Targeting platelet glycoprotein VI attenuates progressive ischemic brain damage before recanalization during middle cerebral artery occlusion in mice

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ABSTRACT

In acute ischemic stroke due to large vessel occlusion (LVO) infarcts rapidly grow into the penumbra, which represents dysfunctional, but still viable brain tissue amenable to rescue by vessel recanalization. However, infarct progression and/or delayed patient presentation are serious and frequent limitations of this so far only acute therapy. Thus, a major goal of translational research is to “freeze” the penumbra already during LVO (before opening the vessel) and thereby extend individual time windows for non-futile recanalization. We used the filament occlusion model of the middle cerebral artery (MCAO) in mice and assessed progressive infarction under occlusion at 2, 3, and 4 h after onset. We show that blocking the activatory platelet receptor glycoprotein (GP)VI substantially delayed progressive neocortical infarction compared to isotype control antibody treated mice. Moreover, the local vascular recruitment of infiltrating neutrophils and T-cells was mitigated. In conclusion, our experimental data support ongoing clinical trials blocking platelet GPVI in acute ischemic stroke.

1. Introduction

Acute ischemic stroke (AIS) is a major cause of death and invalidity worldwide. Reopening of the occluded cerebral artery is a prerequisite to alleviate ischemic stroke injury, but, remarkably, does not guarantee a good clinical outcome (GSR-ET, 2018). The extent of structural brain damage before thrombolysis and/or mechanical thrombectomy (MT) is caused by infarct expansion into the ischemic penumbra nourished by collateral blood flow and progressively hampers therapeutic efficacy (Shuaib et al., 2011). In a mouse model of progressive stroke we could recently show that platelets and leukocytes contribute to penumbral tissue loss under occlusion before recanalization (Schuhmann et al., 2021), but the platelet signaling pathways involved are incompletely understood. Platelets must be slowed down to adhere to the arterial wall under high shear flow conditions, a process named tethering, which depends on their glycoprotein (GP) Ib-V-IX receptor complex. Firm adhesion, however, requires further platelet activation. At sites of endothelial damage this is mainly mediated by GPVI, the principal activating platelet collagen receptor (Nieswandt et al., 2001). The role of GPVI in penumbral tissue loss before recanalization has not yet been addressed previously.

We here report that targeting platelet GPVI substantially decelerates ischemic brain damage early during proximal artery occlusion in mice supporting further clinical development.

2. Material and methods

2.1. Animals

We randomized male C57Bl/6 N mice (6–8 weeks old) and conducted them to a permanent and/or transient middle cerebral artery occlusion (MCAO) (Schuhmann et al., 2021). Focal cerebral ischemia

Abbreviations: glycoprotein, (GP); monoclonal antibody anti GPVI, (JAQ1); large vessel occlusion, (LVO); thrombectomy, (TE); occlusion of the middle cerebral artery, (MCAO); acute ischemic stroke, (AIS); mechanical thrombectomy, (MT); triphenyltetrazolium chloride, (TTC).

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was induced by MCAO in 4 independent groups. In group 1–3 permanent MCAO was performed and the duration of occlusion was varied: 2, 3 or 4 h permanent MCAO. After these varying periods of permanent occlusion read-out was performed as described below immediately, i.e., before and therefore independent of the reperfusion event. In group 4 MCAO endured for 2 h and read-out was after a 6 h period of reperfusion (Schuhmann et al., 2021). Animal studies were approved by the district government of lower Franconia (RUF-55.2.2–2532–2–711) and were conducted in accordance with the US National Institutes of Health Guide for the Care and Use of Laboratory Animals. The experiments were designed, performed, and reported according to the Animal Research: Reporting of In Vivo Experiments guidelines (Kilkenny et al., 2010).

2.2. Stroke volume

Animals were sacrificed after MCAO (Group 1–3; immediately after 2, 3 or 4 h of permanent MCAO, Group 4: not until completing 6 h of reperfusion after recanalization) and the brains were cut in three 2 mm-thick coronal sections. The slices were stained for 20 min at 37 °C with 2% TTC to visualize the infarctions. Edema-corrected infarct volumes were planimetrically calculated (ImageJ software, National Institutes of Health) and also analyzed histologically by Map2a/b (abcam, ab32454) staining as described previously (Schuhmann et al., 2021).

2.3. Animal treatment

To deplete GPVI, mice were given 100 μg anti-GPVI antibody (JAQ1) intraperitoneal, five and four days prior to MCAO induction. JAQ1 is a monoclonal antibody (mAb) against the major collagen-binding site on mouse GPVI, which induces irreversible down-regulation of the collagen receptor GPVI. Control animals received 100 μg rat IgG (Nieswandt et al., 2001).

2.4. Immunohistochemistry

For immunohistochemistry of neutrophils and platelets methanol fixed mouse brain sections were blocked with 10% BSA and stained with antibodies against Ly6G (BioLegend, #127636; dilution 1:100) and GPIX (emfret; dilution 1:100) as described previously (Schuhmann et al., 2021). For CD4+ T-cell quantification identical brain sections at the level of the basal ganglia (0.5 mm anterior from bregma) were selected, fixed with 4% paraformaldehyde, blocked with 5% BSA containing 0.2% Triton X100 and stained with an antibody against CD4 (BioLegend, #100506; dilution 1:50). Cell counting was performed from 3 subsequent slices (Ly6G+) or 1 slice (CD4+) of 5 different animals under a microscope (Leica DMI8 equipped with the DMC 2900 / DFC 3000 G camera control and LAS X software (Leica, Wetzlar, Germany)). Negative controls for all histological experiments included omission of primary or secondary antibody and gave no signals (not shown).

2.5. Statistical analyses

All data from animal experiments are given as box plots including median (Med) with the 25th percentile (25%), the 75th percentile (75%), minimum and maximum. For statistical analysis, the GraphPad Prism 9 software package was used. Data were tested for Gaussian distribution with the D’Agostino-Pearson omnibus normality test and then analyzed by an unpaired, 2-tailed Student t-test, or in the case of non-parametric distribution, the Wilcoxon–Mann–Whitney U test was applied. Probability values <0.05 were considered to indicate statistically significant results.

3. Results

3.1. Targeting platelet GPVI reduces infarct progression under MCA occlusion

First, we assessed if GPVI depletion by anti-GPVI antibody (JAQ1) treatment in wild-type mice influences infarct growth into the penumbra during middle cerebral artery occlusion for 2, 3, or 4 h. Stroke development in GPVI depleted mice was significantly reduced (2 h MCAO: ~61%, 3 h MCAO: ~43%, 4 h MCAO: ~42%) compared to control anti-IgG treated animals as revealed by TTC (Fig. 1A) and MAP2 (Fig. 1B) staining. In addition, reduction of infarct volumes (~ 47%) persisted into the reperfusion phase in GPVI depleted mice after a prolonged primary occlusion time of 2 h followed by 6 h of reflow.

3.2. Blocking the collagen receptor GPVI resulted in reduced neutrophil and T-cell recruitment

Next, we assessed the effect of targeting platelet GPVI on the infiltration of neutrophils and T-cells under MCA occlusion in the ischemic brain hemispheres. Analogous to smaller infarct volumes and improved neuronal survival (Fig. S1), anti-GPVI treated mice demonstrated significantly fewer ipsilesional numbers of neutrophils and T-cells during the occlusion condition and after reperfusion compared to control mice (Fig. 1C,E,F; Fig. S2). We then analyzed the occurrence of co-localizations of neutrophils and platelets. Importantly, the numbers of neutrophils in conjunction with platelets were also significantly reduced in GPVI depleted mice (Fig. 1D; Fig. S2).

4. Discussion

Despite the huge success in restoring blood flow by thrombolysis and/or mechanical thrombectomy after cerebral LVO, a significant number of patients still suffer from severe neurological deficits despite recanalization. This prompted research into pathomechanisms causing rapid infarct growth into the penumbra before recanalization and/or thereafter during the phase of acute ischemia/reperfusion injury (Stoll and Nieswandt, 2019). We have previously shown that progressive brain infarction can be delayed by blocking detrimental leukocyte and GPIb-mediated platelet responses in hyper-acute stroke before recanalization (Schuhmann et al., 2021), as well as after recanalization during I/R injury (Stoll and Nieswandt, 2019). We now extend these studies by showing that depletion of platelet GPVI mitigates primary infarct growth under occlusion (pMCAO) as well as I/R injury upon delayed recanalization beyond 1 h, the short primary occlusion time mostly used in experimental stroke research (Schuhmann et al., 2019; Schuhmann et al., 2020). Blockade of GPVI massively reduced the recruitment of neutrophils and T-cells to the ischemic brain. T-cells are detrimental in ischemic stroke and directly interact with platelets via CD84, a homophilic cell adhesion molecule (Schuhmann et al., 2020). Although neutrophils are among the first immune cells recruited to the ischemic brain, their functional role is less clear (Schabitz and Minnerup, 2019). In our study ~25% of the neutrophils in the brains of untreated control animals were associated with platelets and GPVI depletion significantly reduced the occurrence of neutrophils associated with platelets. There is increasing evidence for a functional heterogeneity of neutrophil subsets, which may account for the conflicting net effects on stroke outcome (Schabitz and Minnerup, 2019). Further studies are underway to specifically characterize the phenotype of neutrophils which form complexes with platelets and to assess their role in the evolution of ischemic lesions.

A number of GPVI inhibitors are currently under development for clinical use. Recently, an antiplatelet GPVI Fab (glenzocimab) has been shown to be safe and well tolerated in healthy volunteers (Voors-Pette et al., 2019). This prompted an ongoing phase 2 trial in acute stroke patients (ACTIMIS-Trial, 2021) as adjunct therapy to thrombolysis and/or MT. Our experimental data support this approach by showing that...
platelet GPVI contributes to acute infarct progression, both before recanalization (this study) as well as during reperfusion, and thereby appears safe even in conjunction with rt-PA treatment (Schuhmann et al., 2019).

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Fig. 1. Blocking of GPVI delays ischemic brain damage and diminished ultra-early neutrophil recruitment in the ischemic brain. Representative images of cortical sections stained with A TTC or B Map2a/b 2, 3 and 4 h after MCAO or after 2 h of MCAO with additional 6 h of reperfusion in mice treated with rat IgG (a-Ctrl IgG) or JAQ1 (a-GPVI mAb) 5 and 4 days before MCA occlusion. Infarcted areas are shown in white. Planimetric analyses were used to quantify the infarct volumes. Results are presented as box plots (n = 8–10). C Representative immunocytologic stainings (left) and quantification (right) of brain-infiltrating Ly6G-positive neutrophils (Alexa594, red), nuclei (DAPI, blue) in the whole ipsilateral hemisphere 2, 3 and 4 h after MCAO or after 2 h of MCAO with additional 6 h of reperfusion in mice treated with rat IgG (a-Ctrl IgG) or JAQ1 (a-GPVI mAb) 5 and 4 days before MCA occlusion using 20× objective. Scale bar 50 μm (n = 5; 3 slices/animal). D Representative immunocytologic stainings (left) and per cental distribution (right) of brain-infiltrating Ly6G-positive neutrophils (Alexa594, red) co-localized with GPIIX-positive platelet aggregates (Alexa488, green), nuclei (DAPI, blue) in the whole ipsilateral hemisphere 2, 3 and 4 h after MCAO or after 2 h of MCAO with additional 6 h of reperfusion in mice treated with rat IgG (a-Ctrl IgG) or JAQ1 (a-GPVI mAb) 5 and 4 days before MCA occlusion using 40× objective. Scale bar 25 μm (n = 5; 3 slices/animal). E Representative immunocytologic stainings (top) and quantification (bottom) of brain-infiltrating CD4-positive T lymphocytes (Cy3, red), nuclei (DAPI, blue) in the ipsilateral hemisphere 2, 3 and 4 h after MCAO or after 2 h of MCAO with additional 6 h of reperfusion in mice treated with rat IgG (a-Ctrl IgG) or JAQ1 (a-GPVI mAb) 5 and 4 days before MCA occlusion using 20× objective. Scale bar 100 μm (n = 5). F (top) Native half mouse brain with schematic overview of ACA and MCA territories. The ROI used for T-cell quantification is shown on a TTC stained coronal brain section (2 h MCAO). (bottom) Quantification of brain-infiltrating CD4-positive T lymphocytes (Cy3, red), nuclei (DAPI, blue) in the ROI at 2, 3 and 4 h after MCAO or after 2 h of MCAO with additional 6 h of reperfusion in mice treated with rat IgG (a-Ctrl IgG) or JAQ1 (a-GPVI mAb) 5 and 4 days before MCA occlusion using 20× objective (n = 5). *P < 0.05, **P < 0.01, ***P < 0.001 between the indicated groups, 2-tailed Student t-test or in the case of nonparametric functional outcome, the Wilcoxon–Mann–Whitney U test was applied. b.d., beyond detection level. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Authors'contributions

Conceptualization and study design: MB, MKS, MP, GS. Data curation, validation and visualization: MB, MKS, AMK, DS, BN, MP, GS. Writing - original draft: MB, MKS, GS. Writing - review and editing: MB, MKS, AMK, DS, BN, MP, GS.

Declaration of Competing Interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.expneurol.2021.113804.

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