to the surface coating shifted the dominant platelet morphology towards the lamellopodial form 56.33 (16.61 ; 64.97) at the expense of the filopodial form 20.89 (18.20 ; 41.27) and inactivated platelets 16.39 (14.71 ; 35.11) **(fig 5)**. The ability of the soluble form of JAM-A to inhibit thrombus formation was investigated by T-TAS. Median with IQR of time to occlusion in control samples 587 (399.5, 728.5) and in the presence of 10ug/ml sJAM-A 470 (391, 631). (n=5). The results do not exhibit statistical significance, but the underlying trend is consistent with the results published by Rath et al. 2022 where published data indicated that sJAM-A lowers the activation threshold and promotes thrombus formation **(fig 6)**, results although promising did not possess statistical significance. To further study processess in which the JAM-A is clearly involved we have confirmed that mechanism of $FeCl_3$ induced thrombosis is at least partially pletelet-dependent. To this end we have introduced a control study using Cangrelor* - a P2Y12 platelet receptor antagonist. Complete abrogation of occlusive thrombosis has indicated that effects observed while using BV11 antibodies should be attributed to platelet related mechanism mediated and modified by JAM-A dimerization of murine platelets.

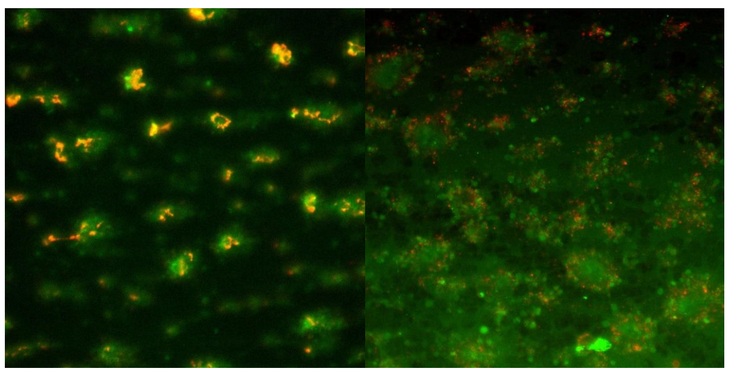

Figure 3
Co-staining of anti-JAM-A (green) and annexin V (red) on a collagen-induced clot formation in flow, did show distinct populations of platelets expressing them. JAM-A staining has proved diffused and largely non-specific in regard to location. Annexin V was staining predominantly edges and apices of the structures. Phosphatidylserine staining distinct for procoagulant platelets does not overlap specifically with JAM-A staining.

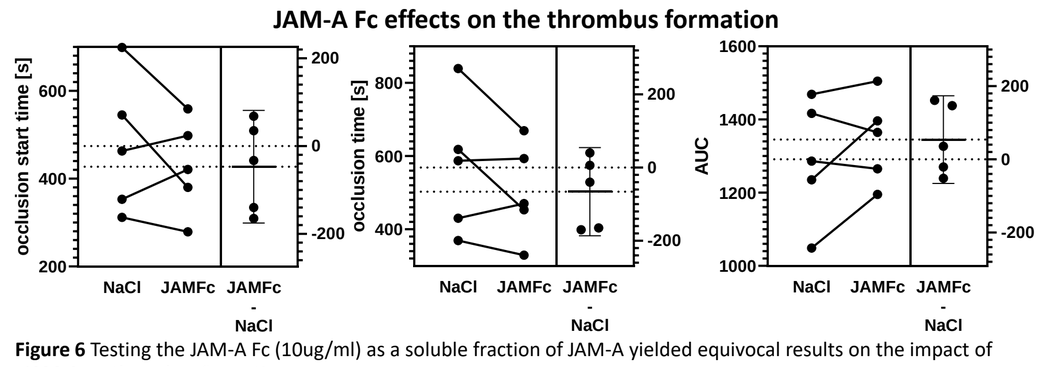

Figure 6 Testing the JAM-A Fc (10ug/ml) as a soluble fraction of JAM-A yielded equivocal results on the impact of sJAM-A on thrombus formation.

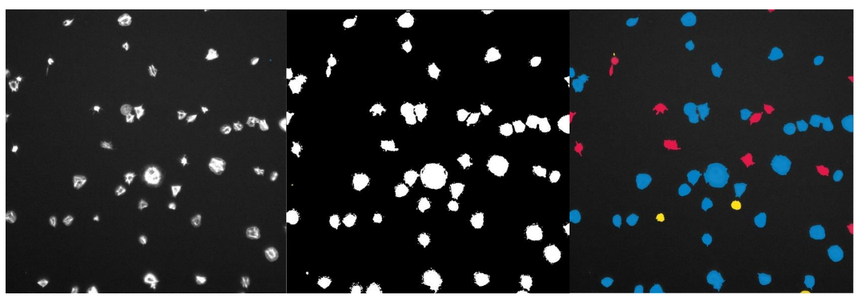

Figure 4
Platelets adhered to the monomeric fibrin, segmented, and sorted into distinct platelet classes (subpopulations). The first picture presents the raw data, the second is an object recognition layer and the third sorts platelets into three morphologically distinct populations of fully spread lamellipodial PLT's (blue), partially spread filopodial PLT's (red), and non-activated PLT's (yellow).

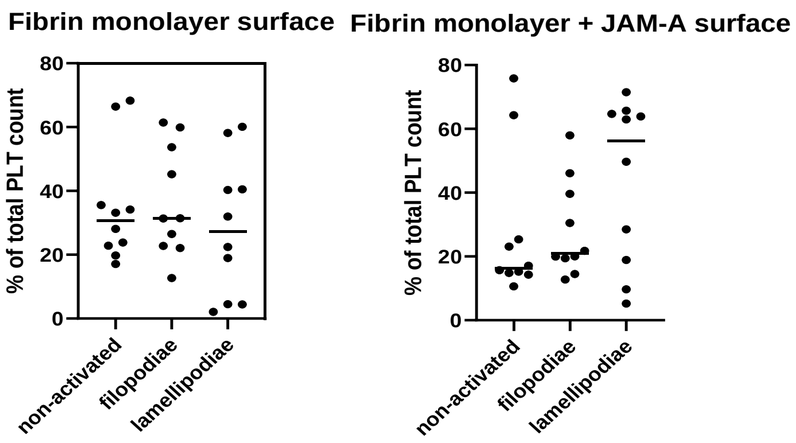

Figure 5
Relative changes in the ratio of distinct platelet populations as recognized and quantified by the machine learning protocol shows decreases in the population of non-activated and filopodial platelets in favour of fully spread lamellipodial platelets.

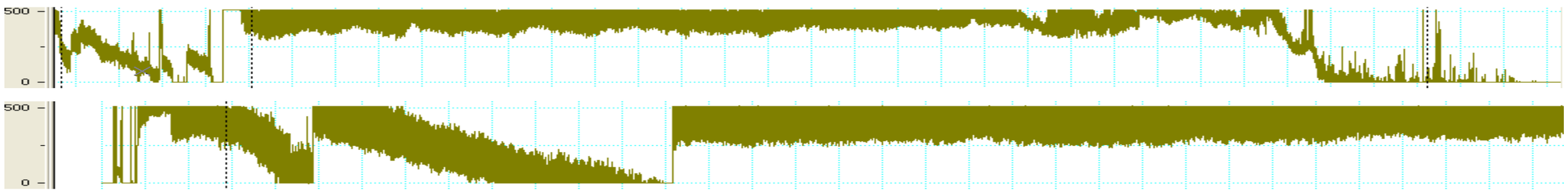

Figure 6 The Control study shows that administration of a $FeCl_3$ soaked filter paper to surgically exposed common carotid artery induces total vessel occlusion within a 30-minute frame. This is visualized as a thinning of the readout and a drop of the values to around 10% of the initial value (upper chart). Pretreatment of the animals with cangrelor (lower chart) stopped the occlusion. This controlled experiment served to determine that the $FeCl_3$ model and changes detected previously are, at least partially, platelet-dependent.

Conclusions

This study was designed to investigate the effect of JAM-A protein on platelet behaviour in different experimental systems reflecting physiological and pathological conditions in the vascular bed. It was found that the addition of JAM-A did not increase platelet activation on fibrinogen-coated surfaces. However, when fibrin was used in the model, increased platelet activation was observed. The effect of JAM-A protein on platelet morphology has been demonstrated. The presence of JAM-A in the substrate regulates the switch of platelet phenotype to lamellopodial with maximized adhesion surface. This may be a platelet population designed to cover the damaged epithelium which, when damaged, relocates JAM-A from tight junctions to the luminal surface. Current and previous studies suggest that JAM-A protein does not play a significant role in fibrin formation because no increased expression of JAM-A was found on procoagulant platelets (which similarily change morphology to maximize active surface area) traditionally considered precursors of fibrin-dependent clot formation. Although previously suggested by stronger staining on filopodial platelets, the current study does not support the notion that JAM-A is expressed preferentially on any known phenotypes of platelets, however, this might be a time-dependent characteristic of platelets, caused by active shedding of surface proteins.

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