Cyclosporine A Has No Direct Effect on Collagen Metabolism by Cardiac Fibroblasts In Vitro

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Background. Cyclosporine A has been implicated in the pathogenesis of myocardial interstitial fibrosis observed in heart transplant recipients. However, other confounding variables such as posttransplantation hypertension and rejection episodes may also be responsible for interstitial fibrosis development and associated abnormalities in ventricular diastolic function. Therefore, we examined whether cyclosporine A directly or indirectly affects fibrillar collagen metabolism by cardiac fibroblasts in vitro.

Methods and Results. Rat cardiac fibroblasts were isolated by collagenase digestion. Subconfluent cultures were then maintained (24 hours) in serum-containing or serum-free medium before addition of cyclosporine A (50–1,000 ng/mL). After an additional 24 hours, total procollagen synthesis, accumulation, and degradation were analyzed by measuring hydroxyproline content in the cell monolayer and in the ethanol-soluble and ethanol-precipitable fractions of the culture medium. mRNA levels for α(I) and α(III) procollagen polypeptides were assessed 2, 6, 12, and 24 hours after cyclosporine A treatment using Northern blot analysis. The results were compared with control cultures maintained in the absence of cyclosporine A. There were no differences in procollagen gene expression, total procollagen synthesis, accumulation, or degradation in cardiac fibroblasts treated directly with cyclosporine A, in concentrations up to 1,000 ng/mL, compared with untreated cells. In additional experiments, we examined whether cyclosporine A might stimulate the production of collagen regulatory substances by cardiac myocytes in culture. However, addition of conditioned media from neonatal myocytes maintained in the absence and presence of cyclosporine A (1,000 ng/mL) also had no effect on collagen deposition by cardiac fibroblasts.

Conclusions. We conclude that cyclosporine A has no direct effect on collagen metabolism by cultured cardiac fibroblasts in vitro. In addition, we have excluded a paracrine effect of ventricular myocytes on collagen production in the presence of cyclosporine A. These results suggest that factors other than cyclosporine A are responsible for the interstitial fibrosis observed in cardiac allografts. (Circulation 1993;86:1368–1377)

Key Words • fibrosis, interstitial • transplantation, heart • cell culture • protein synthesis • cyclosporine A

Cardiac transplantation is a well-established therapy for patients with end-stage heart disease. Although heart transplantation provides effective rehabilitation for most patients, structural and functional abnormalities have been observed in cardiac allografts, which may be related to the therapy required to prevent allograft rejection. Cyclosporine A is an effective immunosuppressant agent that has proved to be extremely beneficial in preventing rejection episodes and increasing graft and patient survival in organ transplantation.1,2 Since the introduction of cyclosporine A in cardiac transplantation, however, an increased frequency of cardiac myocyte hypertrophy and interstitial fibrosis has been noted. Although cardiac systolic function is well maintained,3 Valantine et al4 demonstrated elevated diastolic pressures associated with restrictive-constrictive ventricular physiology in patients treated with cyclosporine A. Similar changes have been reported by other investigators.5,6 These functional abnormalities have been attributed by many authors to the variable degree of myocardial fibrosis observed in endomyocardial biopsies. This fine, diffuse interstitial fibrosis, called “cyclosporine A effect,” has been found in nearly all patients treated with cyclosporine A regardless of whether they have had acute rejection episodes. This form of interstitial fibrosis was not present, however, in patients treated with other immunosuppressive agents.7-9 In this regard, there is provocative histopathological evidence to indicate that cyclosporine A directly affects procollagen accumulation and myocardial interstitial fibrosis in heart transplant recipients because disproportionate collagen accumulation was found in autopsies of liver transplant recipients treated with cyclosporine A.8 In addition, Nast et al10 demonstrated that rats treated with cyclosporine A developed renal cortical fibrosis in the absence of hypertension, which...
was preceded by increased \( \alpha_1 \) mRNA levels, which is the mRNA transcript encoding the most abundant form of fibrillar collagen found in the cardiac extracellular matrix.

One approach has been to study the direct effects of cyclosporine A on cellular function in isolation. In vitro studies of the proliferative effects of cyclosporine A on human fibroblasts and other cell types have been conflicting. Although several studies suggest a proliferative response of fibroblasts in the presence of cyclosporine A,\(^{11,13}\) other investigators have demonstrated a direct inhibitory effect on cellular proliferation\(^{12,13}\) that may be mediated by direct downregulation of growth factor receptors.\(^{14}\) Independent of the effects on proliferation, the production of collagen has been reported to be decreased rather than increased during exposure of cultured fibroblasts to cyclosporine A.\(^{11,13}\) Steinmann et al\(^{13}\) demonstrated that cyclosporine A slowed collagen triple-helix formation and decreased collagen production of cultured skin fibroblasts by increasing collagen degradation. There also is some evidence suggesting an indirect effect of cyclosporine A on collagen production in cell culture and in vivo. The increased production of vasoactive substances such as thromboxane \( A_2 \)\(^{15}\) and endothelin\(^{16}\) by vascular endothelial cells in the presence of cyclosporine A is suggestive of an ischemic cause of the increased interstitial fibrosis observed in renal as well as in cardiac tissue of patients treated with this drug.

In the present report, we have taken a similar approach to evaluate the effects of cyclosporine A on cardiac fibroblasts in long-term culture. Using rat heart fibroblasts maintained in serum-containing and serum-free culture media as a model system, we have investigated the direct effects of cyclosporine A on the regulation of procollagen gene expression and on collagen accumulation. In addition, we have evaluated the potential effects of the drug on the production of paracrine factors by cardiac myocytes that might regulate collagen production by cardiac fibroblasts. Data are presented to indicate that cyclosporine A has no direct or indirect effect on collagen metabolism by cultured cardiac fibroblasts in vitro. These results suggest that factors other than cyclosporine A are responsible for the interstitial fibrosis observed in cardiac allografts.

**Methods**

**Reagents**

Collagenase (type CLS II) was obtained from Worthington Biochemical, Freehold, N.J. Joklik's minimum essential medium (MEM), Earle's salts, Dulbecco's modified Eagle medium—Ham's F-12 medium (1:1, DMEM/F12), RPMI 1640, and antibiotic/antimycotic solution were obtained from Sigma Chemical Co., St. Louis, Mo. \( 4^9\)H(G)Hydroxyproline (5.3 Ci/mmol) was obtained from DuPont NEN Products, Boston, Mass. Fetal calf serum was obtained from Hyclone Laboratories, Logan, Utah. Nu Serum was obtained from Collaborative Biomedical Products, Bedford, Mass. Ventrex PC-1 medium was obtained from Endotronics, Minneapolis, Minn. Phytohemagglutinin (PHA) was obtained from GIBCO BRL, Grand Island, N.Y. Deoxyctydine \( 5'(\alpha-3^P) \) triphosphate (dCTP; 3,000 Ci/mmol) and [methyl-\( ^3H \)]thymidine (46 Ci/mmol) were

![Figure 1. Northern blot analysis of type I and type III procollagen mRNAs after cyclosporine A treatment. Ten micrograms of fibroblast total RNA was size-fractionated on 1% agarose gels and transferred by capillary action to a nylon membrane. The filter was sequentially hybridized with \( 3^P \)-labeled cDNAs encoding \( \alpha_1 \) (panel A), \( \alpha_1 \) (III) (panel B), and GAPDH (panel C). Equal loading conditions were verified by hybridizing the filter with a \( 3^P \)-labeled oligonucleotide probe specific for rat 18S rRNA (panel D). Blots were exposed to Kodak XAR-5 for varying time periods. As is evident from the figure, mRNA levels for both procollagen polypeptides relative to either GAPDH mRNA or 18S rRNA were unaffected by increasing medium concentrations of cyclosporine A (0–1,000 ng/mL). The somewhat reduced signal intensity for \( \alpha_1 \) mRNA in the 1,000-ng/mL lane was due to somewhat reduced loading of this lane with total RNA. The bars to the left in each panel indicate the position of the 28S (upper) and 18S (lower) rRNAs on ethidium bromide-stained agarose gels.](image-url)
obtained from Amersham, Arlington Heights, Ill. Ficoll-Paque was purchased from Pharmacia LKB Biotechnology, Piscataway, N.J. Rabbit anti-rat polyclonal antiserum to type I collagen was obtained from DMI, South Portland, Me. Cyclosporine A was a generous gift of Dr. W. Houlihan, Sandoz Pharmaceuticals Corp., East Hanover, N.J. All other reagents were of the highest grade commercially available and were obtained from Sigma and Baxter S/P, McGaw Park, Ill.

Cardiac Fibroblast Isolation

Left ventricular muscle from Sprague-Dawley rats was minced and digested in perfusion buffer17 containing collagenase (500–800 μg/mL) and CaCl₂ (100 μM). The released cells from three consecutive 10-minute incubations were collected by centrifugation and resuspended in fibroblast growth medium (FGM) (MEM supplemented with Earl’s salts [pH 7.4] containing 2.9 mM CaCl₂, 5% [vol:vol] fetal calf serum, 5% Nu Serum, ITS [0.5 mM], β-aminopropionitrile [300 μM], L-ascorbic acid [250 μM], and antibiotics/antimycotic solution [1% vol:vol]). Subculture was performed as previously described.18 Fibroblasts were then plated onto uncoated plastic Petri dishes or glass coverslips at a density of 100 cells/mm² and allowed to proliferate in FGM for 4–7 days. Subconfluent cultures then were maintained for 24 hours in FGM or serum-free medium (DMEM/F12/PC-1; 1:1:1) before addition of cyclosporine A (50–1,000 ng/mL). After an additional 2–24 hours of maintenance culture, the cell monolayer and conditioned medium were collected for further analysis.

Neonatal Cardiac Myocyte Isolation

Ventricular muscle was isolated from the hearts of 2-day-old rat pups by collagenase digestion as previously described.17 Released cells were collected by centrifugation, resuspended in PC-1 medium at a density of 7.5x10⁶ cells/mL, plated onto collagen-coated 60-mm dishes (3.5x10⁶ cells per dish), and left undisturbed in a 5% CO₂ incubator for 14–18 hours. Unattached cells removed by aspiration and DMEM/F12/PC-1 was added. Spontaneously contracting myocytes were maintained in culture in the presence or absence of 1,000 ng/mL of cyclosporine A for 24 hours. Conditioned media from these cultures were then mixed (25% vol:vol) with either serum-free medium (DMEM/F12/PC-1) or serum-containing medium (FGM) and used to maintain primary fibroblast cultures for an additional 24 hours, at which time the fibroblast cell monolayer was collected for further analysis.

mRNA Analysis

Total RNA was isolated from cultured cardiac fibroblasts 2, 6, 12, and 24 hours after treatment with cyclosporine A in DMEM/F12/PC-1.19 RNA was quantified by absorbance at 260 nm, and the integrity was determined by examining the 28S and 18S rRNA bands in ethidium bromide–stained agarose gels. Total RNA (10 μg) was size-fractionated by electrophoresis under denaturing conditions, transferred to nylon membranes, and immobilized by ultraviolet irradiation. Prehybridization and hybridization with cDNA probes were performed at 42°C in buffer containing 50% formamide, 10% dextran sulfate, 1% sodium dodecyl sulfate (SDS), 1 M NaCl, and 100 μg/mL denatured salmon sperm DNA. Blots were washed in 2xSSC–0.5% SDS (25°C, 20 minutes) and 0.1xSSC–0.1% SDS (65°C, 30 minutes). Prehybridization and hybridization with an oligonucleotide probe were performed at 51°C in a solution containing 6xSSC, 1xDenhard’s solution, 0.1% SDS, 0.05% Na pyrophosphate, 20 μg/mL tRNA, and 100 μg/mL denatured salmon sperm DNA. Blots were washed in 6xSSC–0.1% SDS (65°C, 30 minutes). All blots were exposed to Kodak XAR-5 film with intensifier screens at −80°C for varying time periods. Band intensity was quantified by laser densitometry (LKB Ultrascan XL enhanced laser densitometer interfaced to a personal computer running Gelscan XL Ver. 1.2). The cDNA clones used were human collagen type I, α(I), clone Hf67726; human collagen type III, α(III), clone Hf93421; and human glyceraldehyde 3-phosphate dehydrogenase (GAPDH), clone pHcGAP.22 All cDNA probes were obtained from the American Tissue Type Collection, Bethesda, Md. The inserts were isolated and labeled by random primer extension with (α-32P)dCTP. To ensure equal loading conditions, blots were also hybridized with an oligonucleotide probe specific for the rat 18S rRNA (5'-ACGGTATCTGATCGTCTTC-CGAGGTCTC-3'). The probe was end-labeled with (γ-32P)-ATP using T4 kinase and hybridized as previously described.

Immunofluorescent Staining

Cells grown on glass coverslips were washed with 10 mM Na phosphate, 150 mM NaCl (pH 7.2; PBS) and fixed in 2% (wt:vol) paraformaldehyde in PBS for 10 minutes at room temperature (RT), followed by acetic acid for 15 minutes at −20°C. Cells were incubated with 10% (vol:vol) normal goat serum (1 hour, RT) and washed briefly with PBS. Type I procollagen was identified using a mouse monoclonal antibody specific for the aminoterminal cleavage site of α(I) procollagen polypeptide.25 The hybridoma cell line was obtained from the University of Iowa Hybridoma Bank, and the antibody was purified from conditioned media by protein G–Sepharose 4B affinity chromatography. Fixed cells were incubated with 60 μg/mL of the monoclonal antibody in PBS containing 1% (wt:vol) bovine serum albumin (BSA) overnight at RT. Cells were then washed with PBS (30 minutes, RT) and incubated again with 10% goat serum (1 hour, RT). Excess goat serum was removed and cells were stained with fluorescein isothiocyanate (FITC)
conjugated goat anti-mouse IgG (Cooper Biomedical, Malvern, Pa.) in PBS containing 1% BSA and 0.05% Tween 20 (1 hour, RT). Final washes were performed in PBS containing 0.05% Tween 20 (30 minutes, RT).

Type I collagen in the cell monolayer was identified using a polyclonal rabbit anti-rabbit antibody (1:100 dilution) and FITC-conjugated goat anti-rabbit IgG (Cappel, Westchester, Pa.). The gross morphology and immunofluorescent staining intensity and cellular distribution were evaluated by epifluorescent microscopy.

Quantitative Analysis of Procollagen Synthesis and Degradation

Procollagen synthesis was analyzed by measuring the amount of protein-bound hydroxyproline in the cell monolayer and conditioned medium 24 hours after exposure to cyclosporine A. The cell monolayer was scraped in 67% (vol:vol) ethanol, and the precipitate was collected by centrifugation (30,000g, 30 minutes). The protein sediment was redissolved and hydrolyzed in 6 N HCl (110°C, 16 hours) and decolorized with activated charcoal (70 mg). Similarly, the conditioned medium was mixed with 2 vol of absolute ethanol, and the resulting protein precipitate was hydrolyzed as described above. To estimate the amount of collagen degraded both intracellularly and extracellularly, the ethanol supernate from the conditioned medium was dried, and the residue was acid hydrolyzed. [3H]Hydroxyproline (180,000 dpm) was added to each hydrolysis tube to serve as an internal standard for the recovery of hydroxyproline. Hydrolysates were evaporated to dryness, and the residue was dissolved in 1 mL H2O. After derivatization of imino acids with 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (NBD-Cl), samples were chromatographed by reverse-phase high-performance liquid chromatography as described by Campa et al.26 Hydroxyproline content was determined from the peak area, and derivatization efficiency was assessed by the recovery of [3H]hydroxyproline in the collected hydroxyproline peak. Data are the results of duplicate wells from three different cell isolations.

Thymidine Incorporation Assay

Human peripheral blood mononuclear cells were separated by gradient centrifugation over Ficoll-Paque, washed with PBS, and cultured in 96-well plates (10^3 cells per well) in RPMI 1640 containing 10% heat-inactivated fetal bovine serum, 2 mM glutamine, and antibiotics. Cultures (in triplicate) were then maintained in culture medium alone or in the presence of PHA (1:100 dilution) or in PHA plus cyclosporine A (1,000 ng/mL) for 24, 48, 72, and 96 hours. Mononuclear cells were then labeled with [3H]thymidine (500 nCi/well) for 24 hours, harvested onto filter papers and washed with PBS. [3H]Thymidine incorporation (dpm/well) was then analyzed by scintillation counting (LKB model 1209, Piscataway, N.J.).

Other Assays

Total protein and DNA contents of the cell monolayers were measured as previously described.17

Statistical Analysis

Unless otherwise stated, all results were expressed as mean±SD. Normality was assessed using the Wilk-Shapiro test, and homogeneity of variance was established with Levine’s test. Two-way randomized, blocked ANOVA was used to compare the factors of cyclosporine A and serum on measurements of collagen accumulation in cell monolayers. One-way, blocked ANOVA followed by Dunnett’s test was used for comparisons between multiple groups. Paired and unpaired t tests were used for comparisons between two groups. Data were analyzed using the PROPHET computer system (Division of Research Resources, National Institutes of Health).

Results

Fibrillar Collagen mRNA Levels

Medium concentrations of cyclosporine A up to 1,000 ng/mL had no major effect on the mRNA levels for α1(I) and α1(III) procollagen polypeptides (relative to either GAPDH mRNA or 18S rRNA) in cardiac fibroblasts harvested 12 hours after exposure to the drug (Figure 1). Similar results were obtained at 2, 6, and 24 hours after addition of cyclosporine A.

Intracellular and Extracellular Localization of Type I Collagen

Medium concentrations of cyclosporine A up to 1,000 ng/mL had no effect on the morphology of cultured rat cardiac fibroblasts, as observed by phase-contrast microscopy (Figures 2A and 2B). Total protein and DNA content of the cell monolayers also were unaffected by cyclosporine A treatment, indicating that the drug had no proliferative or toxic effects on these cells (data not shown). Furthermore, cyclosporine A treatment had no apparent effect on the subcellular distribution of newly synthesized type I procollagen (Figures 2C and 2D). The monoclonal antibody to the proaminopeptide domain of α1(I) procollagen polypeptide produced a reticular staining pattern throughout the cytoplasm of both untreated and cyclosporine A-treated fibroblasts. These results indicated that the intracellular level of newly synthesized type I procollagen was unaffected by the drug. Western blot analysis of cell monolayer proteins with this proaminopeptide antibody supported the above findings (data not shown). In addition, extracellular deposition of type I collagen by cardiac fibroblasts was not affected by the presence of cyclosporine A (Figures 2E and 2F). The antibody to type I collagen revealed comparable amounts of mature collagen fibrils occupying the extracellular matrix within the cell monolayer of cyclosporine A-treated and untreated fibroblasts.

Direct Effects of Cyclosporine A on Collagen Production

The direct effect of cyclosporine A on collagen production by cardiac fibroblasts maintained in serum-free medium (DMEM/F12/PC-1) was assessed by measuring hydroxyproline in the cell monolayer as well as in the conditioned medium in the presence and absence of varying concentrations of the drug. As seen in Figure 3A, increasing doses of cyclosporine A did not affect the amount of ethanol-precipitable, hydroxyproline-containing proteins present in the medium after 24 hours. The amount of ethanol-precipitable, hydroxyproline-containing proteins in the cell monolayer was also unaffected by cyclosporine A (Figure 3B). Finally, the
amount of ethanol-soluble, hydroxyproline-containing peptides and free amino acids (produced by the degradation of both newly synthesized and mature collagens) was similar in control and cyclosporine A-treated cells (Figure 3A). Identical experiments were performed in serum-containing medium (FGM) in the presence and absence of a single concentration of cyclosporine A (1,000 ng/mL). The amount of hydroxyproline-containing peptides in all three fractions 24 hours after addition of the drug was found to be similar in both cyclosporine A-treated and untreated cultures (data not shown).

**Indirect Effect of Cyclosporine A on Collagen Production**

The amount of collagen deposited in the extracellular matrix of cultured cardiac fibroblasts (maintained in either DMEM/F12/PC-1 or FGM) was assessed 24 hours after exposure to conditioned media from neonatal myocytes maintained in serum-free culture in the presence or absence of cyclosporine A (1,000 ng/mL). As seen in Figure 4, monolayer hydroxyproline content of fibroblasts maintained in FGM plus myocyte-conditioned medium was significantly greater than that observed in cells cultured in DMEM/F12/PC-1 plus myocyte-conditioned medium (\(p=0.004\) for factor "serum"; two-way ANOVA). However, prior exposure of the myocytes to cyclosporine A had no effect on the fibroblast monolayer hydroxyproline content in either the presence or absence of serum (\(p=0.616\) for factor "CsA"; two-way ANOVA). Data are the mean±SD values of four replicate fibroblast cultures from a single cell isolation.

**Effect of Cyclosporine A on Human Peripheral Blood Mononuclear Cells**

The effectiveness of the cyclosporine A that was used for all of the above experiments was tested for its ability to inhibit the proliferation of peripheral blood mononuclear cells stimulated with PHA in vitro. As seen in Figure 5, cyclosporine A (1,000 ng/mL) markedly inhibited the proliferative response of mononuclear cells to PHA as indicated by its inhibition of \(^{3}H\)thymidine incorporation. Thus, the absence of cyclosporine A effects on fibroblast collagen synthesis and degradation was not due to a lack of potency of the drug.

**Discussion**

In this report, we provide experimental evidence that cyclosporine A has no direct effect on collagen production or accumulation by cardiac fibroblasts in vitro. The concentrations of cyclosporine A used for these cell culture experiments were similar to or higher than the recommended blood levels used to prevent cardiac allograft rejection. With these doses of cyclosporine A, we were unable to demonstrate any alterations in steady-state mRNA levels for fibrillar collagen genes. Collagen production and the rate of collagen degradation also were unaffected by cyclosporine A in either the
presence or absence of fetal bovine serum. Thus, a requirement for serum factors that might interact with cyclosporine A and alter collagen metabolism was excluded. Cyclosporine A also failed to stimulate the production of collagen regulatory substances by cardiac myocytes in culture, excluding the possibility of a paracrine effect of ventricular myocytes on collagen production in the presence of cyclosporine A.

Although we have excluded a direct effect of cyclosporine A on collagen production by cardiac fibroblasts in vitro, indirect effects of the drug may influence collagen accumulation in the cardiac extracellular matrix in vivo. Uncontrolled, sustained hypertension has been shown to produce disproportionate collagen accumulation and myocardial interstitial fibrosis in experimental animals, with subsequent development of left ventricular dysfunction and congestive heart failure. Cyclosporine A is known to induce hypertension in humans, possibly mediated by sympathetic neural activation. Thus, the increased hemodynamic load produced by cyclosporine A treatment probably is related to the increased deposition of fibrillar collagens in the transplanted heart. However, it remains to be determined whether aggressive blood pressure reduction in the heart transplant recipient will reduce fibrillar collagen deposition in cardiac allografts. Furthermore, frequent rejection episodes with the release of cellular and humoral mediators of the immune response also may participate in increased procollagen production and interstitial fibrosis. Several investigators have shown that mononuclear leukocytes can release soluble mediators capable of stimulating or inhibiting collagen production in vitro. In addition, co-culture of mononuclear leukocytes with noncardiac fibroblasts resulted in increased collagen production, suggesting that cell-cell interactions also may regulate collagen production during rejection episodes. It remains to be determined if cyclosporine A influences these cell-cell interactions contributing to the fibrotic response. However, we have excluded an effect of the drug on the production of myocyte regulatory factors that might influence collagen production and accumulation in this model system.

Collagen biosynthesis and turnover are regulated at pretranslational, translational, and posttranslational levels. Recent studies from this laboratory indicate that both pretranslational and posttranslational mechanisms modulate interstitial collagen accumulation in the rat heart during normal growth and hypertrophy. Based on this short-term, in vitro drug study, however, we conclude that cyclosporine A has no effect on any of the steps in procollagen biosynthesis and processing.

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