Blocking of platelet glycoprotein receptor Ib reduces “thrombo-inflammation” in mice with acute ischemic stroke

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Abstract

Background: Ischemic stroke causes a strong inflammatory response that includes T cells, monocytes/macrophages, and neutrophils. Interaction of these immune cells with platelets and endothelial cells facilitates microvascular dysfunction and leads to secondary infarct growth. We recently showed that blocking of platelet glycoprotein (GP) receptor Ib improves stroke outcome without increasing the risk of intracerebral hemorrhage. Until now, it has been unclear whether GPIb only mediates thrombus formation or also contributes to the pathophysiology of local inflammation.

Methods: Focal cerebral ischemia was induced in C57BL/6 mice by a 60-min transient middle cerebral artery occlusion (tMCAO). Animals were treated with antigen-binding fragments (Fab) against the platelet surface molecules GPIb (p0p/B Fab). Rat immunoglobulin G (IgG) Fab was used as control treatment. Stroke outcome, including infarct size and functional deficits as well as the local inflammatory response, was assessed on day 1 after tMCAO.

Results: Blocking of GPIb reduced stroke size and improved functional outcome on day 1 after tMCAO without increasing the risk of intracerebral hemorrhage. As expected, disruption of GPIb-mediated pathways in platelets significantly reduced thrombus burden in the cerebral microvasculature. In addition, inhibition of GPIb limited the local inflammatory response in the ischemic brain as indicated by lower numbers of infiltrating T cells and macrophages and lower expression levels of inflammatory cytokines compared with rat IgG Fab-treated controls.

Conclusion: In acute ischemic stroke, thrombus formation and inflammation are closely intertwined (“thrombo-inflammation”). Blocking of platelet GPIb can ameliorate thrombo-inflammation.

Keywords: Ischemic stroke, Transient middle cerebral artery occlusion, Glycoprotein receptor Ib, Thrombo-inflammation

Introduction

Ischemic stroke (IS) is a leading cause of death and invalidity, and its incidence is increasing [1]. The exact pathophysiological mechanisms underlying IS are still not completely understood. While cerebral ischemia has, for many years, been considered to be a predominantly thrombotic disease, it is currently widely accepted that inflammatory processes also play an important role [2]. IS causes a strong inflammatory response, including transmigration of leukocyte subsets and expression of inflammatory cytokines [3]. We recently introduced the interplay between thrombotic and inflammatory mechanisms at the neurovascular unit as a pathophysiological concept of thrombo-inflammation [4, 5]. A potential molecular target linking inflammation and thrombosis might be the platelet glycoprotein (GP) receptor Ib. Thrombotic pathways include the binding of GPIb to endothelial von Willebrand factor for the initial adhesion of platelets at vascular injury sites. With respect to inflammation, GPIb harbors a binding site for the integrin Mac-1 being expressed on neutrophils and monocytes. Therefore, GPIb has been shown to be involved in the recruitment of immune cells [6]. We have previously
demonstrated that interfering with the early steps of platelet aggregation and activation via blocking platelet GPIb reduces infarct volume and improves stroke outcome in a mouse model of acute experimental stroke, without increasing the risk of intracerebral hemorrhage (ICH) [7]. Furthermore, targeting GPIb was also safe and effective in aged and comorbid mice with IS [8]. Therefore, blockade of GPIb may be a promising target for human stroke studies.

The aim of this study was to investigate whether GPIb blockade also has the potential to mitigate the stroke-associated inflammatory response.

Materials and methods

Animals

In this study, male C57BL/6 wild-type mice were used. The animals were aged 12–14 weeks. Animal experiments were approved by the legal state authorities (Regierung von Unterfranken) and performed according to the recommendations for research in experimental stroke studies [9], and the current ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines (https://www.nc3rs.org.uk/arrive-guidelines).

Animal treatment

Mice were given 100 μg p0p/B antigen-binding fragment (Fab) intravenously after a 60-min transient middle cerebral artery occlusion (tMCAO) at the time point of filament removal to inhibit GPIb. The control animals received 100-μg rat IgG Fab [8].

Real-time polymerase chain reaction (RT-PCR)

Tissue homogenization, RNA isolation, and real-time PCR were performed as described recently [5]. Relative gene expression levels of tumor necrosis factor-α (TNFα) (assay ID: Mm 00443258_m1, Applied Biosystems), interleukin (IL)1β (assay ID: Mm 00434228_m1, Applied Biosystems), and IL6 (assay ID: Mm 00446190_m1, Applied Biosystems) were analyzed with a fluorescent TaqMan technology. As an endogenous control Gapdh (TaqMan® Predeveloped Assay Reagent for gene expression, part number: 4352339E, Applied Biosystems) was used. PCR was performed using StepOnePlus™ Real-Time PCR System (Applied Biosystem).

Immunohistochemistry and thrombosis index

Histology and immunohistochemistry were performed according to standard procedures [13]. Cryoembedded coronal brain sections (2 mm) were cut into 10-μm-thick slices. Every tenth slice was used for evaluation. The following antibodies were used: polyclonal antibody anti-CD31 (ab9498, abcam), monoclonal antibody (mAb) anti-GPIX (generated by B. Nieswandt.), mAb anti-CD11b (MCA711, Serotec), and mAb anti-CD3 (MCA2690A488, BioRad). For quantification of occluded microvessels, brain slices were stained with hematoxylin and eosin. Afterwards, the numbers of occluded and opened vessels per hemisphere were counted to determine the percentage of occlusions as previously described [13]. All immunohistologic stainings were analyzed and acquired using a Nikon Eclipse 50i microscope.

Statistical analysis

All results are given as mean ± standard error of the mean except for the Bederson score and the grip test, which are expressed as ordinal values. For statistical analysis, the GraphPad Prism 5.0 software package (GraphPad Software) was used. Data were tested for Gaussian distribution with the D’Agostino and Pearson omnibus normality test and then analyzed by unpaired, two-tailed Student’s t test. Scores addressing the functional outcome were compared using the Mann–Whitney U test. 
\[ P < 0.05 \] was considered statistically significant.

Results

Consistent with our previous results [7, 8], treatment with anti-GPIb 60 min after tMCAO significantly reduced stroke volumes (51.08 ± 9.52 mm³) compared with IgG Fab-treated controls (90.83 ± 9.07 mm³, \[ P < 0.05 \]) on day 1 (Fig. 1a, b). Importantly, reduced stroke size in the anti-GPIb-treated mice translated into better functional outcome as assessed by the Bederson score (values are the median with 25th and 75th percentiles, respectively, in brackets (IgG Fab, 3.0 (2.25, 3.75); GPIb, 2.0 (2.5, 2.25), \[ P < 0.05 \]) (Fig. 1c), but not in the Grip test (IgG...
Fab, 3.0 (3.0, 3.0); GPIb, 3.0 (3.0, 4.0), \( P > 0.05 \) (Fig. 1d)). Analogous to smaller infarct volumes, anti-GPIb treatment inhibited thrombus formation. The number of GPIX-positive platelet aggregates (number of GPIX-positive dots, IgG Fab 59.32 ± 5.16; GPIb, 35.07 ± 2.10, \( P < 0.01 \)) (Fig. 1e, f) and ipsilesional occluded brain vessels (percentage of occluded vessels, IgG Fab 44.97 ± 1.9; GPIb: 33.62 ± 5.42, \( P < 0.01 \)) (Fig. 1g) was significantly lower in anti-GPIb-treated animals compared with the control group. In the next step, we analyzed if the
inhibition of platelet activation, and therefore thrombus formation, coincides with reduced inflammation. We demonstrated fewer ipsilesional activated microglia/macrophages (Fig. 2a–e, \( P < 0.05 \)) and T cells (Fig. 2f–i, \( P < 0.05 \) and \( P < 0.001 \)) after treatment with anti-GPIb. Cytokine expression analysis revealed significantly less TNFα in the basal ganglia (relative gene expression, IgG Fab 13.33 ± 3.81; GPIb, 2.49 ± 0.89, \( P < 0.05 \)) and a tendency towards

![Fig. 2](image-url)
reduced expression in the cortex as well as reduced expression of IL1β and IL6 in both regions (Fig. 2j, k).

Discussion
In the present study, we identified reduced thrombo-inflammation to be associated with the stroke-protective properties of anti-GPIb treatment in mice. Importantly, in our current and previous studies, GPIb blockade reduced stroke volumes and significantly improved neurologic deficits in a clinically relevant setting, when injected 60 min after tMCAO [7, 8]. Of interest, stroke protection was not accompanied by ICH complications. The observation of an antithrombotic effect when targeting GPIb in an in vivo experimental stroke model is congruent with in vitro findings of platelet thrombus formation under flow [14]. GPIb has a key function in thrombotic processes, as it is essential for the initial adhesion of platelets to the vessel wall under high shear rates [15]. Furthermore, GPIb harbors a binding site for Mac-1. Mac-1 has been shown to be involved in the adhesion of leukocytes to platelets, and leukocyte-platelet complexes might promote inflammation [16]. Therefore, GPIb/Mac-1 interactions might provide a potential mechanistic link between thrombosis and inflammation [4, 6]. Our finding that blocking GPIb reduced the number of ipsilesional leukocytes after experimental stroke strongly supports the concept of thrombo-inflammation and is in accordance with a previous investigation that found GPIb to be involved in the recruitment of immune cells in a model of acute peritonitis [6]. Interestingly, besides reduced numbers of monocytes, the number of CD4-positive T cells invading the ipsilateral hemisphere was also significantly reduced in this study. Importantly, not only the overall number but also the density of infiltrating immune cells in brain areas that are infarcted in both treatment groups was reduced, arguing against an unspecific effect related to smaller infarct volumes.

Tumor necrosis factor, interleukin-1, and interleukin-6 are potent inflammatory cytokines that have been shown to modulate tissue injury in experimental stroke [17]. There is evidence that tumor necrosis factor and interleukin-1 are produced in the ischemic hemispheres of rodents by CD11b-positive cells [18–20]. This is in good agreement with our data revealing reduced TNFα and IL1β expression especially within the basal ganglia regions where blocking of platelet GPIb significantly reduced the number of CD11b-positive cells/mm².

It is widely accepted that T cells have a detrimental role in the acute phase of IS [21, 22]. Brain ischemia rapidly activates the cerebral microvasculature [23]. Endothelial adhesion molecules become rapidly upregulated, and damaged vessels expose subendothelial matrix proteins to the bloodstream, thereby providing an interface for T cell–endothelial as well as platelet–endothelial and T cell–platelet interdependencies. Our mechanistic studies recently revealed that T cells promote stroke due to interactions with the activated endothelium. Thereby, T cells cause microvascular dysfunction and further increase thrombus formation during the early phase after tMCAO [5, 13, 24]. The present study shows, for the first time, that targeting platelets by blocking their early adhesion to vessel walls [25] impacts T cell driven inflammatory processes after stroke.

In summary, our study confirms that in acute IS, thrombus formation and inflammation are closely intertwined. Blocking of platelet GPIb can ameliorate thrombo-inflammation.

Abbreviations
Fab: Antigen-binding fragment; GP: Glycoprotein; ICH: Intracerebral hemorrhage; IgG: Immunoglobulin G; IL: Interleukin; IS: Ischemic stroke; tMCAO: Transient middle cerebral artery occlusion

Acknowledgements
We thank Andrea Sauer for excellent technical assistance.

Funding
This work was supported by the Sonderforschungsbereich 688.

Availability of data and materials
All data generated or analyzed during this study are included in this published article.

Authors’ contributions
CK, PK, and MKS performed the experiments. MKS, JG, and PK interpreted the data. MKS, CK, PK, and MKS planned and conceived the study. MKS, JG, and PK wrote and critically revised the manuscript. All authors have read and approved the final manuscript.

Competing interests
The authors declare they have no competing interests.

Consent for publication
Not applicable.

Ethics approval
Animal experiments were approved by the legal state authorities (Regierung von Unterfranken).

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Received: 13 October 2016 Accepted: 4 January 2017
Published online: 21 January 2017

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