Comparative study of cyclosporin A, cyclosporin G, and the novel cyclosporin derivative IMM 125 in isolated glomeruli and cultured rat mesangial cells: a morphometric analysis

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Abstract

Background. One adverse side-effect of the immunosuppressive drug cyclosporin A (CsA) is a decrease in glomerular filtration rate (GFR). This effect might be the result of increased glomerular contractions. The present study compared the contractile effects of CsA, cyclosporin G (CsG) and the novel cyclosporin derivative IMM 125 in isolated rat glomeruli and primary cultures of rat mesangial cells.

Methods. Interactive image analysis was used to measure glomerular and mesangial cell contraction.

Results. CsA, CsG, and IMM 125 at concentrations of 0, 10⁻⁶, 10⁻⁸, 10⁻⁷, 10⁻⁶ and 10⁻⁵ M caused a time-dependent and a concentration-dependent contraction of isolated glomeruli and mesangial cells 30 min after incubation. In glomeruli, CsA was more potent than CsG and IMM 125. In mesangial cells, IMM 125 also exhibited the lowest contractile activity, while CsA and CsG were almost equally myoreactive. The absolute degree of the glomerular contraction was proportional to the number of contracting mesangial cells in one glomeruli. The number of responding cells after incubation with IMM 125 and CsG were lower compared to CsA, which might explain the different response with CsG and CsA in both models.

Conclusions. Since the concentrations used in these experiments were close to that reached in rat serum after treatment with CsA, the present results suggest that the contractile effects of IMM 125 and CsG in isolated glomeruli were clearly smaller compared to CsA, which might reflect the cyclosporins induced GFR changes in vivo.

Key words: contraction analysis; cultured mesangial cells; cyclosporins; isolated glomeruli; rat

Introduction

The fungus *Tolypocladium inflatum* Gams produces a class of compounds with marked immunological activities. The most famous of these compound is the cyclic undecapeptide cyclosporin A (CsA; Sandimmun, Sandoz, Basel, Switzerland), a hydrophobic substance composed of 11 amino acids. It is a powerful inhibitor of lymphocyte function in vitro and a highly specific immunosuppressive agent in vivo. Many years of clinical use have proved the efficacy of CsA in the treatment of allograft rejection and in therapy for autoimmune diseases [1]. Its application, however, is slightly affected by the induction of reversible side-effects, such as impaired renal function [2]. In order to improve the therapeutic success and the tolerability of cyclosporins, new derivatives with an enlarged therapeutic window and the same or better immunosuppressive activity, but devoid of nephrotoxicity were created [3]. CsG and IMM 125 are examples of such compounds. CsG differs from CsA by substitution of L-nor-valine for a-amino butyric acid at the second amino acid. CsG seems to have in vitro immunosuppressive activity similar to that of CsA. Studies in animals have indicated that CsG is less nephrotoxic than CsA, which might be linked to the higher clearance of the compound [4]. A clinical trial of CsA vs CsG in human cadaveric renal transplantation confirmed the similar efficacy of both drugs and suggested less nephotoxicity in the CsG-treated patients [5]. IMM 125 is the hydroxyethyl derivative of D-serine-CsA with a molecular weight of 1261.7 Dalton. IMM 125 has been shown to be equivalent to CsA in several pharmacological in vitro test systems, without being cytotoxic or cytostatic. Owing to its potentially broader therapeutic dose range, IMM 125 could offer a considerable therapeutic advantage over CsA in transplant recipients and patients with autoimmune diseases [6].

In rat studies, CsA was found to decrease the glomerular filtration rate (GFR). In CsA treated patients the drug causes increased blood urea nitrogen and serum creatinine. It is assumed that there is a
direct link between the CsA-mediated decreased GFR and the altered haemodynamics and vasoconstriction (for review, see [7]). Causes afferent arteriolar constriction, affecting renal blood flow and causing reversible renal insufficiency. Renal mesangial cells have been demonstrated to contract when exposed to CsA and are known to alter glomerular basement membrane tension leading to changes in the ultrafiltration coefficient [8]. The CSA-mediated changes of the basement membrane might lead to GFR reduction. Isolated glomeruli and cultured mesangial cells have proved to be good in vitro models to investigate different drugs and their mechanisms of action [9]. In vitro tests are advantageous because they evaluate general cytotoxic potential of chemicals or drugs by permitting the use of pharmacological concentrations for morphometric studies. Cultured mesangial cells and isolated glomeruli from rat renal cortex without any vascular, nervous or humoral influence, represent useful models to assess possible direct effects of either endogenous or exogenous vasoactive substances [10], such as CsA which induces a time- and dose-dependent decrease in isolated glomeruli and mesangial cell surfaces [11]. The reactivity of the mesangial cell, a smooth muscle-like structure, can modulate the effective renal filtration surface by changing the size and/or patency of glomerular capillaries [12]. CsA might induce mesangial and glomerular contraction by different mechanisms, such as increase in intracellular calcium and production of vasoconstrictive prostanoids, which can be counteracted by calcium blockers and xanthine derivatives [9].

The aim of the present study was to assess the effects of non toxic concentrations of CsA, CsG, and IMM 125 on the reduction of the planar surface area (PSA) in two in vitro glomerular models, cultured rat mesangial cells and isolated rat glomeruli, by using a morphometric analysis method in order to compare their contractile ability.

Subjects and methods

Drugs

CsA and its analogues, CsG and IMM 125, were obtained from Sandoz (Basel, Switzerland), dissolved in DMSO, and kept as stock solutions at room temperature protected from light. Before each experiment, the stock solutions were diluted with Hank’s balanced saline solution (HBSS) at final concentrations ranging from $10^{-5}$ to $10^{-9}$M. Final DMSO concentration for morphometric studies was 0.1%.

Glomeruli isolation

Glomeruli were isolated from the outer renal cortex of male Sprague–Dawley rats weighing 180–200 g (Dépré, France) by means of a sieving technique (successively passing the cortical pulp through calibrated sieves of 180, 125 and 63 μm) according to Martin-Dupont and Cambard [13]. The isolated glomeruli, collected on the 63-μm mesh screen were resuspended in HBSS. More than 85% (86.5 ± 1.2%) of the glomeruli were free of Bowman’s capsule, and tubular contaminations were always less than 3% (2.3 ± 0.4%) of the total number of glomeruli, as checked by counting the number of encapsulated glomeruli and tubular fragments in the isolated glomeruli suspension.

Mesangial cell culture

Mesangial cells were obtained from 21–28-day cultures of freshly isolated glomeruli at 37°C under 95% air and 5% CO₂ in 75 cm² plastic dishes containing 15 ml RPMI 1640 medium supplemented with 20% decomplemented fetal calf serum (FCS), sodium bicarbonate (2 g/l), L-glutamine (2 mM), sodium pyruvate (1 mM), penicillin (100 U/ml), streptomycin sulphate (100 μg/ml), amphotericin (0.25 μg/ml) and buffered with 10 mM N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid (HEPES), pH 7.2 (Sigma, France). In the first days of culture, isolated glomeruli became adherent to the plastic and outgrowth of cells was observed. The early outgrowth from glomeruli contained some endothelial cells and contractile mesangial cells, but glomerular epithelial cells were the predominant cell type. Endothelial cells did not propagate in culture because of the lack of specific culture conditions. After 3 weeks, epithelial cells were no longer observed. Glomeruli were removed during passage with trypsin, leaving mainly mesangial cells as previously described [14]. These cells were investigated between the second and fifth passages to avoid any dedifferentiation processes [14,15]. Positive identification of this cell type was obtained by morphological examination and verification of contraction with $10^{-9}$M CsA. Light-microscopy, mesangial cells cultured in plastic dishes appeared large and stellate with many irregular cytoplasmic projections, while epithelial cells had a polygonal shape. In addition, immunofluorescence staining of Factor VIII antigen, a marker for endothelium, was negative and α-actin, a marker of smooth muscle, was positive, indicating that the cultures were not contaminated with glomerular endothelial and/or epithelial cells [16].

Cytotoxicity evaluation

Cytotoxicity was determined in mesangial cell cultures, plated 48 h previously in 96-well-multidishes (10 000 cells/well). Confluent mesangial cells were incubated for 24 h with different concentrations (16 wells per concentration) of drugs diluted in culture medium supplemented with 5% FCS. Lysosomal integrity was determined by Neutral red (NR) uptake [17]. The mitochondrial integrity was determined by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-di-phenyltetrazolium bromide] (MTT) enzymatic reduction [18]. The toxic effects were expressed by means of IC₅₀ values (concentration at which 50% of the parameters were affected compared to controls) after regression analysis.

Morphometric analysis

Before measuring the glomerular contraction, 35 mm diameter Petri dishes were filled with 200 μl of Poly-L lysine (1 mg/ml) for 10 min at 37°C and 100 μl of the glomerular suspension was added (approximately 2000 glomeruli per dish). After 5 min, glomeruli were attached and the dishes rinsed, then filled with either 2 ml HBSS medium or test compound diluted in HBSS medium. Cultured mesangial cells were directly seeded in these Petri dishes (20 000 cells/ml) and tested before they reached confluence. The dishes were rinsed with 2 ml HBSS medium before any experiments.
Incubations with either control solution or with different concentrations of CsA, CsG or IMM 125 were performed at room temperature (25 ± 2°C) for 30 min.

Approximately 20 suitable glomeruli, which did not contain Bowman’s capsule (identified by less sharp contours under microscope), or 10 mesangial cells per treatment were selected for morphometric analysis. Fields with easily discernible boundaries for each structure were selected for photography (2 fields per dish per concentration). The same glomeruli or mesangial cells (2 same fields, chosen at time 0) were photographed at different incubation times (5, 10, 20, 30 min) under an inverted microscope (Olympus/BH2).

The PSA was determined for each structure at each incubation time by a computerized image analyser (Compaq, Matrox, Alcatel).

The initial PSA of each glomerulus or mesangial cell at the beginning of incubation (T0) with the test compound was expressed as 100%. Contraction was expressed as the decrease of the PSA in comparison to that of each individual initial value.

Statistical analysis

All experiments were performed in duplicate and were carried out under double-blind conditions. Values are expressed as mean value ± standard error to the mean (SEM) of two experiments performed. For cytotoxicity and morphometric studies, variance analysis and Student’s t test were performed. The latter was used to compare between the results of control experiments and experiments with different concentrations of CsA or CsG or IMM 125. Differences at the 5% level or less were considered as statistically significant. Correlations between concentrations, incubation times and decreases in PSA were calculated for each drug and regression analyses were performed by standard statistical methods.

Results

Determination of cytotoxicity in cultured mesangial cells (Table 1)

CsA, CsG and IMM 125 were incubated with cultured mesangial cells for 24 h at concentrations of 0, 10⁻⁸, 10⁻⁷, 10⁻⁶ and 10⁻⁵ M. NR uptake and MTT reduction were measured as parameters of cytotoxicity. A dose-dependent effect of each compound on each parameter was obtained. Regression analysis was used to obtain the IC₅₀ of each compound. In both assays, IMM 125 was found to be the most toxic cyclosporin. CsA was shown to be more toxic in the NR-assay than CsG, while CsA was less toxic than CsG in the MTT-reduction assay. However, cytotoxicity percentages found for 10⁻⁶ M CsA, CsG and IMM 125 after 24 h of incubation were very small: 0.0% for all three as evaluated with NR assay and 7.4% (CsA), 4.4% (CsG), and 1.2% (IMM 125) as evaluated with the MTT test. Under the same conditions 0.1% DMSO did not cause significant cytotoxicity. When CsA, CsG and IMM 125 were incubated for half an hour with mesangial cells at the concentrations mentioned above, none of the cyclosporins was cytotoxic (data not shown).

Contractile effects on isolated rat glomeruli and cultured mesangial cells

PSA of incubated glomeruli were determined at 5, 10, 20 and 30 min after incubation with 10⁻⁶ M cyclosporins. After 30 min of incubation, CsA caused a statistically significant decrease in PSA compared to controls, while there was no significant difference compared to controls after treatment with CsG and IMM 125 (Figure 1). Under similar conditions, CsA caused a significant decrease in PSA in cultured rat mesangial cells, while IMM 125 was less effective compared to control (Figure 2). CsG was also found to induce a significant decrease in PSA of cultured rat mesangial cells compared to control. The decrease in PSA found for the control group at each time point of the incubation period was not statistically different from the initial value at T0: +0.63%, —1.22%, —3.33%, and —3.85% for 5, 10, 20 and 30 min incubation respectively. DMSO seems to cause this slight effect. There was no significant difference between CsA and CsG after 30 min of incubation.

Table 1. The IC₅₀ of each drug in cultured mesangial cells as assessed with two different cytotoxicity assays

<table>
<thead>
<tr>
<th>Assays</th>
<th>NR</th>
<th>MTT</th>
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<tbody>
<tr>
<td>CsA</td>
<td>1.5·10⁻⁴</td>
<td>7.0·10⁻⁴</td>
</tr>
<tr>
<td>CsG</td>
<td>1.8·10⁻⁴</td>
<td>2.8·10⁻⁴</td>
</tr>
<tr>
<td>IMM125</td>
<td>0.5·10⁻⁴</td>
<td>0.7·10⁻⁴</td>
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Values are expressed as M after regression analysis and all are statistically different from those of the control (P<0.001).
Myoreactivity of cyclosporins in vitro

At the concentrations of $0$, $10^{-9}$, $10^{-8}$, $10^{-7}$, $10^{-6}$, and $10^{-5} \text{M}$, CsA, CsG, and IMM 125 caused concentration-dependent contractions of isolated rat glomeruli and cultured rat mesangial cells 30 min after incubation. In glomeruli (Figure 3), CsA was the most potent contractile cyclosporin. Regarding the ratios of the slope of the dose-response curves, CsG and IMM 125 were between 4 and 5 times less potent in causing contractions than CsA. Regarding the absolute degree of mesangial cell contraction (Figure 4), IMM 125 had the least contractile activity, while CsA and CsG were almost equally myoreactive. In addition to the absolute degree of contraction, the number of responding glomeruli and the percentages of contracting glomeruli and mesangial cells were determined (at a decrease in PSA $>8\%$ at T30). There was no significant difference found in glomeruli (Figure 5). All three compounds increased the percentage of responding glomeruli equally. Differences among the compounds were found in the number of responding

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**Fig. 2.** Effects of $10^{-6}$M concentration of each drug and control solute on PSA of cultured rat mesangial cells. Data are expressed as mean $\pm$ SEM for each incubation time point. The levels of significance at T30 for the differences among the drugs are indicated on the graph: NS between CsG and CsA, $P<0.01$ (***) between CsG and IMM 125, $P<0.05$ (*) between CsA and IMM 125. Decreases in PSA are significantly time-dependent after regression analyses with $r=0.995$ ($P<0.0005$), $0.993$ ($P<0.0068$) and $0.984$ ($P<0.005$) respectively for CsA, CsG and IMM 125.

**Fig. 3.** Representative data from two different experiments expressed as mean $\pm$ SEM. All values are significantly different from those obtained at T0 ($P<0.001$). CsA vs control: levels of significance are expressed as *, **, and *** for $P<0.05$, $P<0.01$, and $P<0.001$ respectively. Other symbols are † for CsG and $\dagger$ for IMM 125. The values obtained by regression analysis are $r=0.981$ ($P<0.01$), $0.957$ ($P<0.001$), $0.838$ ($P<0.08$) respectively for CsA, CsG and IMM 125. The slope ratios of CsA/CsG was 1.1 and that of CsA/IMM 125 was 1.4.

**Fig. 4.** Data are expressed as mean $\pm$ SEM. All values are significantly different from those obtained at T0 ($P<0.001$). Levels of significance for CsA versus control are expressed as *, **, and *** for $P<0.05$, $P<0.01$, and $P<0.001$ respectively. Other symbols are † for CsG and $\dagger$ for IMM 125. The values obtained by regression analysis are $r=0.981$ ($P<0.01$), $0.957$ ($P<0.001$), $0.838$ ($P<0.08$) respectively for CsA, CsG and IMM 125. The slope ratios of CsA/CsG was 1.1 and that of CsA/IMM 125 was 1.4.

**Fig. 5.** Percentage of responding isolated glomeruli. Response is defined as a $>8\%$ decrease in PSA of isolated rat glomeruli after a 30 min incubation with one of the three cyclosporins. The values obtained by regression analysis are $r=0.938$ ($P<0.05$), $0.978$ ($P<0.005$), $0.968$ ($P<0.01$) for CsA, CsG and IMM 125. The slope ratios of CsA/CsG was 1.5 and that of CsA/IMM 125 was 1.8.
mesangial cells (Figure 6). CsA caused the greatest increase in individual mesangial cells compared to the total amount of investigated cells, CsG was less effective. The slope of the IMM 125 dose response curve was nearly similar to that of CsG.

**Discussion**

The results obtained by image analysis demonstrate that non-cytotoxic concentrations of CsA and both of its derivatives, CsG and the novel compound IMM 125, can reduce PSA in a dose- and time-dependent way in isolated glomeruli and cultured mesangial cells.

The use of image analysis techniques for the measurement of cell contractions has been extensively described in the current literature [9–11,15]. Cytotoxicity might also cause a decrease in PSAs. In the present study we could exclude such unspecific effects of the applied cyclosporins. After 30 min of incubation, none of the cyclosporins was cytotoxic and no detachment of the cells was observed in any case as described elsewhere [11].

Various studies have shown that results obtained by image analysis correlate very well with biochemical parameters of contraction [15,16]. Our morphometrical data correlate well with results on biochemical mediators of contraction, which were measured by other investigators in mesangial cells after CsG and CsA treatment. In mesangial cells we found a nearly similar absolute decrease in the PSAs with CsG and CsA. This is in accordance with investigations reporting no significant differences between CsA and CsG concerning increased cytosolic Ca$_{2+}$ concentrations [3], decreased PGI$_2$ and endothelin production [19] and the effects of L-arginine pathways [20]. Thus, these results are helpful to validate the present morphometrical technique. These findings clearly demonstrate that non-cytotoxic concentrations of CsA can reduce PSAs in a dose- and time-dependent way in both isolated glomeruli and cultured mesangial cells. In glomeruli, IMM 125 and CsG were less myoreactive, while CsA clearly had the strongest contractile effect. This was demonstrated by slope ratios and by absolute values. In mesangial cells, CsA and IMM 125 behaved in a similar rank order, IMM 125 was less myoreactive than CsA. Although the slopes of all compounds were very similar, CsG had the strongest effects with regard to the absolute extent of contraction. The differences in the responses of glomeruli and in the mesangial cell suggest that mesangial cells are the trigger for the glomerular contraction. However, this apparent contradiction might be explained by the differences in the number of mesangial cells responding generally to the different cyclosporins. It is very obvious that both, the degree and number of contracting mesangial cells determine the glomerular response. In the case of CsG, the degree of contraction was slightly more pronounced, with regard to the slope of the dose response curve, but the number of responding mesangial cells was clearly smaller. Considering these two effects together, CsA has overall greater contractile glomerular activity than CsG.

The contractile status of the glomerulus seems to be a major determinant of renal blood flow and ultrafiltration surface. Decrease in the glomerular ultrafiltration surface by contraction is a plausible explanation for the decrease in glomerular filtration. The findings from the present experimental systems might possibly be extrapolated to GFR. The hypothesis that the present findings might be relevant *in vivo* is supported by the lower nephrotoxicity of CsG or IMM 125 in rats. In these studies, it was shown that both compounds at the same dose as CsA had less effects on GFR. The doses used in this study may be considered relevant for the *in vivo* situation since rats treated daily for 10 days at a dose of 50 mg/kg p.o. achieved kidney and peak plasma concentrations in the range of 10$^{-5}$ M [1]. In renal transplant patients, the CsA plasma trough levels were in the range of 10$^{-5}$–10$^{-8}$ M; however, it was shown that the peak levels are higher, in the range of 10$^{-8}$ M [21]. Therefore, the concentrations, which were applied in the present *in vitro* system were below or in the range of those achieved in the plasma of rats and humans.

In conclusion, since the concentrations of drugs used in the present experiments were close to that found in animals, the results show that the contractile effects of IMM 125 and CsG in isolated glomeruli were clearly smaller than CsA, which is in agreement with *in vivo* rat data concerning GFR changes. The present results suggest that these *in vitro* systems might serve as predictive tools for the evaluation of cyclosporin-induced changes in GFR and that IMM 125 and CsG are less nephrotoxic than CsA.
References