Cyclosporine enhances platelet procoagulant activity

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Abstract

Background. Clinical use of cyclosporine (CsA) was suggested to be associated with an increased risk of thromboembolic complications. The molecular mechanisms underlying these effects remain unresolved.

Methods. We tested the hypothesis that CsA may produce platelet procoagulant activity due to its interaction with the platelet plasma membrane. To verify this hypothesis the possible relationship between platelet morphology, exposure to platelet phosphatidylserine (PS) and platelet procoagulant activity (measured as phospholipid-dependent thrombin generation) was studied.

Results. It was found that CsA (1–100 μg/ml) potentiates collagen-evoked platelet procoagulant response. Platelets treated in vitro with CsA (20–200 μg/ml 20–60 min) expressed procoagulant activity. The CsA-induced platelet procoagulant response was both dose- and time-related and weaker than that produced by collagen. Flow cytometry studies revealed that CsA treatment results in a left shift (decrease) in the forward and side scatter of the entire platelet population. The shift was unimodal, dose-dependent and less pronounced than that elicited by collagen. Using flow cytometry and fluorescein isothiocyanate-labelled annexin V as a probe for PS, we demonstrated an increased binding of this marker to a CsA-treated platelet population. CsA-evoked PS-expression was dose- and time-dependent and smaller than that produced by collagen. CsA, at concentrations similar to those affecting platelet procoagulant response, released lactate dehydrogenase from platelets.

Conclusions. These observations indicate that the thrombogenic properties of CsA may result from the alteration of lipid organization in platelet plasma membrane, leading to externalization of PS and accelerated thrombin generation.

Keywords: cyclosporine; phosphatidylserine; plasma membrane; platelets procoagulant activity

Introduction

Cyclosporine A (CsA) is an immunosuppressive drug used to prevent the rejection of a transplanted organ and to treat diseases of autoimmune origin [1]. However, its clinical use was suggested to be associated with adverse effects, including accelerated atherosclerosis [2], renal arterial and venous thrombosis [3], deep venous thromboses [4] and systemic hypertension [5]. CsA was also reported to affect intravascular haemostatic equilibrium, favouring a prothrombotic state by decreasing the release of prostacyclin [6] and nitric oxide [7] from the endothelium, while increasing thromboxane A₂ synthesis by platelets [8].

The majority of the above-mentioned clinical disorders are likely to be related to activation of platelets and/or to the enhanced generation of thrombin. In fact, it has been found that CsA increases spontaneous activation of platelets [9], enhances platelet aggregation and secretion in response to physiological stimulators [10] and causes increased expression of fibrinogen receptors on their surface [11]. Whether CsA also affects platelet procoagulant response, which is critical for the acceleration of thrombin generation and thrombus formation, is not known.

The rate of thrombin generation in plasma is dramatically increased by the catalytic surfaces provided by regions of the platelet plasma membrane rich in aminophospholipids (phosphatidylserine, PS; phosphatidylethanolamine, PE) [12]. The prothrombinase complex formed on the PS-rich surface is able to synthesize thrombin from prothrombin 300 000 times faster than in the fluid phase of plasma [13]. Normally, (in unstimulated platelets) the aminophospholipid-rich surfaces are not available to the plasma clotting factors since the aminophospholipids are localized in the inner leaflet of an asymmetric plasma membrane [14].
Procoagulant catalytic surfaces, therefore, appear only after deep reorganization of the platelet plasma membrane, which is manifested by the loss of its asymmetry.

CsA is likely to affect both the integrity of the platelet plasma membrane and the loss of its asymmetry. The rationale for this assumption is its structure. This fungal polypeptide is composed of an 11-residue cyclic peptide containing two uncommon amino acids, seven peptid bond N-methylated residues and only one OH group. The CsA molecule has a beta-pleated structure and a loop [15]. Such conformation and composition make the CsA molecule rigid and highly hydrophobic. Experiments performed on model lipid membranes have shown molecule rigid and highly hydrophobic. Experiments performed on model lipid membranes have shown that CsA is located in the membrane interior, where it interacts with the membrane phospholipids and perturbs the bilayer [15,16]. Consequently, this study was performed to test the hypothesis that CsA may produce platelet procoagulant activity due to its interaction with the plasma membrane. The obtained results indicate that CsA may non-specifically alter physicochemical properties of the platelet plasma membrane, which is manifested by the externalization of PS and the acceleration of thrombin formation.

Materials and methods

Chemicals

CsA (Sandimmune) was obtained from Novartis (Basel, Switzerland); collagen (fibrillar, from equine tendon) was from Hormon Chemie, (Munich, Germany). Fluorescein isothiocyanate-labelled annexin V (annexin-FITC), phycoerythrin (PE)-labelled anti-GPIIb/IIIa MoAb (PE-CD41a) and PE-labelled isotypic mouse MoAb were from BD Biosciences-PharMingen. CsA formulation in polyoxyethylated castor oil + ethanol was dissolved in with 0.9% sodium chloride. In the control experiments, equivalent amounts of diluted vehiculum, i.e. castor oil/ethanol solution in 0.9% sodium chloride were used.

Blood collection and platelet preparation

Venous blood was collected with minimum trauma and stasis via a 21-gauge needle (0.8 x 40 mm) into 10 ml polypropylene tubes containing 1 ml of 130 mM trisodium citrate. Platelet-rich plasma (PRP) and washed platelets were prepared as described previously [17].

Measurement of platelet procoagulant activity

The assay system used was similar to that described by Rota et al. [18]. It is based on Russell’s viper venom, which induces thrombin generation by activation of factors V and X, and in the presence of Ca2+ ions, is dependent on the availability of PS. The preparation of defibrinated plasma, activation procedure and assay of phospholipid-dependent thrombin generation was performed as described previously [19]. The amidolytic activity of thrombin was expressed in nanomoles of p-nitroaniline liberated for 1 min and was calculated for 1 ml of platelet suspension.

Platelet activation and flow cytometry

For PS exposure and morphology experiments, 300 μl samples of PRP were placed in a cuvette of an aggregometer and incubated at 37°C for 2 min without stirring. CsA or collagen was added and after an initial mixing (30 s) incubation was continued without stirring at 37°C for 10–60 min. To stop the incubation 60 μl aliquots of incubation mixture were transferred to polystyrene tubes (12 x 74 mm) containing 240 μl Tyrode–Hepes buffer supplemented with bovine serum albumin (BSA; 3.5 mg/ml), CaCl2 (3 mM) and hirudin (2 ATU/ml). Thirty microlitre samples of the diluted suspension were combined with 20 μl annexin-FITC (a marker of PS expression on the surface of platelets) and 10 μl PE-CD41a (a marker of platelets) and incubated for 20 min in the dark. To stop the incubation, the samples were diluted with 1 ml of Tyrode–Hepes buffer supplemented with BSA (3.5 mg/ml) and 2 mM CaCl2. Tyrode–Hepes buffers used in this study were prepared ‘particle-free’. Preparation involved filtration through a 0.2 μm filter (Milipore). Flow cytometry analysis was performed within 45 min after final dilution.

Flow cytometry analysis

Flow cytometry analysis was performed using Coulter EPICS XL flow cytometer. Ten-thousand events were acquired for each sample and analysed for forward light scatter (forward scatter), right angle light scatter (side scatter) and for two colour fluorescent signals. The light scatter and the fluorescence signals were set in a logarithmic gain and were stored in list mode data files. The obtained data were further analysed using Win MDI software program. The events were counted after triggering PE fluorescence of platelet marker CD41a at a preset threshold. The threshold was set above the background fluorescence with PE-labelled isotypic mouse MoAb. The CD41a-positive particle populations were separated by bitmaps (dotplots), where log forward scatter is the x-axis and log side scatter is the y-axis.

Assay of platelet integrity

The extent of platelet lysis following incubation with the studied compounds was estimated in a suspension of washed platelets by measuring the activity of lactate dehydrogenase (LDH) lost from the cells into the suspending fluid. The activity of LDH was measured according to Bergmeyer [20].

Statistical analysis

Data are means ± SD of a minimum of five independent experiments. Differences between means were evaluated by the paired Student’s t-test, with P < 0.05 being taken as the level of significance.

Results

Experiments presented in Table 1 (panel A) were performed to establish whether CsA may potentiate
platelet procoagulant response produced by low collagen concentrations. Collagen is the strongest physiological inducer of platelet procoagulant response [12,14]. Under normal physiological conditions, circulating platelets cannot be stimulated by collagen. However, they do come in contact with collagen fibrils following endothelial damage produced by pathological processes, or extensive surgical procedures (e.g. transplantation). As is seen, a 10 min treatment of platelets with 1–100 μg/ml CsA augmented the procoagulant response induced by subthreshold (5 μg/ml) concentrations of collagen. The degree of this potentiation was dose-relevant. Distinct potentiation (25.2 ± 2.2 vs 16.2 ± 1.5, \( P = 0.05 \)) was observed in the presence of CsA at a concentration of 10 μg/ml, whereas higher (100 μg/ml) concentrations of CsA produced more than a 3-fold increase (55.8 ± 51 vs 16.2 ± 1.5, \( P = 0.05 \)) in platelet procoagulant activity.

As seen from Table 1 (panel B and C), CsA per se induced, in a dose- and time-dependent manner, a procoagulant response in platelets. Compared with control, a 60-min incubation of platelets with CsA (1–200 μg/ml) produced a 1.7–13.2-fold rise in procoagulant activity. This is a relatively weak procoagulant response, since platelets treated for 10 min with collagen (15 μg/ml) express a 22.5-fold rise in procoagulant activity.

Figure 1 shows the results of flow cytometry studies performed to determine how the long (60 min) treatment of platelets with increasing concentrations (50–200 μg/ml) of CsA affects their morphology (i.e. size and granularity). To analyse the changes in platelet morphology, the bivariate scatterplot (forward scatter vs side scatter dot plot) was arbitrarily split into four regions (R1–R4). The splitting of the scatterplot, defining normal untreated platelets, was performed in such a way that one of the regions (here R2) shows the majority (i.e. at least 90%) of the acquired events. The percentage of events of the 10 000 total found (the entire analysed platelet population) is shown in each region. Assuming that forward scatter and side scatter are the criteria of platelet size and granularity, respectively, in the population of normal (untreated) platelets, R1 comprises the subpopulation of small platelets, R2 comprises the platelets with mean and large volume, R3 comprises smaller degranulated platelets and/or cells with changed surface and R4 defines degranulated, mean-volume and large platelets.

As seen from Figure 1, incubation of platelets with 50–200 μg/ml CsA (panels B, C and D) produces a distinctly broader light scatter profile than in control (panel A). This effect was dose-dependent and in the presence of 200 μg/ml of CsA was manifested by a marked increase in the percentage of counts in R1 (by 17%), R3 (by 9.5%) and R4 (by 5%), and a simultaneous drop (by 31.5%) in the percentage of events in R2. Of importance are the not numerous larger objects seen in the upper right corner of R2, which may reflect the presence of a subpopulation of swollen cells in the population of CsA-treated platelets.

Analysis of the forward scatter vs side scatter dot plot of platelets pre-incubated with CsA reveals a unimodal decrease in both forward and side light scatters. Both the left-shifted and down-shifted new events on flow cytometry light scatter plots appear contiguous with the unchanged subfraction of the tested platelet population. In agreement with the considerations of Bode and Hickerson [21], we postulate that the unimodal-shifted population of events appearing in regions R1 and R3 are degranulated platelets with profound changes on their surface.

Next, we have studied how the incubation of platelets with increasing concentrations of CsA affects the PS expression on their surface. The amount of PS expressed on a cell surface is a major determinant of the ability of platelets to support the assembly of a procoagulant complex and augment thrombin generation [12,13]. Therefore, platelet PS expression was directly examined using flow cytometry.
fluorescein-conjugated annexin V. Annexin V binds to aminophospholipids in the presence of calcium with high affinity and strict specificity. As seen from Table 2, CsA induces a rise in the percentage of platelets with PS-exposed on their surface. This effect was dose- and time-dependent. One hour incubation of platelets with 1–200 μM CsA produces a rise in the percentage of PS-expressing cells from 2.3 ± 0.2 to 3.6 ± 0.3 and 30.1 ± 3, respectively. CsA is relatively weak inducer of PS-expression since following 10-min incubation of platelets with collagen, 61% of annexin V-positive cells were found.

Figure 2 shows the result of an experiment which was performed to estimate how collagen affects platelet morphology. As can be seen, a 10-min incubation of platelets with 15 μg/ml collagen produces significant changes in platelet size and granularity. This was manifested by an increase in the events found in region R1 (by 8.5%), R3 (by 43%) and R4 (by 25%) as well as a simultaneous decrease in their number in region R2 (by 76.5%).

Table 3 illustrates the results of experiments which were conducted to determine how CsA affects platelet integrity. The potential disrupting effect to the platelet plasma membrane was measured by the LDH release test. Notably, a 20-min incubation of platelets with increasing concentrations (1–200 μg/ml) of CsA resulted in a dose-dependent LDH release. A statistically significant rise in LDH release was observed following incubation of platelets with CsA added to the final concentration of 30 μg/ml.

Discussion

The molecular mechanism of the immunosuppressive action of CsA is known to be associated with its interaction with lymphocytes T in which cyclosporine specifically binds cyclophilin and inhibits its activity [22,23]. The resulting CsA-cyclophilin complex binds to and inhibits the protein phosphatase, calcineurin [22,23]. Inhibition of calcineurin results in the disruption of T-cell activity, leading to the suppression of the immune response.

![Fig. 1](image-url)
in decreased synthesis of interleukin-2 (IL-2), a major cytokine involved in the activation of lymphocytes.

Much less is known, however, about the mechanisms of the cytotoxic effect(s) of CsA in other cells. Of special importance is the toxicity toward endothelial cells and platelets which seems to be the main feature of CsA-induced endothelial injury in the arterial vasculature and the potential cause of thrombotic events [24,25]. Studies on cultured human umbilical artery endothelial cells (HUVEC) have revealed that treatment of these cells with therapeutical concentrations (0.5–10 μg/ml) of the drug results in cell death, which bears the morphological and biochemical features of primary necrosis [26]. It has also been reported that both therapeutical and pharmacological concentrations (200 μg/ml) of CsA trigger oxidative stress in cultured HUVEC [26]. Oxidative stress, especially that evoked by higher (200 μg/ml) CsA concentrations, has been proposed to cause apoptosis of endothelial cells [26]. Both apoptotic and necrotic cell death is usually associated with profound changes in the plasma membrane, including the loss of its asymmetry, externalization of PS and the shedding of microvesicles (membrane blebbing) [14,27]. It should be stressed that the loss of plasma membrane asymmetry, PS externalization and membrane blebbing has been observed also in platelets stimulated by strong physiological agonists like thrombin and/or collagen and is commonly defined as a

Table 2. Effect of cyclosporine A on the appearance of PS on platelet surface

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Additions</th>
<th>Time of incubation (min)</th>
<th>Annexin V-positive platelets (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>None</td>
<td>60</td>
<td>2.3 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>Vehiculum</td>
<td>60</td>
<td>3.0 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>CsA 1 μg/ml</td>
<td>60</td>
<td>3.6 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>CsA 10 μg/ml</td>
<td>60</td>
<td>5.0 ± 0.4*</td>
</tr>
<tr>
<td></td>
<td>CsA 30 μg/ml</td>
<td>60</td>
<td>8.0 ± 0.7**</td>
</tr>
<tr>
<td></td>
<td>CsA 50 μg/ml</td>
<td>60</td>
<td>11.0 ± 1.0***</td>
</tr>
<tr>
<td></td>
<td>CsA 100 μg/ml</td>
<td>60</td>
<td>20.0 ± 2.0***</td>
</tr>
<tr>
<td></td>
<td>CsA 200 μg/ml</td>
<td>60</td>
<td>30.1 ± 3.0***</td>
</tr>
<tr>
<td></td>
<td>Collagen 15 μg/ml</td>
<td>10</td>
<td>61.2 ± 6.0***</td>
</tr>
<tr>
<td>B</td>
<td>None</td>
<td>60</td>
<td>2.1 ± 0.2</td>
</tr>
<tr>
<td></td>
<td>CsA 200 μg/ml</td>
<td>20</td>
<td>9.5 ± 0.8**</td>
</tr>
<tr>
<td></td>
<td>CsA 200 μg/ml</td>
<td>40</td>
<td>21.0 ± 2.0***</td>
</tr>
<tr>
<td></td>
<td>CsA 200 μg/ml</td>
<td>60</td>
<td>31.2 ± 3.0***</td>
</tr>
</tbody>
</table>

Platelets (PRP) were pre-incubated at 37°C with no stimulator (none) or with cyclosporine A or collagen added to the final concentration as shown. At the indicated time intervals samples of incubating mixture were taken for the measurement of PS expression by means of flow cytometry. The data represent mean percentages of PS-positive platelets (expressed as a percentage of annexin V-positive cells) and ± SD of six experiments each performed on separate platelet preparation (n = 15). *P < 0.05, **P < 0.01, ***P < 0.001.

Table 3. Cyclosporine A-evoked lactate dehydrogenase release

<table>
<thead>
<tr>
<th>Additions</th>
<th>LDH release (% of total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.6 ± 0.2</td>
</tr>
<tr>
<td>Vehiculum</td>
<td>2.7 ± 0.3</td>
</tr>
<tr>
<td>CsA 1 μg/ml</td>
<td>3.0 ± 0.3</td>
</tr>
<tr>
<td>CsA 10 μg/ml</td>
<td>3.4 ± 0.3</td>
</tr>
<tr>
<td>CsA 30 μg/ml</td>
<td>3.8 ± 0.3*</td>
</tr>
<tr>
<td>CsA 50 μg/ml</td>
<td>6.0 ± 0.5**</td>
</tr>
<tr>
<td>CsA 100 μg/ml</td>
<td>8.3 ± 0.8***</td>
</tr>
<tr>
<td>CsA 200 μg/ml</td>
<td>10.4 ± 0.8***</td>
</tr>
<tr>
<td>Collagen 15 μg/ml</td>
<td>3.5 ± 0.3</td>
</tr>
</tbody>
</table>

Samples (0.5 ml) of washed platelets were incubated at 37°C for 20 min without (control) and with the compounds tested added to a final concentration as indicated. Incubation was stopped by centrifugation at 11,000 g for 3 min. LDH activity was determined in the supernatants. To estimate total LDH activity, platelets were lysed with Triton X-100 added to a final concentration of 10% (v/w). The data represent mean values and SD, of four experiments, each performed on a separate platelet preparation (n = 12). *P < 0.05, **P < 0.01, ***P < 0.001.

Fig. 2. The effect of collagen on the platelet morphology. Platelets (PRP) were pre-incubated at 37°C for 10 min with saline (panel A) or with collagen added to the final concentration of 15 μg/ml (panel B). The percentage of events of the 10,000 total found in each region is shown in the inset. Each dot plot is representative of 12 determinations performed on four different preparations. Collagen produced a unimodal left/downward shift in the forward scatter and side scatter of the entire platelet population.
platelet–procoagulant response [13,14]. In this case, in contrast to cells undergoing apoptosis, reorganization of the plasma membrane is relatively rapid (minutes), and PS expression serves to create a catalytic surface required for assembly of the coagulation factors and formation of thrombin [13,14].

In this work, we show for the first time, that CsA may produce profound changes also in the platelet plasma membrane. This is based on the observations that CsA-treated platelets release LDH, express PS on their surface, and accelerate thrombin generation in the presence of factor Xa, Va, prothrombin and Ca²⁺. In addition, CsA potentiates a collagen-evoked, platelet–procoagulant response and initiates morphological changes in the plasma membrane similar to those produced by collagen. Alterations in the morphology of the platelet plasma membrane visible in flow cytometry as changes in a forward scatter signal were postulated to reflect filopod formation [21]. Such morphological changes are likely to be associated with the dramatic increase in the membrane curvature, a phenomenon which has recently been proposed to accelerate PS-related binding of activated factors V and VIII to the platelet surface and thus to promote a platelet–procoagulant response [28].

To sum up, these observations indicate that at least in vitro CsA behaves as a weak inducer of a platelet–procoagulant response and that the thrombogenic properties of CsA may result from the alteration of lipid organization in the platelet plasma membrane, leading to externalization of PS, changes in membrane curvature, and in consequence, in accelerated thrombin generation.

CsA-induced platelet–procoagulant response cannot be produced by a rise in the intracellular Ca²⁺ concentration since the drug per se is not able to induce aggregation—a response which is known to be triggered even by relatively low (nanomolar) and transient (<1 min) calcium signals [29]. Platelet–procoagulant response produced by physiological stimulators is believed to be associated with a high (micromolar) and long-lasting (minutes) rise in the intracellular calcium concentration [12,14]. In fact, CsA was only reported to potentiate some platelet responses (aggregation and secretion) and augment the calcium signal produced by physiological agonists [30]. It is therefore postulated that, CsA-evoked platelet–procoagulant response results from a direct interaction of this drug with the platelet plasma membrane rather than from a rise in the intracellular [Ca²⁺]. This is consistent with the observation of Lambros and Rahman [15], who showed that CsA accumulates in lipid membranes and directly interacts with the acyl chain regions of phospholipids, thus perturbing the bilayer. Such a scenario is also likely to occur in human erythrocytes which have been reported to undergo haemolysis following in vitro CsA-treatment [31]. Accumulation of CsA in blood cells has been confirmed by pharmacokinetic studies. These types of studies have revealed that CsA is highly bound to erythrocytes and plasma proteins, and has a blood to plasma ratio of ~2 [32]. In conclusion, procoagulant properties of CsA observed in vitro may result from the direct interaction of this drug with the platelet plasma membrane.

How are these observations related to the in vivo situation? Assuming that in vitro CsA makes platelets procoagulant at concentrations of 1–200 µg/ml, the question arises whether therapeutically used doses of this drug might affect platelets in vivo. Recommended drug concentrations in the immediate post-transplant period are usually in the range of 1–10 µg/ml, whereas concentrations in the range of 0.6–1 µg/ml are recommended thereafter (long-term therapy) [33].

The observations presented here indicate that (at least, in vitro) CsA at a concentration of 10 µg/ml distinctly augments the platelet–procoagulant response evoked by low collagen doses. Since, in the course of and in the immediate post-transplant period, both pre-activated platelets (due to surgery) and relatively high concentrations of CsA are present in a patient’s blood [33], a rise in the generation of platelet-dependent thrombin is fairly likely. Such a possibility is additionally confirmed by the observations of Reis et al. who showed that platelets of rats treated for seven weeks with CsA doses equal to those used in human clinical practice, demonstrated changes (fillopodia formation) in the plasma membrane morphology visible in electron microscopy [34]. Interestingly, as postulated by Bode and Hickerson [21] this type of morphological change (formation of fillopodia) can be seen in the fluorocytometry dot plot diagrams as a unimodal left shift in forward scatter. It remains to be examined whether such changes in morphology occur also in platelets of patients treated for a long time with CsA.

As shown in this report, CsA affects platelet plasma membrane only at relatively high concentrations suggesting a rather non-specific manner of its action resulting from the accumulation of the drug in the phospholipid bilayer. Elimination of CsA-evoked changes in the platelet plasma membrane is therefore expected following the diminishing of the hydrophobic character of the CsA molecule or through the reduction of its concentrations. Unfortunately, both of these two possibilities are currently excluded. In this instant, a promising solution seems to be replacing CsA with its newer counterpart, tacrolimus. Clinical practice has revealed that as an immunosuppressant tacrolimus is at least as effective as CsA [35], but due to its 50–100 times higher potency in inhibiting calcineurin [36] its effective therapeutic concentration may be significantly lower than that of CsA. Such low concentrations of this drug are therefore not expected to exert non-specific action on platelet plasma membrane. However, this should be proved since tacrolimus molecule is also highly hydrophobic.

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Conflict of interest statement. None declared.
References


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