Cyclosporin Exerts a Direct Fibrogenic Effect on Human Tubulointerstitial Cells: Roles of Insulin-Like Growth Factor I, Transforming Growth Factor $\beta_1$, and Platelet-Derived Growth Factor$^1$

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ABSTRACT

To assess the direct fibrogenic effects of cyclosporin A (CyA) on the human tubulointerstitium, primary cultures of human renal proximal tubule cells (PTC) and renal cortical fibroblasts (CF) were incubated for 24 h with various concentrations of CyA. Cytotoxicity was confirmed in both cell populations by dose-dependent inhibition of thymidine incorporation, viability, and PTC apical sodium-hydrogen exchange activity (ethyliosporopolamidol-sensitive apical $^{22}$Na$^{+}$ uptake). Compared with controls, both 500 and 1000 ng/ml CyA significantly stimulated CF collagen synthesis (proline incorporation 4.6 $\pm$ 0.4, 6.5 $\pm$ 0.8, and 7.1 $\pm$ 1.0%, respectively; $p < .05$) and inhibited matrix metalloproteinase-2 (100%, 85.7 $\pm$ 10.0%, and 38.8 $\pm$ 9.2%) and matrix metalloproteinase-9 activity (100%, 110.6 $\pm$ 9.2%, and 49.9 $\pm$ 12.8%). CyA did not affect CF secretion of transforming growth factor $\beta_1$, but markedly stimulated insulin-like growth factor-I (IGF-I) secretion and inhibited secretion of both IGF-I binding protein-(IGFBP)-3 and IGFBP-2. CyA-induced CF collagen synthesis was abrogated by 5 $\mu$g/ml anti-IGF-I receptor antibody, but not by 5 $\mu$g/ml murine nonimmune globulin. Increasing concentrations of CyA progressively augmented PTC secretion of the fibrogenic cytokines transforming growth factor-$\beta_1$, and platelet-derived growth factor. These results indicate that clinically relevant concentrations of CyA are directly toxic to PTC and CF, irrespective of hemodynamic effects, and promote interstitial fibrosis by inhibiting matrix degradation and stimulating cortical fibroblast collagen synthesis via induction of autocrine IGF-I action. The latter effect may be further accentuated by the ability of CyA to augment secretion of transforming growth factor $\beta_1$, and platelet-derived growth factor by PTCs.

The use of cyclosporin A (CyA) has resulted in substantial improvements in treatment outcomes in organ transplantation and certain forms of autoimmune disease. However, chronic CyA nephrotoxicity, characterized by early disturbances of proximal tubule structure and function progressing to tubular atrophy and interstitial fibrosis, is a universally recognized side effect of CyA therapy (Kopp and Klotman, 1990; Sokol et al., 1990; Wilson and Hartz, 1991; Shehata et al., 1994). Once established, chronic CyA nephropathy is irreversible and may progress to end-stage renal failure (Versluis et al., 1988; Kopp and Klotman, 1990). Although higher CyA levels appear to be associated with more severe degrees of nephrotoxicity (Klintmalm et al., 1984; Versluis et al., 1988), the pathogenesis remains poorly understood.

CyA-induced afferent arteriolar vasoconstriction and chronic ischemia have been postulated as underlying mechanisms of chronic cyclosporin nephropathy (Kopp and Klotman, 1990). However, the demonstration of a clear dissociation between glomerular filtration rate and tubulointerstitial fibrosis in experimental chronic CyA nephropathy suggests that nonhemodynamic factors are important (Elzinga et al., 1993). These factors could include direct effects on tubulointerstitial cells (Becker et al., 1987; Trifillis and Kahng, 1988; Wolf et al., 1990; Sokol et al., 1990; Wilson and Hartz, 1991; Wolf et al., 1990).

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ABBREVIATIONS: CyA, cyclosporin A; aIR, anti-IGF-I receptor neutralizing antibody; CF, renal cortical fibroblast; CM, conditioned media; DMEM/F-12, Dulbecco’s modified Eagle’s medium and Ham’s F-12 (1:1 mixture); IGF-I, insulin-like growth factor-I; IGFBP, IGF binding protein; MMP, matrix metalloproteinase; NHE, sodium-hydrogen exchange; PDGF, platelet-derived growth factor; PTC, proximal tubule cell; TGF, transforming growth factor; TCA, trichloroacetic acid; dpm, disintegrations per minute; ELISA, enzyme-linked immunoassay; RIA, radioimmunoassay.
Ghiggeri et al., 1994) and indirect effects through stimulation of local fibrogenic polyglycopeptide growth factor production (Wolf et al., 1995). Both proximal tubule cell (PTC) and renal cortical fibroblasts (CF) are capable of secreting collagenous proteins, resulting in interstitial fibrosis (Wolf et al., 1990; Ghiggeri et al., 1994), and known fibrogenic cytokines, such as insulin-like growth factor-I (IGF-I; Feld and Hirschberg, 1996), transforming growth factor-β (TGFβ; Shihab et al., 1996), and platelet-derived growth factor (PDGF) (Abboud, 1995), have been shown to be secreted by one or both of these cells (Kartha et al., 1988; Wolf et al., 1995; Abboud, 1995; Johnson et al., 1997c). Moreover, it has recently been demonstrated in our laboratory that CF collagen synthesis is stimulated by proximal tubule-derived TGFβ1, and PDGF (Johnson et al., 1998a).

Unfortunately, attempts to study the direct effects of CyA on tubulointerstitial growth, fibrogenesis, and cytokine secretion have to date been severely hampered by confounding hemodynamic effects, the cellular heterogeneity of the kidney, and the lack of a suitable animal model of chronic CyA nephropathy, which adequately reproduces the exquisite sensitivity of the human renal tubulointerstitium to CyA without having to administer large doses under conditions of salt-depletion (Provoost et al., 1986).

The aims of the present study, therefore, were to determine whether CyA exerted direct toxic and profibrotic effects on primary cultures of human CF and PTC and to evaluate the contributions of secreted growth factors (IGF-I, TGFβ1, and PDGF) to fibrogenesis.

**Materials and Methods**

**Patients.** Segments of macroscopically and histologically normal renal cortex were obtained aseptically from adult human kidneys removed surgically because of small (< 6 cm) renal adenocarcinomas (n = 7), benign angiomylipoma (n = 1), or benign renal cyst (n = 1). The average patient age was 59.6 ± 5.1 years old and the male/female ratio was 5:4. Patients were otherwise healthy and were on no medications. Informed consent was obtained before each operative procedure, and the use of human renal tissue for primary culture was reviewed and approved by the Royal North Shore Hospital Human Medical Research Ethics Committee.

**Cell Culture.** The method for primary culture of human PTC and CF is described in detail elsewhere (Johnson et al., 1997a,c). Briefly, renal cortical tissue was dissected from the medulla, minced, digested with collagenase (class 2, 383 U/mg, Worthington Biochemical Corp., Freehold, NJ), and passed through a 100 μm mesh. Filtered tissue was resuspended in 45% Percoll (Pharmacia, Uppsala, Sweden) and separated into four distinct bands by isopyknic ultracentrifugation. The uppermost band was removed for CF culture and the lowest band for PTC culture. CFs were grown in antibiotic-free, hormone-defined media, consisting of 1:1 (v/v) DMEM/F-12 (ICN Pharmaceuticals, Inc., Costa Mesa, CA) supplemented with 10% fetal calf serum (Trace Biosciences, Sydney, Australia), whereas PTC were cultured in serum-supplemented DMEM/F-12) supplemented with 10% fetal calf serum (Trace Biosciences, Sydney, Australia).

**Experimental Protocol.** All experiments were performed on confluent, quiescent, passage 2 PTC and CF. Cells were made quiescent by three washes followed by incubation for 24 h in basic media (DMEM/F-12 containing 5 μg/ml human transferrin). Basic media containing between 0 and 10,000 ng/ml CyA (Novartis, Basel, Switzerland) were added for an additional 24 h to simulate clinically relevant and toxic tissue CyA concentrations (Rosano et al., 1986; Lensmeyer et al., 1991). A 10 mg/ml stock solution of CyA was prepared in absolute ethanol and subsequently diluted as necessary to provide the required test concentrations. The final concentration of ethanol was standardized at 0.1% for all wells (including controls). This concentration of alcohol has been shown previously to have no effect on tubule cell and fibroblast viability and function (Sokol et al., 1990; Ong et al., 1993). Our results confirmed this for all parameters studied (data not shown).

After the above incubations, studies of cellular viability, growth, and transport were initially performed to confirm that CyA was directly toxic to human tubulointerstitial cells, as has been found in nonhuman animal species (Becker et al., 1987; Raphael and Fish, 1987; Sokol et al., 1990). The direct fibrogenic effects of CyA were then evaluated by measuring tubulointerstitial collagen synthesis, matrix metalloproteinase (MMP) activities, and secretion of fibrogenic cytokines.

**Cytotoxicity Assays.** Cell viability was measured by both exclusion of trypan blue entry (Johnson et al., 1997a) and by release of lactate dehydrogenase (Mitchell et al., 1981). Tritiated thymidine incorporation, an index of DNA synthesis, was assessed according to a previously described method (Johnson et al., 1997b). Apical NHE activity, the major source of PTC oxygen and energy consumption (Johnson et al., 1997b), was determined by measuring the ethylisopropylamidolide-sensitive component of apical 22Na uptake at 1 min after intracellular acidification by a standard 20 mM NH4Cl prepulse. The values of this experimental system for measuring apical NHE activity has been previously confirmed in our laboratory (Johnson et al., 1997a,b).

The effects of CyA on PTC function were further evaluated by measuring the synthesis of transepithelial Na+ and K+ gradients. The presence of these gradients depends on intact monolayers with functional active transport mechanisms (Johnson et al., 1997a).

**Collagen Synthesis Assay.** CF and PTC total collagen synthesis was measured by tritiated proline incorporation (Guida et al., 1993). After 24-h incubation in control or test media, cells were washed and incubated for an additional 3 h under the same conditions in the presence of 20 μCi/ml 2,3-3H-proline (Amersham), 50 μg/ml 1-ascorbic acid (Sigma), and 60 μg/ml β-aminopropionitrile (Sigma). Media containing secreted, noncross-linked collagen were collected on ice and precipitated overnight at 4°C with 1 ml 10% trichloroacetic acid (TCA) containing 0.02% unlabelled proline (Sigma). Pellets obtained by centrifugation at 1250g for 5 min were washed an additional three times in 1 ml of 5% TCA and 0.01% proline and subsequently dissolved in 200 μl 0.2 mol/liter NaOH. The solution was titrated to pH 7.6 with 130 μl 0.2 mol/liter HCl and 100 μl was incubated for 1 h at 37°C with 400 μl Tris-CaCl2-N-ethylmaleimide buffer (50 mM Tris-HCl, 5 mM CaCl2, 2.5 mM N-ethylmaleimide, 0.02% Na3cit) in both the presence and absence of 2 mg/ml collagenase (Worthington class II). The reaction was terminated by precipitation with ice-cold 250 μl 40% trichloroacetic acid and 250 μl 2% tannic acid for 1 h at 4°C. Samples were centrifuged at 1250g for...
5 min. Supernatants were counted in a β-counter (LKB Wallac, Turku, Finland) and counts obtained from samples that were not exposed to collagenase served as background values. Pellets derived from the collagenase group were solubilized in 200 μl 0.2 mol/liter NaOH and counted to measure proline incorporation into collagenase-insensitive proteins. The percentage of total protein synthesized as collagen (% collagen) was calculated as the ratio of collagenase-releasable disintegrations per minute (dpm) divided by total dpm (supernatant plus pellet) as follows:

\[
\% \text{ collagen} = \frac{(C/P)((5.4 \times (1 - C/P)) + (C/P)) 	imes 100}
\]

where \( C \) = collagenase-releasable dpm in supernatants and \( P \) = collagenase-insensitive dpm in pellets.

A correction factor of 5.4 for noncollagen protein was used to adjust for the relative abundance of proline and hydroxyproline in proteins containing collagen (Guarda et al., 1993).

**Gelatin Zymography.** Zymography was used to visualize MMP activities in serum-free media conditioned for 24 h by CF exposed to various concentrations of CyA. After centrifuging to remove cellular debris, media samples containing equal amounts of protein were subjected to SDS-polyacrylamide gel electrophoresis under nonreducing conditions through a 4% polyacrylamide stacking gel and resolved in a 10% separating gel containing 1 mg/ml gelatin (Sigma). Gels were run at 8 to 12 mA/Agel and at a constant 125 V for approximately 90 min at 4°C. After electrophoresis, the gels were incubated in 2.5% Triton X-100 (Sigma) with gentle shaking for 1 h at room temperature with one change of detergent solution. The gels were rinsed in distilled water and incubated overnight at 37°C in substrate buffer (50 mM Tris-HCl, 100 mM NaCl, 10 mM CaCl\(_2\), 0.02% NaN\(_3\), pH 7.5). After incubation, the gels were stained for 15 min in 0.2% Coomassie blue R-250 (Bio-Rad, Hercules, CA) dissolved in 50% ethanol/10% acetic acid followed by destaining for 15 min in 30% ethanol/10% acetic acid. MMP activities appeared as clear lytic bands that were quantitated by densitometry using the Kodak electrophoresis documentation and analysis system (Kodak Scientific Imaging Systems, Rochester, NY).

**Measurement of Growth Factors in Conditioned Media.** We and others have previously demonstrated that CF secrete TGFβ\(_1\), IGF-I, IGF binding protein (IGFBP)-2, and IGFBP-3, but not other IGFBPs or PDGF (Wolf et al., 1985; Johnson et al., 1997c). In contrast, PTC secretes TGFβ\(_1\) and PDGF, but not IGF-I or IGFBPs (Kartha et al., 1988; Abboud, 1995; Johnson et al., 1998a). We have additionally demonstrated in conditioned media and coculture studies that these secreted cytokines are involved in the autocrine and paracrine regulation of interstitial matrix deposition (Johnson et al., 1988a). Levels of these fibrogenic growth factors in CF-conditioned media (CF-CM) and PTC-CM were measured after 24-h exposure to various concentrations of CyA.

CF secretion of IGF-I into culture media was determined by specific RIA after Bio-Spin chromatographic separation of IGFBPs, as previously described (Johnson et al., 1997c). IGFBP-3 and IGFBP-2 levels in CF-CM samples were measured using previously published specific radioimmunoassays (Johnson et al., 1997c).

TGFβ\(_1\) levels in CF- and PTC-CM were measured using a commercially available enzyme-linked immunosassay (ELISA) kit (Promega TGFβ\(_1\), ELISA, Promega, Madison, WI). Total (active plus latent) TGFβ\(_1\) was determined after transient acidification of CM. One ml of CM was incubated with 30 μl 1 mol/liter HCl for 10 min at room temperature, followed by neutralization with an equivalent volume of 1.2 mol/liter NaOH/0.5 mol/l HEPES. The minimum detectable concentration of TGFβ\(_1\) by the assay was 15.6 pg/ml.

PDGF (AB heterodimer) was also measured in CF-CM samples by commercial ELISA kit (human PDGF Biotrak assay, Amersham). Cross-reactivities with the AA and BB dimers of PDGF were 10% and 2%, respectively. The minimum detectable concentration of PDGF by the assay was 8.4 pg/ml. Intra- and interassay coefficients of variation for both ELISA kits were less than 5 and 10%, respectively.

**Role of Autocrine IGF-I Action in Mediating Effects of CyA on CF Collagen Synthesis.** The contribution of CF-derived IGF-I to the effects of CyA on CF collagen synthesis was assessed by incubating CF for 24 h in basic media supplemented with vehicle alone (control), 5 μg/ml mouse monoclonal anti-IGF-I receptor neutralizing antibody (aIR) alone (Santa Cruz Biotechnologies, Santa Cruz, CA) or 1000 ng/ml CyA in the presence or absence of either 5 μg/ml aIR or 5 μg/ml murine nonimmune globulin (Sigma). Collagen synthesis was then measured as described above.

**Statistical Analysis.** All studies were performed in triplicate from PTC and CF cultures obtained from at least three separate human donors. Each experiment contained internal controls originating from the same culture preparation. For the purposes of analysis, each experimental result was expressed as a change from the control value, which was regarded as 100%, and analyzed independently. Results are expressed as mean ± S.E.M. or median (interquartile range), depending on data distribution. Statistical comparisons between groups were made by analysis of variance for parametric data and by the Kruskal Wallis test for nonparametric data. Pairwise multiple comparisons were made by Fisher’s protected least significant differences test. Analyses were performed using the software package Statview version 4.5 (Abacus Concepts Inc., Berkeley, CA). p values less than .05 were considered significant.

**Results**

**CYA Exerts Direct Toxic Effects on CF and PTC at Pharmacologically Relevant Concentrations.** Under control conditions, trypan blue was consistently excluded by greater than 95% of cells. However, CyA exerted dose-dependent cytotoxicity in both cell populations, as evidenced by decreased trypan blue exclusion and increased lactate dehydrogenase release at concentrations of 1000 ng/ml or greater (Table 1). Cell growth was also initially suppressed by CyA at 1000 ng/ml and became more marked at 10,000 ng/ml (Table 1). PTC exposed to CyA exhibited suppressed apical NHE activity (Table 1), diminished transepithelial sodium gradients (values for 0, 100, 500, 1000, and 10,000 ng/ml CyA were 4.4 ± 0.3, 4.6 ± 0.2, 4.2 ± 0.4, 2.9 ± 0.7, and 2.8 ± 0.6 mM, respectively, \( n = 9, p < .05 \)), and reduced transepithelial potassium gradients (1.7 ± 0.1, 1.5 ± 0.0, 1.6 ± 0.1, 1.3 ± 0.1, and 1.2 ± 0.2, respectively, \( n = 9, p < .05 \)).

**CYA Differentially Affects Collagen Synthesis in CF and PTC.** CyA concentrations of 500 and 1000 ng/ml significantly stimulated CF collagen synthesis (Fig. 1). The amounts of secreted total (collagenous + noncollagenous) protein were not significantly different between any of the groups (values for 0, 100, 500, and 1000 ng/ml CyA were 40.5 ± 6.4, 40.9 ± 7.2, 42.1 ± 7.4, and 42.7 ± 7.2 μg, respectively; \( n = 18, p = \) not significant).

Proline incorporation into collagenase-sensitive proteins in PTC-CM containing secreted, noncross-linked collagen was not significantly altered by increasing CyA concentrations (control 9.0 ± 1.4% of total protein synthesis, 100 ng/ml CyA 8.3 ± 1.0%, 500 ng/ml CyA 8.7 ± 1.0%, and 1000 ng/ml CyA 7.6 ± 0.5%; \( n = 18, p = \) not significant).

**CYA Inhibits CF Secretion of MMPs.** Zymography of media conditioned by CF under control conditions demonstrated gelatinolytic bands at 92 kDa and 72 kDa, corresponding to MMP-9 (gelatinase B) and MMP-2 (gelatinase A), respectively (Fig. 2). Exposure of CF to increasing concentra-
tions of CyA resulted in a progressive inhibition of the activities of both of these MMPs. Median (interquartile range) MMP-9 band intensities in CF-CM in the presence of 0, 100, 1000, and 2000 ng/ml CyA were 1119 (284), 1450 (684), 414 (108), and 18 (12) arbitrary densitometric units, respectively (n = 3, p = .01). Corresponding values for MMP-2 band intensities were 1078 (642), 982 (467), 320 (16), and 99 (23) arbitrary densitometric units (n = 3, p = .01).

**CyA Stimulates CF and PTC Secretion of Fibrogenic Growth Factors.** IGF-I secretion by CF was stimulated by CyA in a dose-dependent fashion (Fig. 3) and reached a level 3 times that of controls in the presence of 1000 ng/ml CyA. In contrast, CF secretion of IGFBP-3 and IGFBP-2 was significantly inhibited at this concentrations (Fig. 4). In keeping with a previous study (Johnson et al., 1997c), PTC did not secrete detectable quantities of IGF-I or IGFBPs.

TGFβ11 secretion by CF was not significantly altered over the range of CyA concentrations tested (control 1.14 ± 0.52, 100 ng/ml CyA 1.02 ± 0.50, 1000 ng/ml CyA 1.23 ± 0.71, and 10,000 ng/ml CyA 1.50 ± 0.77 ng/mg protein/day; n = 9, p = not significant). In contrast, PTC secreted markedly increased amounts of TGFβ11 at a CyA level of 500 ng/ml or greater (Fig. 5).

PTC secretion of PDGF was also augmented by CyA in a dose-dependent manner (control 0.34 ± 0.04, 100 ng/ml CyA

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**TABLE 1**

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<th>Toxicity Measure</th>
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*p < .05 versus control.

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**Fig. 1.** Collagen synthesis by CF exposed to CyA. Three-hour proline incorporation into collagenase-sensitive proteins was measured in CF (n = 18) incubated for 24 h with various concentrations of CyA. Results are expressed as a percentage of total protein synthesis. *p < .05 versus control.

**Fig. 2.** Effects of CyA on CF secretion of MMPs. Media conditioned by CF for 24 h in the presence of vehicle (0.1% alcohol) and various concentrations of CyA (0–2000 ng/ml) were subjected to standard gelatin zymography. Equal amounts of total protein were loaded in each lane. Typical MMP-9 and MMP-2 activities are evident as clear, lytic bands at 92 and 72 kDa, respectively.
0.44 ± 0.05, 1000 ng/ml CyA 0.49 ± 0.10, and 10,000 ng/ml CyA 0.60 ± 0.09 ng/mg protein/day; n = 9, p < .05). Significant stimulation of secretion was first observed at 10,000 ng/ml CyA. PDGF was not detected in CF-CM.

**CyA Stimulates CF Collagen Synthesis by Induction of Autocrine IGF-I Action.** As demonstrated previously, proline incorporation into collagenase-sensitive proteins in CF-CM was significantly greater in CF incubated for 24 h with 1000 ng/ml CyA than with vehicle alone (controls; Fig. 6). This stimulation of CF collagen synthesis by CyA was completely and significantly inhibited by coincubation with 5 μg/ml aIR, although aIR exerted no effect on CF collagen synthesis under basal conditions. An identical concentration of murine nonimmune globulin did not affect CF collagen synthesis under basal (98.6 ± 11.9% of controls; n = 12, p = not significant) or CyA-stimulated conditions (134 ± 12.2% of controls; n = 12, p < .01), implying that the effect of aIR on the latter was specifically related to blockade of autocrine IGF-I action.

**Discussion**

Chronic CyA nephrotoxicity is characterized by early structural and functional derangements of the proximal tubule (Kopp and Klotman, 1990), increased cortical expression of polypeptide growth factors such as TGFβ1 (Wolf et al., 1995; Shihab et al., 1996) and PDGF (Shehata et al., 1994), and extracellular matrix accumulation (Kopp and Klotman, 1990; Nast et al., 1991; Wolf and Nielson, 1992). The present in vitro study demonstrates that some or all of these pathologic
events might be attributable to the direct actions of CyA on human PTCs and CF, independent of hemodynamic effects. Furthermore, important novel findings of this study include the demonstration that CyA-induced extracellular matrix accumulation in vitro is likely to be due to a combination of suppressed MMP activity and augmented CF collagen synthesis. The latter is in turn related to facilitated autocrine IGF-I action.

In keeping with previous studies in LLC-PK₁ (Becker et al., 1987), MDCK (Raphael and Fish, 1987), rabbit PTC (Sokol et al., 1990), and human PTC (Trifillis and Kahng, 1988; Wilson and Hartz, 1991), CyA exerted dose-dependent cytotoxicity in human PTC and CF characterized by reduced cellular viability, decreased growth, increased paracellular inulin permeability, impaired maintenance of transepithelial electrolyte gradients, and suppressed apical NHE activity. However, in contrast with previous studies that have used very high concentrations of CyA typically in the range of 10,000 ng/ml (8.3 μM) to 10⁶ ng/ml (830 μM) (Becker et al., 1987; Trifillis and Kahng, 1988; Sokol et al., 1990; Wilson and Hartz, 1991), human CF and PTC cytotoxicity in the present study consistently became evident at a concentration of approximately 1000 ng/ml. This concentration is likely to be clinically relevant because peak whole blood CyA concentrations frequently reach this level (Kovarik et al., 1994). Furthermore, Lensmeyer et al. (1991) demonstrated significant (at least 8-fold) renal concentration of CyA compared to blood, with measured tissue concentrations of the parent compound ranging between approximately 500 and 2000 ng/mg tissue. Studies reported by other investigators have confirmed these findings (Rosano et al., 1986; Kahn et al., 1986), although separate measurements of intra- and extracellular concentrations of CyA in renal tissues have not been made.
The demonstration that CyA significantly inhibits the activity of apical NHE has not been previously described in the kidney, although Rosoff and Terres (1986) have observed an inhibition of NHE1 activity in human lymphocytes by CyA. The mechanism of this inhibition remains uncertain. CyA has been reported to inhibit basolateral bicarbonate transport (Kanli and Terreros, 1991) and Na⁺,K⁺-ATPase (Tumlin and Sands, 1993), which would be expected to raise intracellular pH and sodium thereby reducing the electrochemical driving force for apical NHE. However, this could not account for the inhibition of apical NHE in the present study, because PTC basolateral transporters were inactivated by the use of bicarbonate-free buffers and ouabain. TGFβ has also been reported to inhibit sodium influx into BSC-1 cells (Walsh-Reitz et al., 1984); it is therefore possible that CyA inhibits apical NHE through induction of TGFβ secretion by PTC. However, studies performed in our laboratory suggest that exogenous TGFβ does not influence apical NHE activity in human PTC (Johnson et al., 1998b).

With respect to the pathogenesis of interstitial fibrosis in chronic CyA nephropathy, which was the main focus of the present study, our results suggest that extracellular matrix accumulation is mainly due to an effect of CyA on collagen synthesis by CF, rather than by PTC. CyA stimulated CF collagen synthesis at concentrations of 500 to 1000 ng/ml and inhibited collagen synthesis in both CF and PTC at high concentrations (i.e., 10,000 ng/ml), which were accompanied by reduced cell viability. This contrasts with the findings by Wolf et al. (1990) that 1000 ng/ml CyA stimulates secretion of type 1 and type 4 procollagens by MCT cells. This apparent disparity may relate to species variability or to the reported inability of epithelial cell lines to synthesize extracellular matrix by the normal routes (Ghiggiere et al., 1994). Ghiggiere et al. (1994) also noted that CyA in a narrow range and at very low concentrations (1–5 ng/ml) stimulated collagen synthesis in human PTC. These results are difficult to resolve with those of the present study, except that the former study examined PTC at higher passage numbers (4–12 versus 2) in the presence of streptomycin (a known PTC toxin) and in the possible presence of fibroblast contamination, given the moderately positive staining of their cells for vimentin (a marker of mesenchyme).

The increased synthesis of collagen by human CF in the presence of CyA was accompanied by significant reductions in matrix metalloproteinase activities, thereby favoring the net accumulation of extracellular matrix. Although there have been no previously published studies of the effects of CyA on tubulointerstitial MMP activities, it has been suggested on the basis of findings in murine interstitial fibroblasts (Wolf et al., 1995), that CyA could promote interstitial matrix accumulation by enhancing CF production of TGFβ. CF-derived TGFβ could, in turn, act in an autocrine fashion to promote increased deposition and reduced degradation of interstitial matrix (Shihab et al., 1996). In the present study, however, CyA did not significantly alter human CF secretion of total (active + latent) TGFβ. Some of the disparity between the studies may be explained by species differences. Additionally, the method used to measure TGFβ secretion in the current investigation does not exclude the possibility that CyA-induced collagen deposition occurred on the basis of enhanced activation of CF-derived latent TGFβ, even though total TGFβ secretion was unchanged.

IGF-I is also known to stimulate matrix synthesis by many mesenchymal cell types (Kopp and Klotman, 1990; Feld and Hirschberg, 1996), and its action is regulated by a family of six IGFBPs. In the present study, CyA exerted dose-dependent stimulation of IGF-I and inhibition of IGFBP-3 and IGFBP-2 secretion by CF. IGF-I levels at various CyA concentrations paralleled collagen synthesis, except at 10,000 ng/ml, where reduced CF viability may have counteracted autocrine stimulation by IGF-I. A key role for autocrine IGF-I action in mediating the stimulation of CF collagen synthesis by CyA is suggested by the fact that this stimulation was abrogated by the blockade of IGF-I receptors in CF. The effect of the CyA-induced decreases in IGFBP-3 and IGFBP-2 release on the autocrine action of CF-derived IGF-I is uncertain because both of these binding proteins have been variably reported to either inhibit or potentiate the effects of IGF-I on fibroblasts (Feld and Hirschberg, 1996). Because IGF-I also stimulates growth and apical NHE activity in nearby PTC, it is possible that CyA-associated modifications of the tubulointerstitial IGF-I axis may facilitate regeneration of injured PTC. Although no studies have looked at the effect of CyA on renal IGF-I and IGFBP production in vivo, the renal IGF-I axis has been shown to be involved in other models of progressive renal failure and tubulointerstitial disease, such as subtotal renal ablation and experimental diabetes (Feld and Hirschberg, 1996). Moreover, Ferracioli et al. (1995) reported that CyA, but not hydroxychloroquine, was associated with a significant 2-fold increase in serum IGF-I levels in patients with chronic arthritis over a 2-month period.

In addition to stimulation of autocrine IGF-I action, CF secretion of collagen in chronic CyA nephropathy may be further accentuated by effects of CyA on the cytokine secretory profile of contiguous PTC. CyA directly stimulated human PTC secretion of both TGFβ and PDGF in a concentration-dependent fashion. Wolf et al. (1995) also demonstrated that CyA stimulates TGFβ secretion by MCT cells, but there have been no previous studies looking at the effects of CyA on PDGF secretion. Nevertheless, in vivo studies confirm that renal expression of TGFβ and PDGF is up-regulated in both human and experimental chronic CyA nephropathy (Shehata et al., 1994; Wolf et al., 1995; Shihab et al., 1996). Both cytokines have been shown to induce each other's synthesis and biological activities (Abboud, 1995) and to stimulate fibroblast proliferation and extracellular matrix deposition (Kopp and Klotman, 1990; Wolf et al., 1995; Abboud, 1995; Shihab et al., 1996). Moreover, immunoneutralization studies performed in our laboratory have clearly demonstrated that human PTCs are able to stimulate collagen synthesis in cocultured CF through paracrine mechanisms that involve both TGFβ and PDGF (Johnson et al., 1998a).

These results indicate that the direct effects of CyA on PTC and CF are sufficient to account for the tubular atrophy and interstitial fibrosis found in chronic CyA nephropathy, but clearly do not preclude additional contributing factors. CyA is known to stimulate renal vasoconstriction (Kopp and Klotman, 1990), and chronic renal ischemia has been shown to independently promote tubulointerstitial fibrosis (Kopp and Klotman, 1990). Moreover, Raphael and Fish (1987) demonstrated in MDCK cells that the inhibitory effects of CyA and ischemia on DNA and protein synthesis are synergistic. Other agents induced by CyA, such as endothelin, thrombox-
ane, and platelet activating factor, may also contribute to tubulointerstitial pathology (Kopp and Klotman, 1990).

In conclusion, the present study demonstrates that CyA, in clinically relevant concentrations, directly inhibits human PTC and CF viability and growth, and suppresses PTC-apical NHE activity independently of hemodynamic effects. Interstitial matrix accumulation is promoted by the inhibition of MMP activities and stimulation of CF collagen synthesis by induction of autocrine IGF-I action. Matrix deposition is probably further enhanced by the stimulatory effects of CyA on PTC secretion of TGFβ1 and PDGF. It is likely that these direct CyA effects significantly contribute to the proximal tubular atrophy, tubular dysfunction, and interstitial fibrosis characteristic of chronic CyA nephropathy.

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References


