Role of P-glycoprotein in cyclosporine cytotoxicity in the cyclosporine–sirolimus interaction

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Cyclosporine nephrotoxicity remains a major side effect in solid organ transplantation, and can be exacerbated by concomitant administration of sirolimus. Cyclosporine and sirolimus are P-glycoprotein (Pgp) substrates. We hypothesized that the Pgp activity level may affect cyclosporine cytotoxicity by interfering with the ability of Pgp to remove cyclosporine from within tubular cells, and that an interaction between cyclosporine and sirolimus on Pgp function may explain the enhancement of cyclosporine nephrotoxicity by sirolimus. Cyclosporine cytotoxicity was evaluated in primary cultures of normal human renal epithelial cells (HRECs) by cell viability and cytotoxicity assays. Verapamil, quinine, PSC833, and PGP-4008 were used as Pgp inhibitors. Rhodamine-123 (R-123), a fluorescent substrate of Pgp, was used to assess Pgp-mediated transport. Cellular cyclosporine concentration was measured by high-performance liquid chromatography coupled to tandem mass spectrometry. Pgp expression and function were confirmed in HRECs and cyclosporine and sirolimus were shown to be Pgp inhibitors in this model. Verapamil-induced inhibition of Pgp led to a significant increase in cellular concentration of cyclosporine ($P < 0.05$). Cyclosporine exerted a concentration-dependent cytotoxic effect on HRECs that was significantly increased by inhibition of Pgp activity. Sirolimus exerted an inhibitory effect on R-123 efflux in HRECs and increased cellular cyclosporine concentrations in a dose-dependent manner. These data demonstrate that Pgp plays a critical role in protecting renal epithelial cells from cyclosporine toxicity. The inhibitory effect of sirolimus on Pgp-mediated efflux and the cellular concentration of cyclosporine could explain the exacerbation of cyclosporine nephrotoxicity observed clinically.

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Cyclosporine A, a member of the calcineurin inhibitor class of drugs, remains a mainstay of immunosuppressive regimens to prevent allograft rejection following solid organ transplantation.[1] In renal transplantation, the major limiting factor for use of cyclosporine is nephrotoxicity, which may reduce the overall benefit of cyclosporine therapy with respect to long-term graft survival.[2] In a prospective study of the natural history of chronic allograft nephropathy, cyclosporine appeared to be the chief cause of late ongoing histologic injury, even in grafts with excellent early histologic findings.[3] Two forms of cyclosporine nephrotoxicity have been described. Acute (or 'functional') nephrotoxicity is mediated by an imbalance of vasoconstrictors and vasodilators within the renal vasculature, leading to vasoconstriction and sustained renal ischemia.[2] Chronic (or 'structural') cyclosporine nephropathy is defined as irreversible renal dysfunction associated with morphological injuries such as tubular atrophy, interstitial fibrosis and arteriolar hyalinosis.[4] Cyclosporine may induce these changes by promoting multiple, inter-related pathophysiological processes.[5] A combination of cyclosporine-induced hemodynamic changes and direct toxic effects of cyclosporine on tubular epithelial cells is thought to play a role in the development of interstitial fibrosis via the release of factors such as transforming growth factor-beta, endothelin-1, plasminogen activator inhibitor-type 1, inducing fibroblast proliferation, and matrix synthesis.[6] Cyclosporine may also activate apoptosis genes and increase apoptosis in tubular and interstitial cells, thereby inducing tubular atrophy.[7]

Another mechanism that may contribute to cyclosporine-related nephrotoxicity, but which has not been completely explored, involves the P-glycoprotein (Pgp). The *ABCB1* (previously multidrug-resistance-1) gene product Pgp is a membrane protein, which functions as an ATP-dependent exporter of intracellular xenobiotics. Its importance was first recognized as a consequence of its role in the development of multidrug resistance in cultured tumor cells against various anticancer agents. In the kidney, Pgp is constitutively expressed on the brush border of the proximal tubular cells and on the distal tubule[8] and it has been suggested that Pgp may be instrumental in cyclosporine nephrotoxicity.

Cyclosporine is a substrate of Pgp,[9] and variations in expression and/or function of Pgp could lead to accumulation of cyclosporine, along with other cytotoxic agents, within the tubular cells. In support of this hypothesis, immunohistological studies by Del Moral *et al.*[10] have shown an inverse relationship between cyclosporine deposits in renal tissue and the level of Pgp expression in proximal tubular cells in animal models, suggesting that the normal Pgp response may be defective in patients susceptible to cyclosporine-related nephrotoxicity, leading to retention of excess amounts of cyclosporine in the cells.[11,10] More recently, Koziolek *et al.*[8] showed that low expression of Pgp in renal parenchymal cells was associated with the occurrence of cyclosporine nephrotoxicity. Moreover, a *ABCB1* polymorphism in kidney allograft donors, which is associated with decreased expression of Pgp in renal tissue, has been shown an independent risk factor for the development of cyclosporine-related nephrotoxicity.[12] Together, these findings suggest that factors that modulate Pgp expression may have an impact on cyclosporine-related nephrotoxicity by causing an accumulation of cyclosporine within the renal cells.

The new immunosuppressive agent sirolimus is also a Pgp substrate,[13] and an interaction between cyclosporine and sirolimus is seen clinically. Although perceived as a non-nephrotoxic drug, administration of sirolimus reduces renal function when given concomitantly with cyclosporine.[14] We hypothesized that Pgp may be the site of this interaction. Experimental studies in salt-depleted rats have demonstrated that sirolimus increases intrarenal cyclosporine concentrations to a greater extent than blood concentration of cyclosporine.[15] In addition, even subtherapeutic doses of cyclosporine and sirolimus exert a synergistic effect on the development of chronic nephrotoxicity in rats[16] and recent studies have shown that administration of sirolimus around the time of renal injury can exacerbate the injury and delay repair, an effect that may be due to a potent antiproliferative effect of sirolimus on renal tubular cells.[17]

We therefore postulated that an interaction between cyclosporine and sirolimus could limit Pgp-mediated drug efflux and increase cyclosporine cytotoxicity by limiting the removal of cyclosporine from tubular cells. The present study was undertaken using human renal epithelial cells (HRECs) in primary culture to investigate firstly, whether impaired Pgp function may contribute to nephrotoxicity by interfering with the ability of Pgp to remove cyclosporine from the tubular cell, thereby increasing cyclosporine intracellular toxicity, and secondly, to assess the effect of sirolimus on Pgp-mediated efflux of cyclosporine.

RESULTS

Expression of Pgp in HRECs

Expression of Pgp by the HRECs was confirmed with Western blot analysis of crude membranes using the anti-Pgp monoclonal antibody C219. Results showed varying levels of expression of Pgp on the crude membrane from different kidneys (Figure 1a).

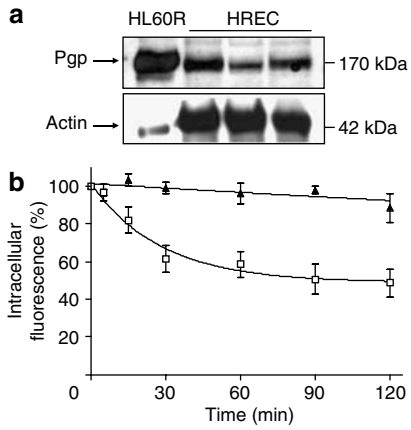

Figure 1 | Expression and function of Pgp in normal HRECs. (**a**) Immunoblot analysis of Pgp in crude membranes from HL60R cells, overexpressing Pgp and used as positive controls (left) and from HRECs derived from three different kidneys using the C219 monoclonal anti-Pgp antibody (10 μg/ml). (**b**) Immunoblotting for actin confirmed equal loading for HREC samples Cellular efflux of R-123. with or without the Pgp inhibitor verapamil. Cells were preloaded for 1 h with 5 μg/ml R-123 and thereafter incubated with R-123-free medium with verapamil 40 μM (triangles) or without verapamil (squares). Intracellular fluorescence was quantified at successive timepoints after the removal of R-123 from the medium. Results are expressed as percentage of the initial intracellular fluorescence, corrected by the total protein content of each sample, before any R-123 efflux. All determinations were performed in triplicate plates. Data shown represent the mean $\pm$ s.e.m. of five independent experiments performed with cells from three different kidneys.

To ensure that the efflux properties of Pgp are not altered by the isolation procedure or culture of HRECs, Pgp activity was evaluated by measuring the effect of Pgp inhibition on cellular efflux of the fluorescent Pgp substrate R123 in the presence or absence of 40 μM verapamil. Figure 1b plots the decrease in intracellular fluorescence in a time-dependent manner. Cellular fluorescence decreased by 40% after an efflux time of 30 min without verapamil, a reduction that was dramatically attenuated in the presence of verapamil. Pgp blockage limited the efflux to $<10\%$ after 120 min.

Effect of cyclosporine on Pgp-mediated efflux

In the absence of cyclosporine, 55% of R123 remained within HRECs after an efflux time of 30 min. Cyclosporine increased cellular retention of R123 in a concentration-dependent manner (Figure 2a). The maximum inhibitory effect was obtained with clinically relevant concentrations of cyclosporine concentrations (>1 μM), above which R123 efflux was almost entirely blocked and R123 intracellular retention was 90%.

In order to analyze the consequences of Pgp inhibition on cyclosporine accumulation, intracellular concentration of cyclosporine was measured after varying durations of cellular efflux, with and without verapamil. Cyclosporine concentration gradually decreased over 60 min (Figure 2b). In the presence of verapamil, this decrease was dramatically