Original Paper

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

Hang Cao^a Rosi Bissinger^a Anja T. Umbach^a Meinrad Gawaz^a Florian Lang^b^aDepartment of Medicine III, ^bDepartment of Physiology I, Eberhard-Karls-University, Tuebingen, Germany**Key Words**CRP • Platelet activation • Degranulation • Integrin • Cytosolic Ca²⁺ 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 Ca²⁺ 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 Ca²⁺-activity ([Ca²⁺]_i) from Fluo-3 fluorescence, platelet degranulation from P-selectin abundance, integrin activation from α_{IIb}β₃ 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 [Ca²⁺]_i, but did not significantly modify P-selectin abundance, activated α_{IIb}β₃ integrin, annexin-V-binding, cell volume, caspase activity and aggregation. Exposure of platelets to CRP was followed by significant increase of [Ca²⁺]_i, P-selectin abundance, α_{IIb}β₃ 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.

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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 ($[\text{Ca}^{2+}]_i$) [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 (Biotinium, 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 $\alpha_{\text{IIb}}\beta_3$ integrin (JON/A-PE). Washed mouse platelets (1×10^6) 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 FACSCalibur.

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 CaCl_2 , staining with 1:20 dilution of Annexin-V FITC (Mabtag, Germany) in Tyrode buffer (pH 7.4) with 2 mM CaCl_2 and incubation at 37°C for 30 minutes. Annexin-V binding reflecting surface exposure of phosphatidylserine was evaluated by flow cytometry utilizing a BD FACSCalibur [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 FACSCalibur (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 $\mu\text{g/ml}$ 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 FACSCalibur (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 $\pm$ 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 $\mu\text{g/ml}$ or 5 $\mu\text{g/ml}$) without and with presence of temsirolimus (40 $\mu\text{g/ml}$, 30 minutes).

Fluo-3 fluorescence, determined by flow cytometry, was employed to estimate cytosolic Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$). Without presence of CRP, platelet $[\text{Ca}^{2+}]_i$ was slightly, but significantly decreased by addition of temsirolimus (Fig. 1). Treatment with CRP was followed by a marked significant increase of $[\text{Ca}^{2+}]_i$, 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

Fig. 1. Tamsirolimus sensitive CRP-induced increase of cytosolic Ca^{2+} concentration. A,B. Original histogram overlays of Fluo-3 fluorescence reflecting cytosolic Ca^{2+} activity in murine platelets without (A) and with (B) a 150 s treatment with CRP (2 $\mu\text{g}/\text{ml}$) without (grey areas) and with (black lines) presence of tamsirolimus (40 $\mu\text{g}/\text{ml}$, 30 minutes). C. Arithmetic means $\pm$ SEM ($n = 6$) of Fluo-3 fluorescence reflecting cytosolic Ca^{2+} activity in murine platelets without (left bars) and with (right bars) a 150 s CRP treatment (2 $\mu\text{g}/\text{ml}$) in the absence (white bars) and presence (black bars) of 40 $\mu\text{g}/\text{ml}$ tamsirolimus. ### ($p < 0.001$) indicates statistically significant difference from absence of CRP, * ($p < 0.05$), *** ($p < 0.001$) indicates statistically significant difference from absence of tamsirolimus.

Fig. 2. Tamsirolimus and CRP sensitive reactive oxygen species. A,B. Original histogram overlays of DCFDA fluorescence reflecting ROS abundance (arbitrary units) in murine platelets without (A) and with a 10 min treatment with (B) CRP (2 $\mu\text{g}/\text{ml}$) without (grey areas) and with (black lines) presence of tamsirolimus (40 $\mu\text{g}/\text{ml}$, 30 min). C. Arithmetic means $\pm$ SEM ($n = 4$) of DCFDA fluorescence reflecting ROS abundance (arbitrary units) in murine platelets in the absence (white bars) and presence (black bars) of 40 $\mu\text{g}/\text{ml}$ tamsirolimus without (resting) and with a 10 min CRP (2 $\mu\text{g}/\text{ml}$) treatment. ### ($p < 0.001$) indicates statistically significant difference from absence of CRP, *** ($p < 0.001$) indicates statistically significant difference from absence of tamsirolimus.

decrease of the ROS abundance (Fig. 2). The ROS abundance was significantly enhanced by treatment of the platelets with CRP. Tamsirolimus 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 tamsirolimus. CRP treatment markedly and significantly increased P-selectin abundance, an effect significantly blunted in the presence of tamsirolimus (Fig. 3).

Fig. 3. Tamsirolimus 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 tamsirolimus (40 µg/ml, 30 minutes). C. Arithmetic means $\pm$ 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 tamsirolimus. ### (p < 0.001) indicates statistically significant difference from absence of CRP, *** (p < 0.001) indicates statistically significant difference from absence of tamsirolimus.

Fig. 4. Tamsirolimus 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 tamsirolimus (40 µg/ml, 30 minutes). C. Arithmetic means $\pm$ 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 tamsirolimus. ### (p < 0.001) indicates statistically significant difference from absence of CRP, *** (p < 0.001) indicates statistically significant difference from absence of tamsirolimus.

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 tamsirolimus (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 tamsirolimus (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 tamsirolimus. CRP significantly increased the caspase activity, an effect significantly blunted by tamsirolimus 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

Fig. 5. Tamsirolimus sensitive CRP-dependent caspase activation. A,B. Original histogram overlays of fluorescence reflecting caspase-3 activity in murine platelets without (A) and with (B) a 10 minutes CRP (5 μ g/ml) treatment without (grey areas) and with (black lines) presence of tamsirolimus (40 μ g/ml, 30 minutes). C. Arithmetic means $\pm$ SEM (n = 4) of fluorescence reflecting caspase-3 activity in murine platelets without (left bars) and with (right bars) a 10 minutes CRP treatment (5 μ g/ml) in the absence (white bars) and presence (black bars) of 40 μ g/ml tamsirolimus. ### (p<0.001) indicates statistically significant difference from absence of CRP, * (p<0.05) indicates statistically significant difference from absence of tamsirolimus.

Fig. 6. Tamsirolimus sensitive CRP-dependent cell membrane scrambling. A,B. Original histogram overlays of annexin-V-binding reflecting phosphatidylserine abundance at the surface of murine platelets without (A) and with (B) a 10 minutes CRP (5 μ g/ml) treatment without (grey areas) and with (black lines) presence of tamsirolimus (40 μ g/ml, 30 minutes). C. Arithmetic means $\pm$ SEM (n = 6) of annexin-V-binding reflecting phosphatidylserine abundance at the surface of murine platelets without (left bars) and with (right bars) a 10 minutes CRP treatment (5 μ g/ml) in the absence (white bars) and presence (black bars) of 40 μ g/ml tamsirolimus. ### (p<0.001) indicates statistically significant difference from absence of CRP, ** (p<0.01) indicates statistically significant difference from absence of tamsirolimus.

of the absence or presence of tamsirolimus. CRP treatment markedly and significantly enhanced the percentage of annexin-V binding platelets, an effect again significantly blunted in the presence of tamsirolimus (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 tamsirolimus. CRP treatment markedly and significantly decreased forward scatter, an effect again significantly blunted in the presence of tamsirolimus (Fig. 7).

To elucidate the effect of tamsirolimus on platelet aggregation, platelets were labeled with two distinct dyes and the coincidence of the two dyes estimated by flow cytometry. As illustrated

Fig. 7. Tamsirolimus sensitive CRP-dependent cell shrinkage. A,B. Original histogram overlays of forward scatter reflecting cell volume of murine platelets without (A) and with (B) a 10 minutes CRP (5 μ g/ml) treatment without (grey areas) and with (black lines) presence of tamsirolimus (40 μ g/ml, 30 minutes). C. Arithmetic means $\pm$ SEM (n = 6) of forward scatter reflecting cell volume of murine platelets without (left bars) and with (right bars) a 10 minutes CRP treatment (5 μ g/ml) in the absence (white bars) and presence (black bars) of 40 μ g/ml tamsirolimus. ### (p < 0.001) indicates statistically significant difference from absence of CRP, ** (p < 0.01) indicates statistically significant difference from absence of tamsirolimus.

Fig. 8. Tamsirolimus sensitive CRP-dependent platelet aggregation. A. Original dot plots reflecting platelet aggregation without (a, b) and with (c, d) 4 min treatment with CRP (2 μ g/ml) in the absence (a, c) and presence (b, d) of 40 μ g/ml tamsirolimus. B. Arithmetic means $\pm$ SEM (n = 4) of platelet aggregation without (white circles) and with (black circles) prior 40 μ g/ml tamsirolimus treatment as a function of time after addition of CRP (2 μ g/ml) * (p < 0.05) indicates statistically significant difference between absence and presence of tamsirolimus.

in Fig. 8, aggregation was markedly and significantly enhanced by treatment of the platelets with CRP. Tamsirolimus significantly blunted the effect of CRP on the aggregation (Fig. 8).

Discussion

The present observations reveal a novel effect of tamsirolimus, i.e. its effect on platelet function and survival. Tamsirolimus significantly decreases cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$) and the abundance of reactive oxygen species (ROS) and significantly blunts or virtually abrogates the effect of collagen related peptide (CRP) on $[\text{Ca}^{2+}]_i$, ROS, degranulation,

$\alpha_{\text{IIb}}\beta_3$ integrin activity, caspase 3 activity, cell membrane scrambling, cell volume, and aggregation.

Platelet activation and apoptosis are a function of cytosolic Ca^{2+} activity ($[\text{Ca}^{2+}]_i$), a powerful regulator of platelet activation involved in the pathophysiology of arterial thrombosis [72]. An increase of $[\text{Ca}^{2+}]_i$ 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, tamsirolimus may counteract platelet activation, hemostasis and thrombosis. It is tempting to propose the use of tamsirolimus 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 tamsirolimus 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 tamsirolimus in the physiology and pathophysiology of platelets.

In conclusion, tamsirolimus blunts the CRP-induced increase of $[\text{Ca}^{2+}]_i$, ROS, P-selectin abundance, $\alpha_{\text{IIb}}\beta_3$ integrin activity, caspase activity, annexin-V-binding, cell volume and aggregation, and may thus counteract platelet activation and apoptosis.

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Disclosure Statement

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

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