Enhancement of human platelet aggregation and secretion induced by rapamycin

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Abstract

**Background.** Rapamycin is a new immunosuppressive drug of the macrolide type. Despite binding to one of the FK-binding proteins as the initial step in intracellular action, further effects differ from those of the other fungally derived macrolides, cyclosporine and tacrolimus. We have previously demonstrated an enhancement of agonist-mediated platelet activation by cyclosporine and tacrolimus which was associated with increased phosphorylation of two intracellular platelet proteins, p20 and p40. Because rapamycin utilizes the same class of binding proteins as tacrolimus, but its action is not associated with the inhibition of calcineurin, we postulated that if the stimulatory effect of cyclosporine or tacrolimus was due to calcineurin inhibition, rapamycin should not affect platelets in a similar fashion.

**Methods.** Normal, washed human platelets were treated with various concentrations of rapamycin (from ng to \(\mu\)g/ml), and pre-incubated at 37°C with rapamycin for various periods (1–30 min). Several platelet functional parameters were measured in samples treated with rapamycin and these parameters were compared with control platelet samples treated with the vehicle for the same period: Platelet aggregations following exposure to ADP or to the thrombin equivalent, TRAP-6, were measured as changes in optical transmission in a Chronolog lumi-aggregometer. Each experiment was repeated at three or more times and the mean results were used for statistical comparison.

**Results.** Rapamycin-treated platelets demonstrated an increase in their dose- and time-dependent sensitivity to ADP, resulting in a significantly enhanced primary wave of ADP-induced platelet aggregation followed by a secondary wave of aggregation, indicative of granule secretion. Furthermore, rapamycin-treated platelets showed significantly enhanced sensitivity to TRAP-6 as demonstrated by an increase in the initial velocity of aggregation, an increase in their maximal extent of aggregation and an enhancement of granular ATP secretion. Concentrations of rapamycin in the ng range, as well as short pre-incubation times (within min), were sufficient to cause significant enhancement of agonist-induced platelet aggregation and secretion \((P<0.001)\) as compared with their vehicle controls.

**Conclusions.** Rapamycin significantly potentiates agonist-induced platelet aggregation in a time- and dose-dependent manner. As these findings are similar to those observed with the other fungal macrolides, we hypothesize that inhibition of calcineurin may not be necessary for the increase in intracellular protein phosphorylation observed following exposure of platelets to cyclosporine or tacrolimus. Whether the rapamycin-induced enhancement of sensitivity to agonists and platelet hyperaggregability explains the thrombocytopenia observed in patients when high doses of rapamycin are administered in the clinical setting, and whether these effects are synergistic with cyclosporine, are questions which remain to be investigated.

**Key words:** ADP; ATP secretion; cyclosporine; platelet aggregation; rapamycin; tacrolimus; thrombin receptor agonist

Introduction

Atherosclerosis has become the leading cause of death in long-term (>10 years) transplant survivors and progresses at an accelerated rate [1]. Chronic, low-grade platelet activation may play a role in the generation of atherosclerosis and potentially in the genesis of chronic rejection, which remains the leading cause of renal allograft loss [2]. Platelets become activated by a number of physiological agonists including ADP, epinephrine, thrombin, collagen, platelet-activating factor and stimulatory antibodies directed against specific platelet membrane proteins [3]. Once activated, platelets synthesize thromboxane A\(_2\), which has been implicated in the pathogenesis of cyclosporin (CSA) toxicity, and they release platelet-derived growth factor.
(PDGF) and TGF-β, agents which have been implicated in the genesis of progressive renal diseases [4].

The use of CSA has been associated with a variety of clinical disorders which may be related to platelet activation, including accelerated atherosclerosis and increased thrombotic episodes, deep venous thromboses, pulmonary embolism, and renal arterial and venous thrombosis [5–10]. In addition, other less well-characterized entities have been reported in which platelet activation appears to play a critical role, most prominently, a syndrome of acute renal failure with platelet plugs and fibrin deposition in the glomerulus observed on renal biopsy, which resembles the haemolytic-uremic syndrome [7]. This entity has also been noted in patients treated with tacrolimus (FK 506) [11], as has an increase in thrombotic episodes, primarily deep venous thromboses [12].

Our previous studies have shown that both CSA and tacrolimus significantly enhance platelet aggregation and secretion in response to physiological agonists [13,14]. The agonist-stimulated response of platelets is greater following tacrolimus exposure than following exposure to CSA, which parallels the drugs’ immunosuppressant potencies [12].

Rapamycin is a newer immunosuppressive drug which is structurally similar to tacrolimus [15–17], and binds to one of the same family of rotamases as tacrolimus, the FK-binding proteins (FKBP) [17]. However, the rapamycin–FKBP complex does not inhibit calcineurin, an intracellular phosphatase, which is similar to a calcium-binding regulatory protein present in platelets [18]. We investigated the effects of rapamycin in vitro on both ADP and thrombin–peptide (TRAP)-stimulated platelet aggregation in order to determine whether their effects differ from those produced by the other fungal macrolide immunosuppressants, which would imply a calcineurin-dependent inhibition as etiologic in previous observations, or whether rapamycin also enhances platelet responses to ADP and to TRAP, which would imply a calcineurin-independent mechanism and might explain the dose-dependent fall in platelet counts which has been observed in patients when the drug is used clinically [15].

Materials and methods

Preparation and washing of platelets

Whole blood was collected by venipuncture from healthy donors as described previously in accordance with protection of human subjects as per the Institutional Review Board [3]. Donors were drug-free for at least 2 weeks prior to blood donation. Blood (7 vol) was collected in the anticoagulant acid–citrate–dextrose (1 vol) and was mixed gently at 22 °C. Platelet-rich plasma (PRP) was obtained from blood by centrifugation 200 g for 10 min at 22 °C. The inhibitors PGE₁ (1 μM), apyrase (1 unit/ml) and heparin (2 units/ml) were added to the PRP. The washed platelets were pelleted by centrifugation at 1100 g for 10 min. The platelets were washed as described previously [3]. In brief, the platelet pellet was washed three times using a Tyrode’s albumin solution containing NaCl (137 mM), KCl (2.7 mM), MgCl₂ (1 mM), NaH₂PO₄ (0.36 mM), NaHCO₃ (12 mM), CaCl₂ (2 mM), glucose (5.5 mM) and albumin (0.35%), and adjusted to pH 7.35 using 1 M citric acid. The first wash solution contained heparin, apyrase and PGE₁, the second wash solution contained apyrase and PGE₁, and the third wash solution contained only PGE₁. The final platelet pellet was resuspended in albumin-free Tyrode’s buffer (pH 7.35) in the absence of any inhibitors of platelet aggregation. Platelets were counted spectrophotometrically [19] or microscopically using a hemocytometer and diluted with Tyrode’s buffer to a final platelet count of 2–4 x 10⁹/ml. Platelet suspensions were maintained at 37 °C for all subsequent experiments.

Platelet aggregation, ATP secretion and preparation of rapamycin

The platelet aggregation experiments were carried out at 37 °C under stirring conditions in a Chronolog lumaggregometer (Chronolog Corp., Havertown, PA). Aggregation tracings were plotted on a dual channel recorder as a change in optical transmission following the addition of the platelet agonist ADP which was prepared from the stock solution of rapamycin in 80% DMSO, as a stock solution at a concentration of rapamycin (1 mg/ml) and maintained at −20 °C until use. The final concentrations of DMSO in the platelet suspensions varied from 0.01 to 2.2%. Control (vehicle) solutions were prepared in the same manner as those described above, except that rapamycin was omitted and identical volumes of the vehicle containing DMSO were added to platelet suspensions. Washed platelet suspensions were incubated with gentle mixing with either rapamycin or the vehicle for various periods, from 1 to 30 min, at 37 °C. Quotations of the maximal extent of ADP- or TRAP-induced platelet aggregation were determined by measuring light transmission units (LTU) displayed on a chart recorder. The initial velocity of platelet aggregation was measured from the initial slope of the platelet aggregation tracing and expressed as LTU/min. In parallel, ATP secretion was measured by adding the luciferin/luciferase reagent (Chronolog Corp.) during platelet aggregation.

Statistical analysis

Statistical analysis was performed by the SUNY Scientific and Academic Computing Center, Brooklyn, NY, using SPSS for Windows Release 6.1. and by use of Sigma Stat.

Results

A representative example of the response of washed platelets to the agonist ADP (in the absence of rapamycin) is shown in Figure 1A. The initial slope of the
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ADP-induced aggregation curve represents the initial rate (velocity) of platelet aggregation (LTU/min), and the increase in light transmission is indicative of the extent of ADP-induced platelet aggregation (LTU) from which the maximal extent of aggregation is determined. Figure 1B shows the response of platelets pre-incubated for 1 min with rapamycin, to the addition of the agonist ADP. On the one hand, as shown in these experiments, the addition of ADP, in the presence of the vehicle control solution (Figure 1A), resulted in a very low degree of platelet aggregation, which was observed to be monophasic and reversible. On the other hand, pre-incubation of platelet suspensions with rapamycin (Figure 1B) resulted in a significant robust increase (600%) in the initial velocity of platelet aggregation and a significant increase in the extent of platelet aggregation in response to identical concentrations of ADP. As demonstrated in Figure 1B, we consistently observed a transition from a low monophasic reversible response in the absence of rapamycin, to an enhanced response to ADP (shown here by the biphasic response in the presence of rapamycin) consisting of a primary wave of aggregation followed by a secondary wave of aggregation representing active induction of granular secretion. Such enhancement of platelet aggregation (termed hyperaggregability) by rapamycin resulted in an irreversible platelet aggregation. As these experiments were conducted with washed platelets free of additional plasma proteins (other than exogenously added fibrinogen), our results indicate that rapamycin exerts its procoagulant effects in vitro by interacting directly with platelets, and does not require the presence of additional plasma factors.

The dose–response relationship of the effect of rapamycin in the induction of agonist-induced platelet hyperaggregability, following a 1 min pre-incubation period, is shown in Figure 2. The data points represent the mean ± standard error (SEM) for ADP-induced platelet aggregations tested at various concentrations of rapamycin. Concentrations of rapamycin of 100 ng, 220 ng and 2.2 μg/ml produced an increase in platelet aggregation in response to ADP ranging from 150 to 300% over control values. Concentrations of rapamycin of 100 ng and 2.2 μg/ml produced a significant (P < 0.05) increase in platelet hyperaggregability, and treatment of platelets with a concentration of 220 ng/ml also resulted in an approximate 200% increase in platelet hyperaggregability in response to ADP with a P-value very close to significant (P < 0.053). Approximately 50% of maximal hyperaggregability was achieved at a rapamycin concentration of 220 ng/ml, and a rapamycin concentration of 2.2 μg/ml resulted in approximately 86% of maximal hyperaggregability. A plateau in hyperaggregability was achieved at concentrations of rapamycin between

Fig. 1. Enhancement of ADP-induced platelet aggregation induced by rapamycin. (A) The response of washed human platelets (0.45 ml) to the agonist ADP (arrow) (11 μM, final concentration) in the presence of the control vehicle. (B) Enhanced response of washed human platelets to ADP (11 μM, final concentration) in the presence of rapamycin (1 μg/ml) depicting the induction of both a primary and secondary wave of aggregation associated with secretion. This figure is representative of three or more separate experiments.

Fig. 2. Dose–response relationship of ADP-induced platelet aggregation in the presence of various concentrations of rapamycin. Washed human platelets (450 μl; 2–4 × 10^6/ml) were incubated with the final concentrations of rapamycin (100 ng, 220 ng, 2.2 μg, 7.3 μg, 11.1 μg and 22.2 μg per ml) (or with an identical volume of the DMSO vehicle) for 1 min at 37°C under stirring conditions. Aliquots (10 μl) of the agonist, ADP (11 μM), were added to initiate platelet aggregation and the extents of aggregation [measured in light transmission units (LTU)] were determined for all samples treated with rapamycin and compared with samples treated with vehicle alone. The extent of platelet aggregation in the absence of rapamycin was set at 100%. One sample t-tests were used to compare the extent of platelet aggregation at each dose of rapamycin relative to its control for three experiments. A P-value of <0.05 was considered significant. The asterisk (*) indicates significant differences between treated and untreated samples. At a rapamycin concentration of 0.22 μg/ml, the P-value was very close to significant, P = 0.053. In addition, a Bonferonni correction was performed for small group size. The values obtained at 2.2 and 7.3 μg/ml rapamycin were combined, and the values obtained at 11.1 and 22.2 μg/ml rapamycin were combined and compared with the control values. The values of the rapamycin-treated samples were found to be significantly different from the vehicle-treated samples at P < 0.0125.
7.3 and 11.1 μg/ml. The highest concentration of rapamycin examined, 22.2 μg/ml, also resulted in a significant increase in the extent of ADP-induced platelet aggregation.

Figure 3 represents a dose–response curve of rapamycin-induced platelet hyperaggregability (at concentrations of 25–200 ng/ml), as might be encountered at peak plasma levels clinically. After a 30-min pre-incubation with rapamycin, we observed an enhancement of ADP-stimulated platelet aggregation at all concentrations of rapamycin examined, with significant differences observed at concentrations of 50 ng/ml (P < 0.006) and 200 ng/ml (P < 0.001); significance at 100 ng/ml was not observed owing to the large variance in the results at this concentration.

The effect of pre-incubation time on rapamycin-induced platelet hyperaggregability is shown in Figure 4. One concentration of rapamycin was selected (1 μg/ml), and the pre-incubation time with rapamycin was examined. We observed that a short incubation of 1 min with rapamycin resulted in a 150% increase in the extent of ADP-induced platelet aggregation. At longer periods of incubation with rapamycin (10 and 30 min), rapamycin treatment caused further increases in the extent of ADP-induced platelet aggregation.

Since ADP provides information concerning only one pathway of platelet activation, the agonist TRAP (a thrombin equivalent, termed the thrombin receptor agonist peptide) was examined for its effects on platelet function in the presence of rapamycin. Figure 5A shows the potentiating role of rapamycin when TRAP is used as the agonist. Incubation of platelets with rapamycin (2 min at 37°C) resulted in a 600% increase in TRAP-induced platelet aggregation which was associated, in parallel, with a potent induction of granular secretion as measured by the release of dense-granule-containing ATP (lower panel of Figure 5A). In the absence of rapamycin (Figure 5B), only a small, reversible platelet aggregation was evident in response to TRAP, and no ATP secretion could be detected (lower panel of Figure 5B). The dose–response relationship of rapamycin-induced platelet sensitivity was examined by using the extent of platelet aggregation as a measure of platelet hyperaggregability. Figure 6 demonstrates that incubation of platelets with rapamycin at a concentration of 430 ng/ml produced a significant (P < 0.001) enhancement (of approximately 300%) in the extent of TRAP-induced platelet aggregation. The enhancement of platelet aggregation at this concentration of rapamycin was approximately 43% of the maximal level, and approximately 80% of the maximal response was observed at a concentration of 1.1 μg/ml. Lower concentrations of rapamycin of 110 and 220 ng/ml did not reveal significant differences between control vehicle-treated samples and rapamycin-treated samples, however a tendency for an increase in the extent of platelet aggregation could be observed at these lower concentrations of rapamycin. Maximal hyperaggregability induced by rapamycin was observed at a concentration of 2.2 μg/ml and resulted in a significant enhancement in the extent of platelet aggregation, of up to 10- to 14-fold (Figure 6). Rapamycin also increased the TRAP-induced initial velocity of platelet aggregation as shown in Figure 7. Concentrations of rapamycin in the ng range significantly increased (by 2-fold) the initial velocity of platelet aggregation induced by TRAP, whereas μg concentra-
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Fig. 5. Enhancement by rapamycin of thrombin receptor agonist (TRAP)-induced platelet aggregation and secretion. (A) The enhanced aggregation and secretion of washed human platelets in response to TRAP (1 µg/ml, final concentration) in the presence of rapamycin (abbreviated as RAP) (4.3 µg/ml). (B) The response of washed human platelets to the agonist TRAP (1 µg/ml, final concentration) in the presence of the control vehicle (DMSO). Incubation at 37°C under stirring conditions was carried out for 2 min with either rapamycin or the vehicle prior to the addition of TRAP. Simultaneous measurements of granular release of ATP (ATP secretion) were performed using the luciferin–luciferase (L–L) reaction. The addition of the L–L reagent and the measurement of the secreted ATP are shown by the arrows on the bottom-half of panels A and B.

Discussion

Our data strongly suggest that rapamycin is a potent enhancer of platelet aggregation and secretion at concentrations which may occur at peak plasma levels, and that this effect does not require the presence of plasma proteins, but is a direct effect of rapamycin on platelet function. Pre-incubation times as short as 1 min resulted in increased platelet aggregation and secretion, thus, the exposure of platelets to transient rises in rapamycin levels, which occur following human ingestion of the compound, could have profound clinical effects.

Preclinical data have demonstrated a dose-dependent association of rapamycin administration with decreased platelet count [15]. Presently it is not known whether such decreases in platelet count occurred due to enhanced platelet clumping and aggregation in vivo, whether the decrease in platelet count was due to the destruction of platelets, or whether such a decrease resulted from a direct effect on the suppression of megakaryopoiesis. These clinical studies [15] were performed using cyclosporine and rapamycin concomitantly, and cyclosporine has been demonstrated to have an independent effect responsible for the enhancement of platelet aggregation [8–10]. Therefore, future studies will require the evaluation of the effects of cyclosporine and rapamycin together in vitro, in order to determine whether these compounds produce profound synergistic effects on platelet activation.

The present study suggests that significant alterations in platelet function can occur following rapamycin exposure in a pattern which resembles that observed following pre-incubation with other fungally derived immunosuppressants, cyclosporine or tacrolimus [13,14]. Although rapamycin binds to a class of FK-binding proteins, the FK-BP–rapamycin complex does not inhibit calcineurin [17] suggesting that the inhibition of calcineurin is not the mechanism by which the enhanced intracellular protein phosphorylation and subsequent platelet activation occur following exposure of platelets to cyclosporine.

Low-grade platelet activation has been implicated in many long-term effects, including atherogenesis and
The enhancement of the initial velocity of thrombin receptor agonist (TRAP)-induced platelet aggregation by rapamycin. Washed human platelets (450 µl; 2–4 × 10⁸/ml) were incubated in the presence of various concentrations of rapamycin (as shown above) for 1 min at 37°C under stirring conditions. Control samples were incubated with the vehicle in an identical manner. Aliquots (6 µl) of the agonist, TRAP (1 µg/ml, final concentration), were added to initiate platelet aggregation. The extents of aggregation measured in light transmission units (LTU) were determined for all samples treated with rapamycin and compared with samples treated with vehicle alone. The P-values were determined using two-tailed unpaired Student’s t-test. A P-value of < 0.05 was considered significant. The asterisk (*) indicates significant differences (P < 0.001) between the two groups. Values are the mean ± SEM for three or more separate experiments.

Fig. 6. Enhancement of the extent of thrombin receptor agonist (TRAP)-induced platelet aggregation by rapamycin. Human platelets (450 µl; 2–4 × 10⁸/ml) were incubated in the presence of various concentrations of rapamycin (as shown above) for 1 min at 37°C under stirring conditions. Control samples were incubated with the vehicle in an identical manner. Aliquots (6 µl) of the agonist, TRAP (1 µg/ml, final concentration), were added to initiate platelet aggregation. The extents of aggregation measured in light transmission units (LTU) were determined for all samples treated with rapamycin and compared with samples treated with vehicle alone. The P-values were determined using two-tailed unpaired Student’s t-test. A P-value of < 0.05 was considered significant. The asterisk (*) indicates significant differences between the treated and control samples (P < 0.001). Values are expressed as the mean ± SEM for three or more separate experiments.

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