Stimulation of Platelet Death by Vancomycin

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Key Words
Platelets • Phosphatidylserine • Caspase-3 • Apoptosis • Cell membrane scrambling • Mitochondrial potential • Ca\textsuperscript{2+}

Abstract

Background/Aims: Side effects of vancomycin, a widely used antibiotic, include thrombocytopenia. The vancomycin-induced thrombocytopenia has been attributed to immune reactions. At least in theory, thrombocytopenia could result in part from the triggering of apoptosis, which results in cell shrinkage and cell membrane scrambling with subsequent phosphatidylserine exposure at the cell surface. The cell membrane scrambling could be initiated by a signaling involving increase of cytosolic Ca\textsuperscript{2+} activity, ceramide formation, mitochondrial depolarization and/or caspase activation. Vancomycin has indeed been shown to trigger neutrophil apoptosis. An effect of vancomycin on platelet apoptosis has, however, never been tested. The present study thus explored the effect of vancomycin on platelet activation and apoptosis. Methods: Human blood platelets were exposed to vancomycin and forward scatter was utilized to estimate cell volume, annexin V-binding to quantify phosphatidylserine (PS) exposure, Fluo-3 AM fluorescence to estimate cytosolic Ca\textsuperscript{2+} activity ([Ca\textsuperscript{2+}]\textsubscript{i}), antibodies to quantify ceramide formation and immunofluorescence to quantify protein abundance of active caspase-3. Results: A 30 minutes exposure to vancomycin (≥1 µg/ml) decreased cell volume, triggered annexin V-binding, increased [Ca\textsuperscript{2+}]\textsubscript{i}, activated caspase 3, stimulated ceramide formation, triggered release of thromboxane B\textsubscript{2}, and upregulated surface expression of CD62P (P-selectin) as well as activated integrin α\textsubscript{IIb}β\textsubscript{3}. Annexin V-binding and upregulation of CD62P (P-selectin) and integrin α\textsubscript{IIb}β\textsubscript{3} was significantly blunted by removal of extracellular Ca\textsuperscript{2+}. Annexin V-binding was not significantly blunted by pan-caspase inhibitor zVAD-FMK (1 µM). In conclusion, vancomycin results in platelet activation and suicidal platelet death with increase of [Ca\textsuperscript{2+}], caspase-3 activation, cell membrane scrambling and cell shrinkage. Activation and cell membrane scrambling required the presence of Ca\textsuperscript{2+}, but not activation of caspases. Conclusion: Vancomycin exposure leads to platelet activation and apoptosis.

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Introduction

Vancomycin is widely used in the treatment of infections with Gram-positive pathogens [1]. Side effects of vancomycin include thrombocytopenia [2-4], which has been attributed to an immune reaction [5-8]. Alternatively, thrombocytopenia could result from excessive suicidal platelet death or apoptosis [9]. As a matter of fact, vancomycin has been reported to trigger apoptosis of neutrophil granulocytes [10]. To the best of our knowledge, an effect of vancomycin on platelet apoptosis has never been reported.

The present study explored the effect of vancomycin on platelets. Platelet apoptosis is apparent from cell membrane scrambling [11-13]. Mechanisms involved in the triggering of platelet apoptosis include increase of cytosolic Ca\(^{2+}\) activity [14], ceramide formation [12], and caspase-3 activation [11-13]. Platelet activation is followed by stimulation of exocytosis leading to CD62P selectin and activated integrin \(\alpha_{\text{IIb}}\beta_3\) expression at the platelet surface [15, 16]. Moreover, following platelet activation integrin \(\alpha_{\text{IIb}}\beta_3\) changes its conformation into a high-affinity state [17, 18].

Platelet activation and apoptosis are two simultaneous and inter-dependent mechanisms [19]. Platelet apoptosis may result in thrombocytopenia, platelet activation in thrombosis.

The present observations indeed revealed that exposure of platelets to vancomycin triggers cell shrinkage, cell membrane scrambling and increase of CD62P (P-selectin) as well as activated integrin \(\alpha_{\text{IIb}}\beta_3\) (CD41/61) abundance at the platelet surface. The cell membrane scrambling was paralleled by caspase-3 activation, but was not abrogated by pan-caspase inhibitor zVAD-FMK (Z-Val-Ala-Asp-fluoromethyl ketone). Vancomycin increased cytosolic Ca\(^{2+}\) activity and stimulated ceramide formation. Removal of Ca\(^{2+}\) virtually abolished vancomycin induced cell membrane scrambling and CD62P (P-selectin) as well as activated integrin \(\alpha_{\text{IIb}}\beta_3\) abundance.

Materials and Methods

Isolation and stimulation of human platelets

Fresh ACD-anticoagulated blood was obtained from healthy volunteers between the age of 22 to 50 years with informed consent according to the Ethics Committee of the Eberhard Karl University Tübingen, Germany (184/2003V). The blood was centrifuged at 200 g for 20 minutes at 25°C. The platelet rich plasma was separated, Tyrode buffer added (137 mM NaCl, 2.8 mM KCl, 12 mM NaHCO\(_3\), 5 mM glucose, 0.4 mM Na\(_2\)HPO\(_4\), 10 mM HEPES, 0.1% BSA, pH 6.5) in a 1:6 volumetric ratio and centrifuged at 900 g and 25°C for 10 minutes. The platelet pellet was resuspended in 250 µl of Tyrode buffer (pH 7.0). Care was taken not to expose platelets to excess mechanical stress or oxygen.

10\(^6\) platelets were stimulated in a total volume of 1ml Tyrode buffer (pH 7.4 with 2mM CaCl\(_2\)). Where indicated, vancomycin (synthesized by the Department of Microbiology and Biotechnology, Eberhard Karl University Tübingen) was added at the indicated concentrations (1, 5, 10 and 15 µg/ml) for 30 minutes at 37°C. A negative control without vancomycin and a positive control with ionomycin (1 µM) was analyzed simultaneously with each set of experiment. Since ionomycin was dissolved in DMSO, a solvent control was measured in addition.

LDH release

To determine the vancomycin-induced LDH release platelets were centrifuged and LDH concentration in the supernatant determined by ELISA (Promega, CA, USA).

Phosphatidylserine (PS) exposure

Phosphatidylserine exposure was measured following stimulation with vancomycin, centrifuging the cells at 1000 g for 2 minutes followed by washing once in Tyrode buffer (pH 7.4) with 2 mM CaCl\(_2\). Annexin V-Fluos (1:20 dilution, Immunotools, Germany) staining in the same buffer and incubating at 37°C for 30 minutes. The fluorescence was measured in FL-1 of a BD FacsCalibur (BD Biosciences, CA, USA).
Calcium measurements

Intracellular Ca\(^{2+}\) concentration was measured following stimulation with vancomycin as indicated, washing once in Tyrode buffer (pH 7.4) with 2 mM CaCl\(_2\) and staining with 5 µM Fluo-3AM (Biotium, USA) in the same buffer and incubating at 37°C for 30 minutes. The fluorescence was measured in FL-1 of a BD FacsCalibur (BD Biosciences, CA, USA).

Mitochondrial membrane potential

Platelets were first stimulated with vancomycin as described before and 10\(^7\) platelets were suspended in phosphate buffered saline (PBS) (Invitrogen, CA, USA) supplemented with 1 mM MgCl\(_2\), 5.6 mM glucose, 0.1% BSA and 0.1 mM HEPES (pH 7.4) in a total volume of 1 ml and stained with 10 nM DiOC\(_6\) (Invitrogen, CA, USA) for 10 minutes. The stained cells were centrifuged at 1000 g for 5 minutes at 20°C, resuspended in PBS and measured in FL-1 [11].

Caspase-3 activity

Active caspase-3 produced in the cells was measured by CaspGlow Fluorescein Active Caspase-3 Staining kit from BioVision (CA, USA) as per the manufacturer’s instruction. Fluorescence was measured in FL-1 in BD FACS Calibur (BD Biosciences, CA, USA). Two sets of experiments were done to determine caspase-3 activity. One set contained Tyrode buffer (pH 7.4) with 2 mM CaCl\(_2\), the second set contained Tyrode buffer (pH 7.4) with 0.5 mM EGTA. In each set of experiments 10\(^6\) platelets/ml Tyrode buffer (pH 7.4 with 2 mM CaCl\(_2\)) were stimulated for 30 minutes at 37°C with vancomycin (1-15 µg/ml), washed once in Tyrode buffer (pH 7.4) and stained with CaspGlow Fluorescein Active Caspase-3 according to manufacturer’s instructions.

Immunofluorescence

Fresh isolated platelets were adhered to a fibrinogen surface (20 µg/ml) on chamber slides, stimulated as described before and fixed with 2 % paraformaldehyde for 2 hours at 37°C. Then paraformaldehyde was removed and the platelets were washed with PBS by centrifuging at 1000 g for 2 minutes at 25°C and blocked with 2 % bovine serum albumin for 30 minutes, followed by a triton X-100 (0.1% in PBS) treatment for permeabilization. Then the platelet-coated slides were stained with Annexin V-Fluos (1:100 dilution, Roche, Mannheim, Germany) or 1:50 dilution of caspase-3 rabbit monoclonal antibody (Cell Signaling Technology) by incubating for 2 hours at room temperature. Chamber slides were washed and incubated with secondary antibody labeled with FITC (Santa Cruz, USA) in the case of caspase-3 staining. The actin cytoskeleton was stained with rhodamine-phalloidin (Invitrogen). Confocal microscopy was performed using a Zeiss LSM5 EXCITER Confocal Laser Scanning Microscope (Carl Zeiss Micro Imaging, Jena, Germany) with a A-Plan 63x ocular. Colocalization of annexin V with actin cytoskeleton was analysed in order to define the location of annexin V binding on the cell.

Ceramide formation

For detection of ceramide formation, 10\(^8\) platelets/ml were stimulated for 60 minutes as described before and centrifuged at 2000 g for 2 minutes and the pellet was incubated with 50 µl of mice antibody to human ceramide (1:5 dilution, Alexis, USA) in PBS with 1% BSA for 1 hour at 37°C and 5% CO\(_2\). Then primary stained cells were centrifuged at 1000 g for 2 minutes and the pellet was stained with 50 µl of secondary goat anti-mouse IgG (1:50 dilution, BD Pharmingen, Hamburg, Germany) for 20 minutes. The reaction was stopped with 200 µl PBS and measured immediately in FACS analysis. The mean fluorescence of the FITC-labeled secondary Ig was measured.

Expression of activated integrin α\(_{\text{IIb}}\)β\(_3\) and CD62P (P-selectin)

10\(^6\) platelets/ml were suspended in Tyrode buffer (pH 7.4) with 2 mM CaCl\(_2\), or in Tyrode buffer (pH 7.4) with 0.5 mM EGTA and stimulated with vancomycin or 1 µM ionomycin for 30 minutes at 37°C, stained with 1:10 dilution of PAC-1 FITC antibody targeted against the active conformation of integrin α\(_{\text{IIb}}\)β\(_3\) and anti-CD62P FITC (both from BD Pharmingen, USA) for 30 min at 37°C, washed once and analyzed in FACS.
Detection of Thromboxane B2 (TxB2) with ELISA

The vancomycin-treated platelets were centrifuged at 1000 g for 2 minutes at 25°C and 1000 μl of the supernatant was mixed with 200 μl methanol (Merck, Germany) and vortexed briefly. 50 μl of the sample was added to each of the wells of the coated (Thromboxane B2) ELISA plates (Oxford Biosciences, UK) and incubated at room temperature with shaking for 2 hours. The plate was washed three times with 300 μl washing buffer (1:10 dilution in PBS) (Thromboxane B2 ELISA kit, Oxford Biosciences) and incubated 1 hour with 50 μl HRP-conjugated TxB2 antibody. After washing again three times with 1:10 dilution of the washing buffer, incubation for 30 minutes with 150 μl of TMB substrate (Thromboxane B2 ELISA kit, Oxford Biosciences) and blocking with 1N HCl, measurements were done immediately at 450 nm in a Biotek Power Wave XS2 ELISA reader against a standard of 0, 0.1, 0.2, 0.4, 1, 2, 4 and 10 ng/ml thromboxane B2 (thromboxane B2 ELISA kit, Oxford Biosciences). The amount of TxB2 formed in the samples was determined from a curve of concentration vs actual absorbance with Microsoft Excel 2007. The experiment was repeated with Tyrode buffer (pH 7.4) containing 0.5 mM EGTA to determine the role of extracellular calcium on thromboxane metabolism.

Effect of caspase on phosphatidylserine exposure

10^6 platelets were suspended in a total volume of 1 ml Tyrode buffer (pH 7.4) in either the presence of 2 mM extracellular CaCl2 or in the presence of extracellular calcium chelator 0.5 mM EGTA (Carl Roth, Germany). Platelets were incubated for 30 minutes at 37°C, centrifuged and washed with Tyrode buffer (pH 7.4) at 1000 g, 25°C for 2 minutes and resuspended in 1 ml of the same buffer, stimulated with 10 and 15 µg/ml concentration of vancomycin at 37°C for 30 minutes, washed once in Tyrode buffer (pH 7.4) and stained with Annexin V-Fluos (1:20 dilution, Immunotools, Germany) in Tyrode buffer (pH 7.4) 2 mM CaCl2 for 30 minutes at 37°C. Immediate measurement of fluorescence was done in FL-1 with FACS Calibur. To define the role of caspase on PS exposure, 10^6 platelets/ml were pre-treated with zVAD-FMK (1 µM) for 10 minutes at 37°C in Tyrode buffer (pH 7.4) 2 mM CaCl2, washed once, stimulated with 10 and 15 µg/ml concentration of vancomycin at 37°C for 30 minutes and stained with Annexin V-FITC and active caspase-3 FITC antibody as mentioned before.

Statistical analysis

Data are provided as arithmetic means ± SEM, statistical analysis was made by one-way ANOVA or Student’s t-test, where applicable. Comparisons were made in absence or presence of vancomycin comparing blood from the same donors. Thus, where possible, paired t-test was performed.

Results

In order to determine the effect of vancomycin on platelet apoptosis, cell membrane phospholipid scrambling with subsequent exposure of phosphatidylserine at the cell surface was estimated from binding of fluorescent annexin V-Fluos. As revealed by confocal microscopy, a 30 minutes exposure to vancomycin resulted in annexin V-Fluos binding in human platelets (Fig. 1A), an effect reaching statistical significance at >1 µg/ml vancomycin when estimated with FACS analysis (Fig. 1B). The cell membrane scrambling was associated with decrease in platelet volumes, estimated from forward scatter in FACS (Fig 1C). In order to determine cell membrane integrity of the platelet cell membrane, lactate dehydrogenase (LDH) release was determined. As a result, the LDH concentration in the supernatant was similar following treatment with 10 µg vancomycin/ml (0.33 ± 0.05 nM , n = 3) as without treatment (0.26 ± 0.03 nM, n = 3).

In search for the mechanism triggering cell membrane scrambling, the effect of vancomycin on cytosolic Ca2+ was determined utilizing Fluo-3AM fluorescence as an indicator of cytosolic Ca2+ activity. As illustrated in Fig. 2A and B, a 30 minutes exposure of human platelets to vancomycin tended to increase intracellular Ca2+ in platelets, an effect reaching statistical significance at 1 µg/ml. Vancomycin-treated platelets also undergo mitochondrial depolarization (Fig. 2C). Vancomycin treatment was followed by ceramide
formation, an effect reaching statistical significance at 10 µg vancomycin/ml as revealed with FACS analysis (Fig. 2D).

In additional experiments, caspase-3 activity was estimated utilizing FACS and immunofluorescence. As illustrated in Fig. 3A, a 30 minutes exposure to vancomycin was followed by an increase of caspase 3 activity, an effect reaching statistical significance at ≥1 µg/ml vancomycin (Fig 3B).

In order to test, whether the effect of vancomycin on cell membrane scrambling depended on caspase-3 activity, platelets were exposed to vancomycin in the presence and absence of the pan-caspase inhibitor zVAD-FMK (1 µM). As shown in Fig. 4A, zVAD-FMK did not significantly blunt the increase of annexin V-binding following vancomycin exposure. As shown in Fig. 4B, 1µM zVAD-FMK did significantly decrease caspase-3 activity following vancomycin treatment. Accordingly, the stimulation of PS exposure in platelets upon vancomycin treatment does not require caspase-3 activation.
In order to explore the role of Ca\(^{2+}\) in the effect of vancomycin on cell membrane scrambling and caspase-3 activation, platelets were exposed to vancomycin in the presence and absence (0.5 mM EGTA) of extracellular CaCl\(_2\). As illustrated in Fig. 5A and B, the removal of Ca\(^{2+}\) (and addition of 0.5 mM EGTA) virtually abolished the effect of vancomycin (10 µg/ml) on phosphatidylserine exposure and caspase-3 activation.

In an additional series, experiments were performed to test, whether thromboxane B\(_2\) was released and CD62P (P-selectin) and activated integrin \(\alpha\)IIb\(\beta\)3 were expressed on the platelet membrane surface. As shown in Fig. 6A, 10 µg vancomycin/ml triggered the release of thromboxane B\(_2\), an effect not requiring the presence of extracellular Ca\(^{2+}\). As shown in Fig. 6B and C, vancomycin (1 µg/ml) treatment was followed by significant upregulation of CD62P (P-selectin) and activated integrin \(\alpha\)IIb\(\beta\)3 activation at the platelet surface, an effect dependent on extracellular Ca\(^{2+}\). Vancomycin thus triggers apoptosis-like events paralleled by activation of platelets. Both apoptosis and activation were dependent on extracellular Ca\(^{2+}\).
Discussion

The present observations reveal a novel effect of vancomycin, i.e. the stimulation of platelet activation and apoptosis. The concentration required for this effect is achieved during antibiotic treatment [20-28]. Vancomycin treatment may be required from 10 up to 28 days [29]. Vancomycin has previously been shown to inhibit platelet aggregation [30-34].
To the best of our knowledge, an effect of vancomycin on platelet activation and apoptosis or thrombosis has never been reported. Vancomycin did not significantly increase lactate dehydrogenase (LDH) release, indicating that vancomycin did not affect cell membrane integrity.

The vancomycin-induced apoptosis is paralleled by depolarization of the mitochondria, activation of caspase-3 and scrambling of the cell membrane. The vancomycin induced platelet apoptosis and activation are virtually abolished in the absence of extracellular Ca\(^{2+}\). Thus, the presence of Ca\(^{2+}\) is apparently a prerequisite for the stimulation of cell membrane scrambling. Further experiments were performed to elucidate whether vancomycin triggers the formation of ceramide, which has previously been shown to sensitize erythrocytes to the scrambling effect of cytosolic Ca\(^{2+}\) [35, 36]. As a matter of fact, the exposure of platelets to vancomycin was indeed followed by significant ceramide formation. Stimulation of platelets leads to release of Ca\(^{2+}\) from intracellular stores and subsequent activation of calpain [37] which in turn modifies several substrates in platelets and triggers platelet activation, migration, apoptosis and adhesion [38].

Ca\(^{2+}\) influx into platelets is regulated by SGK-1 and NF-κB [39]. Removal of extracellular Ca\(^{2+}\) blunted the upregulation of CD62P (P-selectin) and activated integrin αIIbβ3, an effect possibly again involving calpain. Ca\(^{2+}\)-independent thromboxane B\(_2\) release may be due to Ca\(^{2+}\)-independent phospholipase A\(_2\) accomplishing Ca\(^{2+}\)-independent arachidonic acid release from platelets [40, 41]. Thromboxane B\(_2\) is a stable hydrolysis product of the bioactive but unstable thromboxane A\(_2\). Thromboxane is involved in vascular permeability and inflammation [42].

Somewhat surprisingly, the vancomycin-induced platelet apoptosis was not abrogated by the pan-caspase inhibitor zVAD-FMK. Apparently, Ca\(^{2+}\) and ceramide trigger cell membrane scrambling at least in part by mechanisms other than caspase-3 activation. According to earlier studies [43], caspase-independent but Ca\(^{2+}\)-dependent phosphatidylserine exposure in platelets is more typical of agonist-mediated activation-associated events resembling apoptosis [44]. Use of ionomycin as a positive control shows that the effect of vancomycin on platelets is similar to that of a calcium ionophore. Other than activation of platelets by
Fig. 6. Activation of platelet function by vancomycin. A. Arithmetic mean ± SEM (n = 3-4) of thromboxane B<sub>2</sub> secretion from platelets after 30 minutes of vancomycin treatment compared to negative control (white bar) and positive control (ionomycin 1 μM). ** (p<0.01) and *** (p<0.001) indicate statistically significant difference from negative control by one-way ANOVA and ### (p<0.001) indicates statistically significant difference from respective value in the presence of calcium (Student’s paired t-test). Ionomycin (1 μM) was used as a positive control. B. Arithmetic mean ± SEM (n = 3-4) of % CD62P (P selectin) positive platelets after 30 minutes of vancomycin treatment compared to negative control (white bar) and positive control (ionomycin 1 μM). ** (p<0.01) and *** (p<0.001) indicate statistically significant difference from negative control by one-way ANOVA and ## (p<0.01) and ### (p<0.001) indicate statistically significant difference to value in the presence of calcium (Student’s paired t-test). Ionomycin (1 μM) was used as a positive control. C. Arithmetic mean ± SEM (n = 3-4) of % integrin α<sub>IIb</sub>β<sub>3</sub> positive platelets after 30 minutes of vancomycin treatment compared to negative control (white bar) and positive control (ionomycin 1 μM). ** (p<0.01) and *** (p<0.001) indicate statistically significant difference from negative control by one-way ANOVA, ## (p<0.01) and ### (p<0.001) indicate statistically significant difference from respective value in the presence of calcium (Student’s paired t-test). Ionomycin (1 μM) was used as a positive control.

The agonists thrombin or collagen-related peptide (CRP) the stimulation of platelets by the Ca<sup>2+</sup>-ionophore ionomycin is limited to Ca<sup>2+</sup> entry and subsequent triggering of Ca<sup>2+</sup>-sensitive platelet functions [37].

Phosphatidylserine exposing apoptotic platelets are expected to be engulfed by phagocytosing cells and thus to disappear from circulating blood. Excessive apoptosis is thus expected to result in thrombocytopenia. The thrombocytopenia following treatment with vancomycin may, however, largely result from immune reactions [2-4]. On the other hand, vancomycin causes thrombocytopenia even in the absence of drug-dependent antibodies [45]. Thus, at least in theory, the influence of vancomycin on platelet number may in part reflect a direct effect of vancomycin on platelets.
In conclusion, vancomycin treatment of human blood platelets triggered ceramide formation, caspase-3 activation, cell shrinkage and cell membrane scrambling. The cell membrane scrambling required the presence of Ca^{2+}. Vancomycin-induced cell membrane scrambling presumably accelerates the clearance of platelets from circulating blood.

Acknowledgements

The authors acknowledge the meticulous preparation of the manuscript by Lejla Subasic. This study was supported by the Deutsche Forschungsgemeinschaft (KFO 274 and SFB766).

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