Temsirolimus Sensitive Stimulation of Platelet Activity, Apoptosis and Aggregation by Collagen Related Peptide

Hang Cao\textsuperscript{a}  Rosi Bissinger\textsuperscript{a}  Anja T. Umbach\textsuperscript{a}  Meinrad Gawaz\textsuperscript{a}  Florian Lang\textsuperscript{b}

\textsuperscript{a}Department of Medicine III, \textsuperscript{b}Department of Physiology I, Eberhard-Karls-University, Tuebingen, Germany

Key Words
CRP • Platelet activation • Degranulation • Integrin • Cytosolic Ca\textsuperscript{2+} concentration • Caspase • Phosphatidylserine translocation • Aggregation

Abstract

Background/Aims: The mammalian target of rapamycin (mTOR) inhibitor temsirolimus stimulates apoptosis of tumor cells and is thus therapeutically used for the treatment of diverse malignancies. On the other hand, temsirolimus has been shown to protect against apoptosis of hippocampal neurons. Similar to nucleated cells, blood platelets may enter suicidal death characterized by cell shrinkage and cell membrane scrambling. Platelet apoptosis is frequently preceded by Ca\textsuperscript{2+} entry, degranulation, integrin activation and stimulation of caspases. Those events could be triggered by collagen related peptide (CRP). The present study explored whether treatment of platelets with temsirolimus modifies platelet activation, caspase activity, platelet shrinkage, and phosphatidylserine abundance.

Methods: Platelets isolated from wild-type mice were exposed for 30 minutes to temsirolimus (40 µg/ml) without or with additional CRP (2 µg/ml or 5 µg/ml) treatment. Flow cytometry was employed to estimate cytosolic Ca\textsuperscript{2+}-activity ([Ca\textsuperscript{2+}]i), platelet degranulation from P-selectin abundance, integrin activation from α\textsubscript{IIb}β\textsubscript{3} integrin abundance, caspase activity utilizing an Active Caspase-3 Staining kit, phosphatidylserine abundance from annexin-V-binding and relative platelet volume from forward scatter.

Results: In the absence of CRP, the administration of temsirolimus (40 µg/ml) significantly decreased [Ca\textsuperscript{2+}]i, but did not significantly modify P-selectin abundance, activated α\textsubscript{IIb}β\textsubscript{3} integrin, annexin-V-binding, cell volume, caspase activity and aggregation. Exposure of platelets to CRP was followed by significant increase of [Ca\textsuperscript{2+}]i, P-selectin abundance, α\textsubscript{IIb}β\textsubscript{3} integrin activity, annexin-V-binding, ROS, caspase activity and aggregation, effects significantly blunted in the presence of temsirolimus. CRP further decreased forward scatter, an effect again significantly blunted by temsirolimus. Conclusions: Temsirolimus is a powerful inhibitor of platelet activation and suicidal platelet death.

© 2017 The Author(s)  Published by S. Karger AG, Basel
Introduction

Temsirolimus, a selective inhibitor of the kinase mammalian target of rapamycin (mTOR) [1-9], is used for the treatment of malignancies, such as metastatic renal cell carcinoma [2, 10-37], diverse further solid tumors [38-42], refractory mantle cell lymphoma [1, 43-48], non-Hodgkin lymphomas [38, 48], and multiple myeloma [38]. Temsirolimus is effective by triggering apoptosis of tumor cells [9, 49-59] with activation of caspases [49]. On the other hand, temsirolimus has been shown to protect against apoptosis of hippocampal neurons [60].

Side effects of temsirolimus treatment include thrombosis [61, 62], which may involve blood platelets [63, 64]. Activation of platelets contributes to the pathogenesis of a variety of clinical disorders including arterial thrombosis, vascular inflammation and atherosclerosis [65-68] and conversely, impaired platelet function may result in bleeding disorders [69-71]. Platelets are activated by an increase of cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_i\)) [72] due to Ca\(^{2+}\) release from intracellular stores [73] and subsequent activation of Ca\(^{2+}\) release-activated channel Orai1 in the plasma membrane by the Ca\(^{2+}\) sensing proteins STIM1 or STIM2 [74]. Platelets could enter apoptosis, characterized by caspase activation, cell shrinkage, and cell membrane scrambling with phosphatidylserine translocation to the cell surface. Stimulators of platelet activity and apoptosis include collagen related peptide [75]. To the best of our knowledge, nothing is known about an effect of temsirolimus on platelet apoptosis.

The present study thus explored whether temsirolimus triggers or modifies platelet activation and apoptosis. In order to avoid the variability of platelet function in human individuals, experiments were performed in murine platelets.

Materials and Methods

Mice

All animal experiments were conducted according to the German law for the welfare of animals and were approved by the authorities of the state of Baden-Württemberg. Experiments were performed with blood platelets isolated from wild type mice. The mice had free access to water and control chow (Ssniff, Soest, Germany).

Preparation of mouse platelets

Platelets were obtained from 10- to 12-week-old mice of either sex. The mice were anesthetized and 800 µl blood was drawn from the retro-orbital plexus into tubes with 200 µl acid-citrate-dextrose buffer before the mice were sacrificed [76]. Platelet rich plasma (PRP) was obtained by centrifugation at 260 g for 5 minutes. Afterwards, PRP was centrifuged at 640 g for 5 minutes to pellet the platelets. Where necessary, apyrase (0.02 U/ml; Sigma-Aldrich) and prostaglandin I\(_2\) (0.5 µM; Calbiochem) were added to the PRP to prevent activation of platelets during isolation [77]. After two washing steps, the pellet of washed platelets was resuspended in modified Tyrode-HEPES buffer (pH 7.4, supplemented with 1 mM CaCl\(_2\)). Where indicated, CRP (Roche, Basel Switzerland) was added at the indicated concentrations [78].

Cytosolic calcium

For the measurement of the cytosolic Ca\(^{2+}\) concentration, the platelet preparation was washed once in Tyrode buffer (pH 7.4), stained with 3 µM Fluo-3AM (Biotium, USA) in the same buffer and incubated at 37°C for 30 minutes. Following the indicated experimental treatment, relative fluorescence was measured utilizing a BD FACS Calibur (BD Biosciences, Heidelberg, Germany) [79].

P-selectin and activated integrin abundance

Fluorophore-labeled antibodies were utilized for the detection of P-selectin expression (Wug.E9-FITC) and the active form of \(\alpha_\text{IIb}\beta_\text{3}\) integrin (JON/A-PE). Washed mouse platelets (1x10\(^8\)) were suspended in modified Tyrode buffer (pH 7.4) containing 1 mM CaCl\(_2\) and antibodies (1:10 dilution) and subsequently...
subjected to the respective treatments and for the indicated time periods at room temperature (RT). The reaction was stopped by addition of PBS and the samples were immediately analyzed on a BD FACS Calibur.

**Phosphatidylserine exposure and forward scatter**

For determination of phosphatidylserine exposure, the platelet preparation was centrifuged at 660 g for 5 minutes followed by washing once with Tyrode buffer (pH 7.4) with 1 mM CaCl₂ staining with 1:20 dilution of Annexin-V FITC (Mabtag, Germany) in Tyrode buffer (pH 7.4) with 2 mM CaCl₂ and incubation at 37°C for 30 minutes. Annexin-V binding reflecting surface exposure of phosphatidylerine was evaluated by flow cytometry utilizing a BD FACS Calibur [80]. In parallel, the forward scatter (FSC) of the platelets was determined by flow cytometry as a measure of platelet size [75].

**Reactive oxidant species (ROS)**

The abundance of ROS was determined utilizing 2',7'-dichlorodihydrofluorescein (DCFDA) diacetate. Washed platelets were stained with DCFDA (10 µM; Sigma, Schnelldorf, Germany) in Tyrode buffer at 37°C for 30 min and washed once in Tyrode buffer. The DCFDA-loaded platelets were resuspended in 200 µl Tyrode buffer and ROS-dependent fluorescence intensity was measured at an excitation wavelength of 488 nm and an emission wavelength of 530 nm on a BD FACS Calibur.

**Caspase-3 activity**

Caspase 3 activity was determined utilizing a CaspGlow Fluorescein Active Caspase-3 Staining kit from BioVision (CA, USA) according to the manufacturer’s instruction. Fluorescence intensity was measured at an excitation wavelength of 488 nm and an emission wavelength of 530 in a BD FACS Calibur (BD Biosciences, USA).

**Platelet aggregation**

Aggregation was determined utilizing flow cytometry as previously described [75]. To this end, platelets were labeled with CD9-APC and CD9-PE monoclonal antibodies (1:100 dilution, Abcam) for 15 minutes at room temperature. Following incubation, differently labeled samples were washed twice, mixed 1:1, and then pre-incubated for 30 min at 37°C while shaking at 600 rpm for 10 minutes. Pre-incubated platelets were activated with 2 µg/ml CRP at 37°C while shaking at 1000 rpm. At the indicated time points, samples were fixed by addition of 0.5% paraformaldehyde (Carl Roth, Germany) in phosphate-buffered saline. The fixed samples were measured utilizing a BD FACS Calibur (BD Biosciences, Heidelberg, Germany). For quantification, a quadrant was set in the dot plot of respective channels on non-stimulated platelets. The appearance of double-colored events in the upper right quadrant (Q2) was quantified as percentage of total amount of labeled events (Q1+Q2+Q4) at every time point analyzed.

**Statistical analysis**

Data are provided as means ± SEM; n represents the number of independent experiments. All data were tested for significance using ANOVA with Tukey’s test as post-test or unpaired student’s t-test as appropriate. Results with p<0.05 were considered statistically significant.

**Results**

The present study explored whether temsirolimus modifies platelet activity and apoptosis without and with additional stimulation by collagen related peptide (CRP). Murine platelets were isolated from wild type mice and exposed to CRP (2 µg/ml or 5 µg/ml) without and with presence of temsirolimus (40 µg/ml, 30 minutes). 

Fluo-3 fluorescence, determined by flow cytometry, was employed to estimate cytosolic Ca²⁺ concentration ([Ca²⁺]ₖ). Without presence of CRP, platelet [Ca²⁺]ₖ was slightly, but significantly decreased by addition of temsirolimus (Fig. 1). Treatment with CRP was followed by a marked significant increase of [Ca²⁺]ₖ, an effect significantly blunted in the presence of temsirolimus (Fig. 1).

The abundance of reactive oxygen species (ROS) was quantified utilizing DCFDA fluorescence. In the absence of CRP, treatment with temsirolimus was followed by a significant
decrease of the ROS abundance (Fig. 2). The ROS abundance was significantly enhanced by treatment of the platelets with CRP. Temsirolimus virtually abrogated the effect of CRP on the ROS abundance (Fig. 2).

Increase of P-selectin abundance at the platelet surface, determined by flow cytometry utilizing specific antibodies, was taken as evidence for platelet degranulation. As illustrated in Fig. 3, without presence of CRP, the P-selectin abundance at the platelet surface was negligible in both, absence and presence of temsirolimus. CRP treatment markedly and significantly increased P-selectin abundance, an effect significantly blunted in the presence of temsirolimus (Fig. 3).
The abundance of active integrin $\alpha_{IIb}\beta_3$, determined by flow cytometry utilizing specific antibodies, was again negligible in the absence of CRP irrespective of the absence or presence of temsirolimus (Fig. 4). The abundance of active integrin $\alpha_{IIb}\beta_3$ was markedly and significantly increased by CRP treatment, an effect again significantly blunted by temsirolimus (Fig. 4).

A kit has been used for the detection of activated caspase 3. As illustrated in Fig. 5, without presence of CRP, caspase activity was negligible in both, absence and presence of temsirolimus. CRP significantly increased the caspase activity, an effect significantly blunted by temsirolimus treatment (Fig. 5).

Phosphatidylserine abundance was estimated from annexin-V-binding. As shown in Fig. 6, the percentage of annexin-V positive platelets was negligible in the absence of CRP irrespective of the absence or presence of temsirolimus (Fig. 6). The percentage of annexin-V positive platelets was negligible in the absence of CRP irrespective of the absence or presence of temsirolimus. CRP significantly increased the annexin-V+ platelets, an effect significantly blunted by temsirolimus (Fig. 6).

**Fig. 3.** Temsirolimus sensitive CRP-induced platelet degranulation. A,B. Original histogram overlays of P-selectin related fluorescence in murine platelets without (A) and with (B) a 15 minutes CRP (2 µg/ml) treatment without (grey areas) and with (black lines) presence of temsirolimus (40 µg/ml, 30 minutes). C. Arithmetic means ± SEM (n = 6) of the P-selectin related fluorescence (arbitrary units) in murine platelets without (left bars) and with (right bars) a 15 minutes CRP treatment (2 µg/ml) in the absence (white bars) and presence (black bars) of 40 µg/ml temsirolimus. ### (p<0.001) indicates statistically significant difference from absence of CRP, *** (p<0.001) indicates statistically significant difference from absence of temsirolimus.

**Fig. 4.** Temsirolimus sensitive CRP-induced $\alpha_{IIb}\beta_3$ integrin activation. A,B. Original histogram overlays of active $\alpha_{IIb}\beta_3$ integrin related fluorescence in murine platelets without (A) and with (B) a 15 minutes CRP (2 µg/ml) treatment without (grey areas) and with (black lines) presence of temsirolimus (40 µg/ml, 30 minutes). C. Arithmetic means ± SEM (n = 6) of the $\alpha_{IIb}\beta_3$ integrin related fluorescence (arbitrary units) in murine platelets without (left bars) and with (right bars) a 15 minutes CRP treatment (2 µg/ml) in the absence (white bars) and presence (black bars) of 40 µg/ml temsirolimus. ### (p<0.001) indicates statistically significant difference from absence of CRP, *** (p<0.001) indicates statistically significant difference from absence of temsirolimus.
of the absence or presence of temsirolimus. CRP treatment markedly and significantly enhanced the percentage of annexin-V binding platelets, an effect again significantly blunted in the presence of temsirolimus (Fig. 6).

Platelet volume was estimated from forward scatter, which was determined by flow cytometry. As illustrated in Fig. 7, in the absence of CRP, the forward scatter was similar in the absence and presence of temsirolimus. CRP treatment markedly and significantly decreased forward scatter, an effect again significantly blunted in the presence of temsirolimus (Fig. 7).

To elucidate the effect of temsirolimus on platelet aggregation, platelets were labeled with two distinct dyes and the coincidence of the two dyes estimated by flow cytometry. As illustrated
in Fig. 8, aggregation was markedly and significantly enhanced by treatment of the platelets with CRP. Temsirolimus significantly blunted the effect of CRP on the aggregation (Fig. 8).

Discussion

The present observations reveal a novel effect of temsirolimus, i.e. its effect on platelet function and survival. Temsirolimus significantly decreases cytosolic Ca\(^{2+}\) activity ([Ca\(^{2+}\)]) and the abundance of reactive oxygen species (ROS) and significantly blunts or virtually abrogates the effect of collagen related peptide (CRP) on [Ca\(^{2+}\)], ROS, degranulation,
α_{IIb}β_{3} integrin activity, caspase 3 activity, cell membrane scrambling, cell volume, and aggregation.

Platelet activation and apoptosis are a function of cytosolic Ca^{2+} activity ([Ca^{2+}]), a powerful regulator of platelet activation involved in the pathophysiology of arterial thrombosis [72]. An increase of [Ca^{2+}], further stimulates breakdown of the phosphatidylserine asymmetry of the cell membrane with translocation of phosphatidylserine to the platelet surface. Phosphatidylserine at the platelet surface fosters coagulation and is thus a decisive stimulator of hemostasis [81]. Phosphatidylserine exposing platelets are further bound to and engulfed by macrophages [82]. In view of the present observations, temsirolimus may counteract platelet activation, hemostasis and thrombosis. It is tempting to propose the use of temsirolimus for the treatment of conditions with enhanced platelet reactivity, such as arterial thrombosis, vascular inflammation and atherosclerosis [65-68]. On the other hand, the present observations advocate caution when using temsirolimus in clinical conditions associated with impaired platelet function, such as hepatic failure [70]. However, additional experimental effort particularly in vivo is required to fully define the effect of temsirolimus in the physiology and pathophysiology of platelets.

In conclusion, temsirolimus blunts the CRP-induced increase of [Ca^{2+}], ROS, P-selectin abundance, α_{IIb}β_{3} integrin activity, caspase activity, annexin-V-binding, cell volume and aggregation, and may thus counteract platelet activation and apoptosis.

Acknowledgements

We thank Efi Faber for providing technical assistance as well as Lejla Subasic for meticulous preparation of the manuscript. This study was supported by the Deutsche Forschungsgemeinschaft and Open Access Publishing Fund ofTuebingen University - Klinische Forschergruppe [DFG-KFO 274] 'Platelets—Molecular Mechanisms and Translational Implications', as well as the Tuebingen Platelet Investigative Consortium (TuePIC). Work of R.B. is supported by the Institutional Strategy of the University of Tübingen (Deutsche Forschungsgemeinschaft, ZUK63).

Disclosure Statement

The authors of this manuscript state that they have no conflicts of interest to declare.

References


52 Carew JS, Espitia CM, Zhao W, Mita MM, Mita AC, Nawrocki ST: Targeting Survivin Inhibits Renal Cell Carcinoma Progression and Enhances the Activity of Temsirolimus. Mol Cancer Ther 2015;14:1404-1413.
Cao et al.: Temsirolimus Sensitive Platelet Function

1262


Cell Physiol Biochem 2017;42:1252-1263

DOI: 10.1159/000478954
Published online: July 11, 2017

Cao et al.: Temsirolimus Sensitive Platelet Function

© 2017 The Author(s). Published by S. Karger AG, Basel
www.karger.com/cpb


