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Calcineurin Inhibitors Alter the Osmotic Properties of Erythrocytes in Patients After Renal Transplantation*

Inhibitory kalcyneuryny zmieniają własności osmotycznych erytrocytów u chorych po przeszczepie nerki

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Abstract

Background. Renal transplant recipients treated with calcineurin inhibitors are known to demonstrate abnormalities in the physical properties of red blood cell membranes.

Objects. The aim of the present study was to determine whether the osmotic properties of erythrocytes are altered in patients after renal transplantation treated with cyclosporin A (CsA) or tacrolimus (TAC).

Material and Methods. Venous blood samples were collected from 34 renal transplant recipients with good allograft function (serum creatinine concentration < 1.3 mg/dl). Among them, 13 were treated with tacrolimus and 21 with cyclosporin. Erythrocytes from 31 healthy donors were also examined.

Results. Osmotic fragility (osmolality at 50% hemolysis) was determined spectrophotometrically in solutions of varying NaCl concentration. The rate of hemolysis was determined in hypotonic solution (62 mM NaCl) by the stopped-flow method. CsA and TAC cause the migration of the hemolysis curve towards lower osmolarities. Statistically significant decreases in relative osmotic fragility were observed both in patients receiving CsA compared with controls (0.409 ± 0.004 vs. 0.420 ± 0.003) %NaCl and in those receiving TAC (0.407 ± 0.004 vs. 0.420 ± 0.003) %NaCl. A statistically significant decrease in the rate of hemolysis during tacrolimus treatment (0.103 ± 0.027 vs. 0.127 ± 0.015) s⁻¹ was found. There was negative correlation between cyclosporin trough level and osmotic hemolysis rate, but no such correlation for tacrolimus was seen.

Conclusions. These results suggest that both calcineurin inhibitors alter the osmotic properties of erythrocytes towards decreased susceptibility to hypotonic hemolysis (Adv Clin Exp Med 2008, 17, 2, 213–216).

Key words: erythrocytes, renal transplantation, osmotic fragility, hemolysis, calcineurin inhibitors.

Streszczenie

Wprowadzenie. Leczenie inhibitorami kalcyneuryny wpływa na fizyczne własności błon erytrocytów biorców przeszczepu nerki.

Cel pracy. Zbadanie, czy zmiany własności osmotycznych błony erytrocytów zachodzą po zastosowaniu różnych inhibitorów kalcyneuryny u biorców przeszczepu nerki leczonych cyklosporyną lub takrolimusem.

Materiały i metody. Krew żylną pobrano od 34 biorców przeszczepu nerki z dobrą czynnością przeszczepu (stężenie kreatyniny w surowicy poniżej 1,3 mg/dl). 13 chorych pobierało takrolimus, a 21 cyklosporynę. Do badań kontrolnych użyto erytrocyty od 31 zdrowych ochotników. Do badania szybkości hemolizy osmotycznej w roztworze hipotonicznym (62 mM NaCl) wykorzystano metodę pomiaru kinetyki szybkich reakcji (*stopped flow*). Podatność osmotyczną (osmolarność przy 50% hemolizie) wyznaczono spektrofotometrycznie w roztworach o różnych stężeniach NaCl.

Wyniki. Cyklosporyna i takrolimus wpływały na przesunięcie krzywej hemolizy w kierunku niższych osmolarności. Istotne zmniejszenie podatności osmotycznej obserwowano u chorych leczonych cyklosporyną w stosunku do

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osób zdrowych ($0,409 \pm 0,004$ vs $0,420 \pm 0,003$) %NaCl i takrolimusem ($0,407 \pm 0,004$ vs $0,420 \pm 0,003$) %NaCl. Obserwowano istotne zmniejszenie szybkości hemolizy podczas leczenia takrolimusem ($0,103 \pm 0,027$ vs $0,127 \pm 0,015$)(s⁻¹).

Wnioski. Oba inhibitory kalcyneuryny zmieniają własności osmotyczne erytrocytów, zmniejszając podatność na osmotyczną hemolizę (*Adv Clin Exp Med* 2008, 17, 2, 213–216).

Słowa kluczowe: erytrocyty, inhibitory kalcyneuryny, hemoliza, przeszczepianie nerek, podatność osmotyczna.

Calcineurin inhibitors are widely used in immunosuppressive treatment in transplant recipients. After administration, most of them are detected in erythrocytes. Within therapeutic concentration ranges, 58% of cyclosporin A (CsA) and 95% of tacrolimus (TAC) is taken up by erythrocytes [1, 2]. Renal transplant recipients treated with calcineurin inhibitors are known to demonstrate abnormalities in the physical properties of red blood cell membranes. Interactions of CsA with the erythrocyte membrane may lead to changes in erythrocyte shape [3], fluidity [4], deformability [5, 6] and trigger suicidal erythrocyte death (eryptosis) [7]. These events may change blood flow in the microcirculation [8], which partially explains the common side effects of calcineurin inhibitor treatment, i.e. anemia and thrombosis [9]. As demonstrated in the present authors' previous study using electron paramagnetic resonance (EPR), CsA-treated recipients exhibited an increase in red cell membrane fluidity but TAC-treated recipients did not [10]. The aim of the present study was to determine whether the osmotic properties of erythrocytes are altered in patients after renal transplantation treated with cyclosporin A or tacrolimus.

Material and Methods

Kidney graft recipients transplanted between 1989 and 2005 (19 females, 15 males, age range: 23–68 years, mean: 48 ± 12 years) were enrolled in the study. Venous blood samples were collected from the 34 recipients with good allograft function (serum creatinine concentration < 1.3 mg/dl). All the recipients remained on triple immunosuppressive therapy consisting of prednisone, azathioprine or mycophenolate mofetil, and one of the calcineurin inhibitors. Among them, 13 recipients were treated with tacrolimus (the TAC group) and 21 patients with cyclosporine (the CsA group). Hypertension was defined as treatment with anti-hypertensive drugs or mean blood pressure greater than 140/90 mm Hg. The two groups of recipients did not differ in terms of demographic or clinical profiles with the exception of a longer post-transplant period in the CsA group. The clinical profiles of the renal transplant recipients are presented in Table 1. For comparison, venous blood samples from 31 healthy donors (22 females, 9 males, aged range: 24–56

years, mean: 39 ± 9 years) with no history of hypertension, diabetes, or drug use were also examined.

CsA and TAC concentrations in whole blood were measured using a Fluorescence Polarization Immunoassay (Abott IMX, Abott TDX). For the examination of osmotic properties, erythrocytes were isolated from fresh venous blood (anticoagulated with K₂EDTA) by centrifugation (4°C, $2000 \times g$, 5 min), purified by three cycles of resuspension, and washed with isotonic solution (147 mmol/l NaCl, 5.6 mmol/l phosphate buffer, pH 7.4). The osmotic fragility was determined spectrophotometrically by measuring the absorbance from hemoglobin release at 545 nm in solutions varying in NaCl concentration. The dependence of the fraction hemolysed (FH) vs. NaCl concentration ([NaCl] in %w/v) was fitted to the equation

$$FH = p3 \operatorname{erfc}\{([NaCl] - p1)/p2\},$$

where $p1$ is the mean [NaCl] corresponding to 50% hemolysis (osmotic fragility), $p2$ the hemolytic dispersion, and $p3$ the half value of the fraction hemolysed at total hemolysis [11]. The time-course of osmotic hemolysis was measured in hypotonic solution (62 mM NaCl) by recording the change in turbidity of the erythrocyte suspension (0.05% hematocrit) at 690 nm by means of the stopped-flow method. The apparent rate constant for hemolysis was defined as the steepest slope of the first-order semilogarithmic plot of the decrease in absorbance versus time [12]. Data are expressed as mean values $\pm$ SD. The level of statistical significance was set at $P < 0.05$.

The project was conducted after approval by the Bioethics Committee of Silesian Piasts University of Medicine in Wrocław and all aspects of the study were in accordance with the World Medical Association's Declaration of Helsinki.

Results

The results indicate that both drugs cause migration of the hemolysis curve towards lower osmolarities. Statistically significant decreases in osmotic fragility were observed both in the patients receiving CsA compared with controls ($0,409 \pm 0,004$ vs. $0,420 \pm 0,003$) %NaCl and in those receiving tacrolimus ($0,407 \pm 0,004$ vs. $0,420 \pm 0,003$) %NaCl (Table 2). A statistically si-

Table 1. Clinical profiles of cyclosporin A (CsA)- and tacrolimus (TAC)-treated renal transplant recipients**Tabela 1.** Dane kliniczne biorców przeszczepu nerki leczonych cyklosporyną (CsA) i takrolimusem (TAC)

	TAC patients (Pacjenci leczeni TAC) mean $\pm$ SD, n = 13	CsA patients (Pacjenci leczeni CsA) mean $\pm$ SD, n = 21
Time post tx – years (Czas po przeszczepie – lata)	3.2 $\pm$ 1.7	6.4 $\pm$ 4.2
Age – years (Wiek – lata)	47 $\pm$ 14	49 $\pm$ 10
Gender M : F (Płeć M : K)	5 : 8	10 : 11
Body mass – kg (Masa ciała)	73 $\pm$ 11	71 $\pm$ 11
Hypertension – 1 : 0 (Nadciśnienie tętnicze)	10 : 3	18 : 3
Diabetes – 1 : 0 (Cukrzyca)	1 : 12	5 : 16
Creatinine – mg/dl (Kreatynina)	1.1 $\pm$ 0.3	1.1 $\pm$ 0.3
Urea – mmol/l (Mocznik)	6.3 $\pm$ 1.4	6.7 $\pm$ 1.9
Albumin – g/l (Albumina)	43.2 $\pm$ 4.4	43.9 $\pm$ 3.0
HGB (g/dl)	14.1 $\pm$ 1.7	13.7 $\pm$ 1.8
HCT (%)	42.5 $\pm$ 4.7	40.9 $\pm$ 5.0
RBC (T/l)	4.5 $\pm$ 0.6	4.3 $\pm$ 0.7
Cholesterol	8.4 $\pm$ 2.8	5.2 $\pm$ 1.1
Triglycerides (Trójglicerydy)	1.4 $\pm$ 0.5	1.6 $\pm$ 0.7
CsA–C ₀ or TAC conc. – ng/ml (Stężenie CsA–C ₀ lub TAC)	8.1 $\pm$ 2.2	122.9 $\pm$ 49.5
Prednisone – mg/d (Prednizon)	4.5 $\pm$ 2.5	5.4 $\pm$ 1.8
Prednisone – mg/kg (Prednizon)	0.06 $\pm$ 0.04	0.07 $\pm$ 0.03

gnificant decrease in the rate of hemolysis during tacrolimus treatment (0.103 ± 0.027 vs. 0.127 ± 0.015) s⁻¹ was found. There was negative correlation between the CsA through level (C₀) and the rate of osmotic hemolysis ($R^2 = -0.63$), but no such correlation was seen for tacrolimus.

Discussion

Decreased values of osmotic fragility for both CsA- and TAC-treated renal transplant recipients point to an increased resistance of their erythrocytes against hypotonic shock. Osmotic fragility is mainly determined by the excess of surface area to initial volume of the erythrocyte. The observed decreases in this parameter during CsA and TAC treatment might be caused by erythrocyte shrinkage following changes in membrane transport properties. Niemoeller et al. showed that exposure of erythrocytes to cyclosporin *in vitro* caused disorders in ion membrane transport leading to erythrocyte shrinkage [7]. This is in accordance with the present *in vivo* observations that administration of CsA and TAC leads to a significant decrease in osmotic fragility, which might result from a changed surface area to volume ratio in erythrocytes. This erythrocyte shrinkage is one of the features of erythrocyte suicidal death (eryptosis). CsA can evoke other hallmarks of eryptosis *in vitro*, e.g. increased phosphatidylserine exposure [7]. Cells exposing phosphatidylserine on their surface are rapidly eliminated from the circulation by phagocytosis. Enhanced sensitivity to eryptosis has also been observed in patients with hemolytic uremic syndrome [13]. It is possible that administration of CsA, and probably TAC, may augment patient susceptibility to eryptosis and thus trigger hemolytic uremic syndrome. After renal transplantation, patients treated with calcineurin inhibitors are known to be at risk of that serious complication.

The rate of osmotic hemolysis, another parameter tested in the present study, measures a diffe-

Table 2. Osmotic fragility and osmotic hemolysis rates in normal controls and CsA- and TAC-treated renal transplant patients**Tabela 2.** Oporność osmotyczna i szybkość hemolizy u biorców przeszczepu nerki leczonych CsA i TAC oraz u zdrowych ochotników

	Normal controls mean $\pm$ SD, n = 31 (Grupa kontrolna)	CsA group mean $\pm$ SD, n = 21 (Grupa CsA)	TAC group mean $\pm$ SD, n = 13 (Grupa TAC)
Osmotic fragility %NaCl (w/v) (Oporność osmotyczna)	0.420 $\pm$ 0.003	0.409 $\pm$ 0.004*	0.407 $\pm$ 0.004*
Osmotic hemolysis rate (s ⁻¹) (Szybkość hemolizy)	0.127 $\pm$ 0.015	0.125 $\pm$ 0.022	0.103 $\pm$ 0.027**

* $P < 0.05$ for patients vs. normal controls.** $P < 0.01$ for patients vs. normal controls.* $p < 0,05$ dla pacjentów względem zdrowych ochotników.** $p < 0,01$ dla pacjentów względem zdrowych ochotników.

rent process from that of the osmotic fragility curve. The measurement of osmotic fragility is a static method of investigating hemolysis, whereas the rate of osmotic hemolysis reflects dynamic aspects of hemolysis. The rate of osmotic hemolysis is determined by the rupturing of erythrocyte membranes which follows erythrocyte reshaping toward a spherical form in hypotonic solutions [12]. In the present study a statistically significant decrease in

the rate of osmotic hemolysis during TAC treatment was demonstrated. Mean hemolysis rates did not differ between the CsA group and the controls. This phenomenon requires further investigation.

In conclusion, the present observations provide evidence that both calcineurin inhibitors, CsA and TAC, alter the osmotic properties of erythrocytes towards decreased susceptibility to hypotonic hemolysis.

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