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Cyclosporine Has a Direct Effect on the Differentiation of a Mucin-Secreting Cell Line

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Cyclosporine is a potent immunosuppressant used in the treatment of ulcerative colitis and keratoconjunctivitis sicca. Neither the etiologies of these diseases nor the mechanism by which cyclosporine exerts its therapeutic effect is well understood. Since both diseases are linked by a common decrease in mucin-filled goblet cells, this study tests a hypothesis that cyclosporine acts directly on goblet cells to promote their differentiation and production of secretory mucins. The HT29-18N2 human colon adenocarcinoma cell line, which is capable of forming monolayers of well-differentiated goblet cells, was used as a model system. Cyclosporine induced a dose-dependent increase in intracellular mucin stores. A 2-week exposure to 1 μM cyclosporine resulted in an average increase in mucin volume of 94%. This increase resulted from both a higher percentage of cells with mucin stores and an increased volume of mucin per cell. PSC-833, a nonimmunosuppressive analog of cyclosporine, also increased mucin production. The intracellular accumulation of mucin was not a result of reduced secretion, since the time required for the release of pulse-radiolabeled glycoproteins was similar for both control and cyclosporine-treated monolayers. The effect of cyclosporine was not mediated by the drug's previously documented abilities to decrease cellular proliferation rates, inhibit calmodulin, antagonize prolactin receptor binding, or modulate prostaglandin production. J. Cell. Physiol. 184:400–408, 2000.

Cyclosporine (CsA) is an undecapeptide with potent immunosuppressive actions on T-helper lymphocytes. Following its initial successful use in preventing allograft rejection in organ transplantation, CsA was shown to be useful in treating the increased epidermal proliferation and chronic inflammation associated with psoriasis (Ellis et al., 1986). CsA's ability to directly modulate DNA synthesis and cellular proliferation in normal and transformed keratinocytes in vitro led to the suggestion that the drug's therapeutic effect in psoriasis is at least partially mediated via a direct action on epithelial keratinocytes (Furue et al., 1988).

More recently, CsA has been used in treating ulcerative colitis and keratoconjunctivitis sicca. These two diseases are linked by a decrease in mucin-filled goblet cells in the mucosa of the colon and conjunctiva, respectively. The etiology of ulcerative colitis is poorly understood but the disease is characterized by depletion of intracellular mucin stores in epithelial goblet cells (McCormick et al., 1990). Furthermore, the absence of a particular mucin glycoform has been demonstrated to lead to a predisposition to acquire the disease but not to cause the disease itself (Tysk et al., 1991). High-dose CsA is effective in severe cases of ulcerative colitis that are unresponsive to conventional steroid treatments (Lichtiger et al., 1994). Unfortunately, the value of a high-dose CsA treatment for ulcerative colitis is limited to a bridging therapy for rescuing severe cases, since the treatment is associated with a high incidence of nephropathy and severe opportunistic infections (Sandborn, 1995).

Keratoconjunctivitis sicca (KCS), also known as "dry eye syndrome," is characterized by chronic dryness of the cornea and conjunctiva. Dryness of the eye can result from a decrease in any of the aqueous, mucin, or lipid components of tears. In KCS, lymphocytic infiltration of lacrimal gland acini and ducts can lead to a decrease in the aqueous component of tears (Wieczorek et al., 1988), but it has also been shown that there is up to a 75% decrease in conjunctival goblet cells (Ralph, 1975). Topical CsA has been shown to cause corneal improvement that is not dependent on increased lacrimation (Kaswan et al., 1989). Kaswan and coworkers (1989) noted that the positive effect of CsA on spontaneous KCS is unlikely the result of its immunosuppressive action and suggested a direct epitheliotrophic mechanism as an alternative hypothesis.

The known ability of CsA to directly influence kera-

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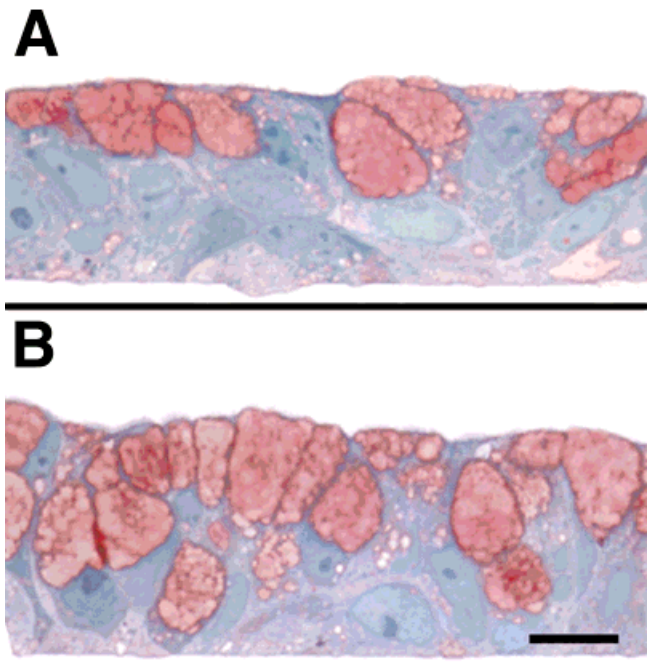

Fig. 1. Light microscopic views of HT29-18N2 cells viewed in 0.9-μm-thick cross sections. **A:** Control cells, grown in the absence of CsA, form monolayers in which some, but not all, of the highly polarized, columnar cells have large accumulations of mucous secretory granules in their apical halves. **B:** Following a 2-week exposure to 1 μM CsA, a higher percentage of the columnar cells contain mucous secretory granules and the number of granules in a typical cell appears greater. Scale bar = 20 μm.

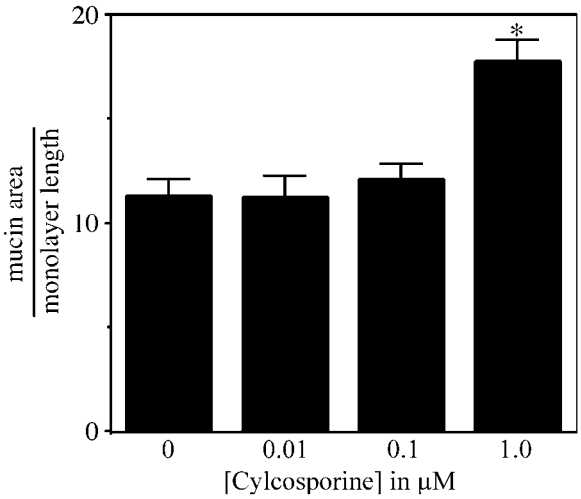

Fig. 2. CsA evokes a dose-dependent increase in mucin stores. Ratio of μm^2 of intracellular mucous secretory granule stores per linear μm for monolayers exposed to either 0, 0.01, 0.1, or 1.0 μM CsA for 2 weeks. There was a 57% increase in intracellular mucin stores at 1.0 μM CsA but the effective dose range was very narrow. Concentrations of 0.1 μM or less had no effect, whereas 10 μM caused vacuolization and cell death. In this figure and all subsequent histographs, each bar represents the mean ± SEM of three identically matched monolayers; six nonadjacent sections were analyzed for each monolayer. *Responses that were significantly different ($P < 0.01$) from the corresponding control monolayers; all other responses were not significantly different ($P > 0.1$) as assessed by the Mann-Whitney *U*-test.

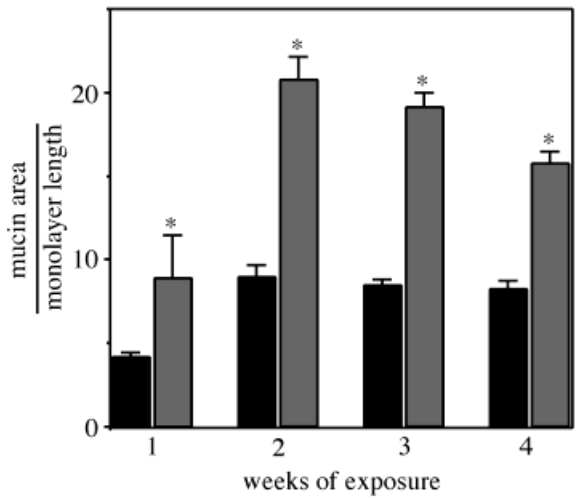

Fig. 3. Time course of CsA enhancement of goblet cell differentiation. The ratio of μm^2 of intracellular mucous secretory granule stores per linear μm was quantified at 1, 2, 3, and 4 weeks for monolayers grown in PFHM-II only (black bars) or PFHM-II + 1 μM CsA (gray bars). For both conditions, granule stores increased through the first 2 weeks and then remained essentially constant. The CsA-treated cells consistently had higher mucous granule stores than their matched control cells. *Responses that were significantly different ($P < 0.01$) from the corresponding control monolayers.

tinocytes (Ramirez-Bosca et al., 1990), endothelial cells (Zoja et al., 1986), fibroblasts (Ghiggeri et al., 1994), mesangial cells (Ghiggeri et al., 1994), and a mouse colon carcinoma cell line (Saydjari et al., 1987) raised the possibility that the drug might be having a direct action on goblet cell proliferation or differentiation. To test this hypothesis, the effects of CsA and its nonimmunosuppressive analog PSC-833 on the HT29-18N2 human adenocarcinoma cell line, which is capable of forming monolayers of predominately mucin-filled goblet cells, were examined.

MATERIALS AND METHODS

Chemicals

CsA was initially obtained from the University of Missouri School of Veterinary Medicine pharmacy; dissolved in a proprietary vehicle (Sandoz Pharmaceuticals), consisting of 12.5% alcohol, olive oil, and Labrafil M 1944CS (polyoxyethylated oleic glycerides); and kept as a 100 mg/ml stock at 4°C. The results reported in Figures 1 through 4 were performed using CsA in this proprietary mixture designed for oral administration in a clinical setting. Although the control samples in these experiments did not contain the vehicle, the highest concentration of vehicle in any of these initial studies with 1 μM CsA was only 0.0012%. To ensure the vehicle was having no effect, however, all subsequent studies were made using a fresh 3 mg/ml stock of CsA (Calbiochem, San Diego, CA), dissolved in a 9:1 mixture of 100% ethanol:Tween 80, including the vehicle in the mock controls. The concentration of the ethanol: Tween vehicle was never higher than 0.02% in any of the experiments and was found to have no statistically significant ($P > 0.1$) effect on intracellular mucin stores. PSC-833 was a generous gift of Novartis

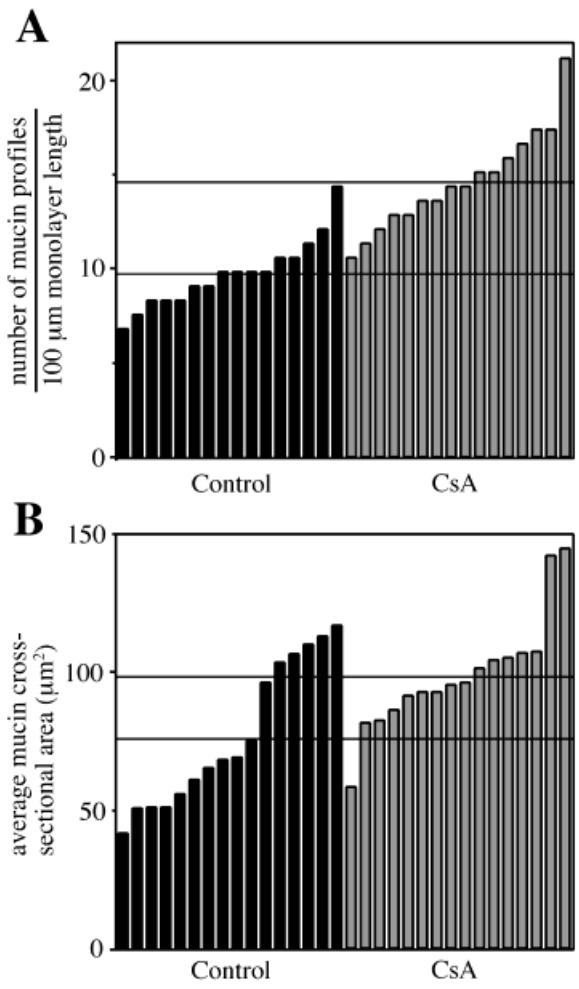

Fig. 4. CsA effect on the number and size of intracellular mucin profiles. **A:** To estimate the number of cells expressing the fully differentiated, goblet cell phenotype, the number of individual profiles of mucous secretory granules per 100 μm of monolayer length analyzed was determined. Each bar represents the number of profiles in a single section. Five to six sections from three identically treated monolayers were analyzed for each experimental condition. The horizontal lines indicate the average number of profiles in all sections for each treatment. CsA-treated monolayers (gray bars) averaged 14.6 profiles/100 μm compared with 9.7 profiles/100 μm in control monolayers (black bars). **B:** To determine whether the average cross-sectional profile of mucous granules in a typical cell was increased by CsA treatment, the average profile size in each of the slides used in (A) was measured. The horizontal line indicates the average profile size in all sections for each condition. CsA treatment (gray bars) resulted in an average cross-sectional profile size of 99.4 μm^2, which was 29% greater than the 77.1 μm^2 profile size in controls (black bars). The data in each graph have been independently rank ordered to more clearly show the distribution of sizes; the order of slides in the first graph does not directly correspond with the order in the second.

Pharma AG (Basel, Switzerland) and was dissolved at 1.5 mg/ml in a 9:1 mixture of 100% ethanol:Tween 80. Stock solutions of 0.5 mg/ml indomethacin (Cayman Chemicals, Ann Arbor, MI) and 3 mg/ml trifluoperazine (TFP; SmithKline Beecham Pharmaceuticals, Philadelphia, PA) were prepared in ethanol and stored at 4°C. Fresh dilutions of indomethacin and TFP in culture medium were prepared immediately before the culture medium was replaced on an alternating-day schedule. Control monolayers in the indomethacin and TFP experiments were grown in culture medium plus the vehicle at concentrations equal to the amount present in monolayers treated with the highest concentration of the drug (i.e., 0.07% ethanol).

Prostaglandins and cyclooxygenase inhibitors were obtained from Cayman Chemical (Ann Arbor, MI). Prolactin (superpure human pituitary prolactin; 30 IU/mg) was generously supplied by Dr. A.F. Parlow of the NIDDK's National Hormone and Pituitary Program (Torrance, CA). Small aliquots of prolactin were dissolved in culture medium + 1 mg/ml bovine serum albumin (Sigma, St. Louis, MO) to minimize loss through nonselective adherence to vessel walls; control media also contained 1 mg/ml BSA in these experiments. Prolactin stocks were kept at 4°C for no longer than 4 days and freshly diluted into culture medium immediately before the cell culture medium was changed on an alternating-day cycle.

Cell culture

HT29-18N2 cells were generously provided by Dr. Daniel Louvard (Pasteur Institute, Paris) and grown as previously described (Phillips et al., 1995). Essentially, stock cultures were maintained in Dulbecco's modified Eagle's medium (DMEM) + 5% fetal bovine serum (FBS). To evaluate the effect of CsA on differentiation, cells were seeded onto 12-mm glass coverslips in 24-well tissue-culture dishes at a concentration of 800,000 per well. After 24 h, the coverslips were transferred to 12-well dishes and fed every other day with 1 ml/well protein-free hybridoma medium II (PFHM-II; Life Technologies, Gaithersburg, MD) in the presence or absence of the drugs indicated in the text. In DMEM + 5% FBS, HT29-18N2 cells grew as multilayers of mostly undifferentiated cells with only occasional interspersed mucin-containing goblet cells. In PFHM-II, they predominately formed monolayers in which most, but not all, of the columnar cells contained mucin-filled secretory granules and looked like well-differentiated goblet cells (Phillips et al., 1995).

Proliferation

Log-phase HT29-18N2 cells, growing in DMEM + 5% FBS were seeded, in the same medium, at a concentration of 50,000 cells/well in six-well culture dishes. After 24 h, the culture medium was replaced with PFHM-II containing either (1) no additive, (2) 0.02% of the ethanol:Tween 80 vehicle, (3) 0.1 μM PSC-833, or (4) 1.0 μM CsA. The medium was replaced every other day with 2 ml of fresh medium. On alternate days, the cells in three wells from each treatment group were removed using trypsin/EDTA and counted using a hemacytometer. The number of cells/well for the three wells of each group per day had an average coefficient of variation of 9.1% (range 3.7–20.1). For each drug concentration, a plot of the log of the average number of cells/well over time could be fit with a straight line that had a correlation coefficient ≥ 0.92. The doubling times were calculated based on the growth rate between days 2 through 10.

Microscopy

Cells were fixed in PFG fixative (2% paraformaldehyde, 2.5% glutaraldehyde, 70 mM NaCl, 30 mM

HEPES, 2 mM $CaCl_2$, pH 7.4) for 1 h at room temperature, rinsed three times in HWB (70 mM NaCl, 30 mM HEPES, 2 mM $CaCl_2$, pH 7.4), postfixed in 1% osmium in HWB, and rinsed three times in dH_2O. Following dehydration with a series of ethanol, they were embedded in EmBed 812 (Electron Microscopy Sciences, Fort Washington, PA). Semithin sections (0.9 μm) were stained with hematoxylin and Safranin O and intracellular mucin stores quantified using a computer-assisted morphometric technique (Phillips and Wilson, 1993; Phillips et al., 1995). Six distinct regions of each monolayer were examined and a total of approximately 1,000 μm of monolayer length used to estimate the average intracellular mucin stores of each monolayer. All measurements were made on randomized, coded sections by an observer unaware of the experimental treatment. A two-tail Mann-Whitney *U*-test was used to determine statistical significance.

Prostaglandin ELISA

Total cellular prostaglandin E_2 concentrations (intracellular + secreted) were measured using the Biotrak prostaglandin E_2 (PGE_2) competitive enzyme immunoassay system (Amersham Pharmacia, Piscataway, NJ). The assay had a sensitivity range of 50–6,400 pg/ml.

Radiolabeled glycoprotein release

Cells were grown in PFHM-II + either the vehicle or 1 μM CsA for 2 weeks and then deprived of all sugars for 1 h by incubation in sugar-free DMEM (Sigma). Each monolayer was then exposed to 45 μCi of ^{3}H-glucosamine (American Radiolabeled Chemicals, Inc., St. Louis, MO) in sugar-free DMEM for 1 h at 37°C. At the end of the pulse, the cells were rinsed twice and incubated in 1 ml of prewarmed PFHM-II. At 1, 2, 4, 6, 8, 12, and 24 h after the pulse, the medium was replaced with 1 ml of fresh, prewarmed PFHM-II. Aliquots of the conditioned medium were diluted into ReadySafe scintillation fluid (Beckman Instruments, Fullerton, CA) and counted in a liquid scintillation counter.

RESULTS

Morphometric quantification of CsA effects on mucin stores

Intracellular mucin stores, an indicator of the degree of goblet cell differentiation, were measured in HT29-18N2 cells that had been grown in PFHM-II + 0, 0.01, 0.1, 1.0, and 10 μM CsA for 2 weeks (Figs. 1 and 2). In control monolayers that were not exposed to CsA, the ratio of μm^2 of intracellular secretory granules to μm of monolayer length averaged 11.3 ± 0.8 (mean ± SEM). Exposure to 0.01 or 0.1 μM CsA had no significant effect ($P > 0.1$) on intracellular mucin stores, whereas 1.0 μM CsA resulted in a 57% increase to 17.7 ± 4.2 $\mu m^2/\mu m$ ($P < 0.01$). In 10 separate experiments, a 2-week exposure to 1 μM CsA resulted in intracellular mucous granule stores that averaged 194% (range 142 to 263%) of the corresponding control level. A 10 μM CsA treatment resulted in significant cell death and detachment from the glass substrate and the remaining cells were highly vacuolated.

One mechanism by which CsA could induce an increase in intracellular mucin stores would be simply to shorten the time it took the HT29-18N2 cells to reach some maximal level that would eventually be reached in the untreated control monolayers. Alternatively, CsA might have been acting to increase mucin stores to levels that could never be achieved in the absence of the drug. To distinguish between these two possibilities, the effect of 1 μM CsA was examined at weekly intervals for 4 weeks (Fig. 3). By week 2, the control cells had reached a level that was essentially constant through week 4; there was no significant difference ($P > 0.1$) between mucin stores in control monolayers between weeks 2 through 4. As early as 1 week postconfluency, CsA-treated cells had 213% ($P < 0.01$) of the control mucin level. Similar to control monolayers, CsA cells reached their maximal level at week 2 (232% of control) and remained constant at week 3 (227%). At 4 weeks, some of the CsA-treated cells had begun to detach from the monolayer and caused what is likely an artificial drop in intracellular mucin stores compared with those of the previous week (191% of control). Although the greater degree of differentiation of the CsA-treated cells may have contributed to their detachment, we do not believe the detachment reflects a cytotoxic response to CsA at 4 weeks, since the remaining cells showed no increased vacuolization or other morphological changes associated with apoptosis or necrosis. In our experience with the HT29-18N2 cell line over 14 years, we have commonly observed that differentiated cells begin to detach from the substratum between 4 and 6 weeks.

An increase in intracellular mucin stores can result from either a greater percentage of cells having accumulations of mucin-filled secretory granules or from an increase in the volume of secretory granules per goblet cell. Following a 2-week exposure to 1 μM CsA, the number of cells with distinct profiles of intracellular mucous granules was 145% of that of control cells ($P < 0.01$; Fig. 4A). Furthermore, the average cross-sectional area of mucous granules in individual cells increased by 29% from 77.1 to 99.4 μm^2 ($P < 0.01$; Fig. 4B).

Nonimmunosuppressive analog of CsA mimics its effect on HT29-18N2 cells

PSC-833 is an analog of CsA, which has no immunosuppressive activity but, like CsA, can sensitize multidrug resistant (MDR) cell lines to chemotherapeutic agents (Gaveriaux et al., 1991). In an experiment comparing CsA and PSC-833 (Fig. 5), 1 μM CsA increased mucin stores to 184% ($P < 0.01$) of the level seen in untreated control monolayers, whereas 1 μM PSC-833 raised mucin stores to 138% ($P < 0.01$). Higher concentrations of PSC-833 were either inhibitory or toxic, since intracellular mucin stores in cells exposed to 10 μM PSC-833 were unchanged (99.8%, $P > 0.8$). Although PSC-833 was less efficacious than equimolar concentrations of CsA, it was more potent, as shown by its ability to increase mucin stores to 115% of control levels ($P < 0.01$) at a concentration of 0.1 μM. The increased potency of PSC-833 was confirmed by the results of two additional experiments in which 0.1 μM PSC-833 increased intracellular mucin stores by 58.5% ($P < 0.01$) and 33.6% ($P < 0.01$) over control levels.

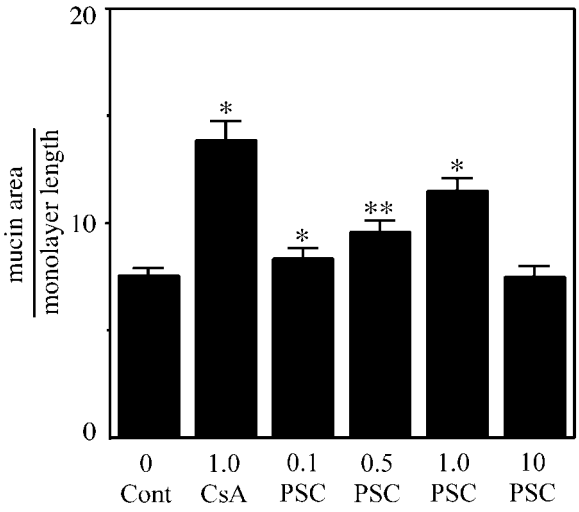

Fig. 5. Effect of the nonimmunosuppressive CsA analog PSC-833 on goblet cell differentiation. HT29-18N2 cells were grown in PFHM ± 1 μM CsA or 0.1, 0.5, 1.0, or 10.0 μM PSC-833 for 2 weeks. Like CsA, PSC-833 promoted a dose-dependent increase in goblet cell differentiation as measured by the ratio of μm^2 of intracellular mucous secretory granule stores per linear μm of monolayer analyzed. Responses that were significantly different from the corresponding control monolayers: $^*P < 0.01$, $^{**}P < 0.05$; all other responses were not significantly different ($P > 0.1$) as assessed by the Mann-Whitney *U*-test.

Effect of CsA on doubling times and proliferation of HT29-18N2 cells

To determine whether the CsA and PSC-833 induced changes in differentiation were the result of reduced proliferation, the doubling times of HT29-18N2 cells growing in either PFHM-II only, PFHM-II + vehicle only, PFHM-II + 0.1 μM PSC-833, or PFHM-II + 1.0 μM CsA were measured over the course of 10 days (Fig. 6A). After 24-h exposure to the new media, the CsA-treated wells contained only 14,815 ± 807 cells/well (mean ± SEM) compared with 30,370 ± 2,253 cells/well in the control wells or 26,104 ± 849 cells/well in the PSC-833 wells. This initial toxic effect of CsA on recently seeded cells was confirmed in other experiments. Despite the initial decrease in cell number, cells grown in CsA had doubling times (61.3 h) similar to those grown in control (66 h), vehicle only (56.6 h), or PSC-833 (69.2 h) media.

Although these results allowed us to conclude that the enhanced differentiation in the presence of CsA was not the result of a slowing of the doubling rate, the initial decrease in cell number induced by CsA raised the possibility that the enhanced differentiation was the result of a selection of a subpopulation of cells by differential toxicity. We examined this possibility in two ways. First, we looked at CsA's effect on cells seeded at a high density in a manner identical to that used in the standard differentiation assays. Glass coverslips in 24-well plastic trays were seeded with 745,000 cells/well in 1 ml of medium in a manner identical to that used in the standard differentiation assays. After 24 h, the coverslips were transferred to PFHM-II ± 1 μM CsA in fresh 12-well trays. Immediately after this transfer step, there were 337,200 ± 10,600 cells/coverslip; most of the remaining cells from the initial seeding were lost when they attached to the plastic substratum of the original 24-well tray (Fig. 6B). After 24 h in PFHM ± 1 μM CsA, there were 541,000 ± 42,100 cells/coverslip in control wells but only 388,300 ± 14,500 cells/coverslip in the CsA-treated wells. The number of dead cells present in the culture medium of either treatment was small enough to make precise quantification difficult (<25,000/well) but appeared to be similar for both control and CsA wells. This suggests that the fewer attached cells in the

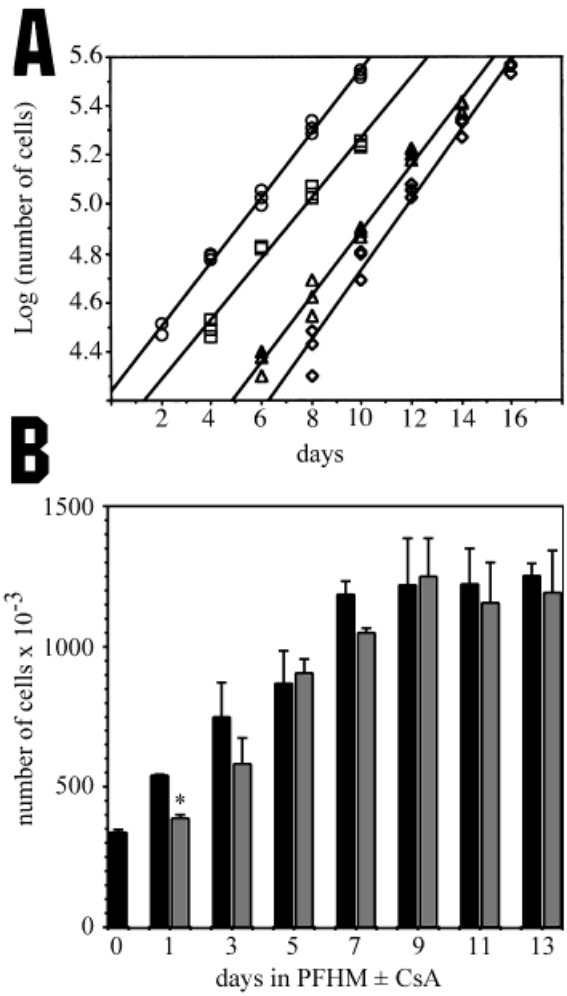

Fig. 6. Cellular proliferation in the presence of CsA and PSC-833. **A:** To determine the doubling time HT29-18N2 cells were grown in PFHM-II only (circles), vehicle control (squares), 1 μM CsA (triangles), or 0.1 μM PSC-833 (diamonds) for 10 days. The growth curves are offset to show that they have similar slopes and, therefore, similar doubling times (approximately 63 h). **B:** The effect of CsA on proliferation of HT29-18N2 cells seeded at high density was examined to see whether CsA was altering proliferation independent of an effect on the doubling rate. One day after the cells are transferred to PFHM ± 1 μM CsA, the number of cells in the CsA-treated wells (gray bars) is significantly less than in control wells (black bars). The limited number of detached cells found in culture medium on day 1 suggests this difference results from an initial lag in the onset of proliferation. After 3 days of exposure to CsA, the number of cells recovers to the control level and remains similar to control monolayers for the remainder of the 2-week exposure. *Responses that were significantly different ($P < 0.01$) from the corresponding controls.

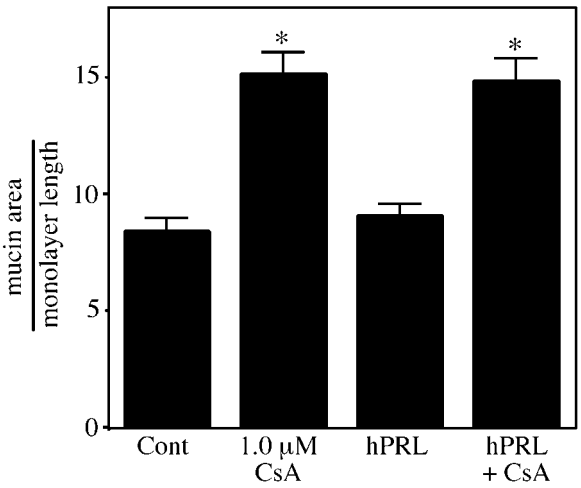

Fig. 7. Effect of prolactin on baseline and CsA-enhanced differentiation of HT29-18N2 cells. Morphometric quantification of the effect of 100 ng/ml prolactin (4.35 nM), 1 μM CsA, or 100 ng/ml prolactin + 1 μM CsA on the ratio of μm^2 of intracellular mucous secretory granule stores per linear μm of monolayer analyzed. Prolactin had no effect on either baseline differentiation or the enhanced differentiation evoked by CsA. *Responses that were significantly different ($P < 0.01$) from the corresponding controls.

CsA wells resulted from decreased mitotic doubling during the initial 24 h, rather than cell detachment. By day 4 after switching to PFHM-II ± 1 μM CsA, there was no difference ($P > 0.1$) in the number of cells in the control and CsA-treated wells. On day 14, the control coverslips had $1.25 \pm 0.04 \times 10^6$ cells, whereas the CsA-treated coverslips had $1.19 \pm 0.15 \times 10^6$ cells ($P > 0.1$).

We also examined the effect of growing cells in PFHM-II ± 1 μM CsA for 12 or 13 days and then switching them to the opposite treatment conditions for 24 to 48 h. Control cells switched to CsA for 24 h did not show a significant increase in intracellular stores (8.3 ± 1.8; 110%; $P > 0.2$) but a 48-h exposure to CsA did rise to 142% (10.6 ± 3.4; $P < 0.01$) of the level in 14-day controls (7.5 ± 1.7). The positive response of these fully confluent monolayers would argue against the effect's being a result of selective toxicity. Cells grown in CsA for 12–13 days and switched to control media for the final 24 h (13.9 ± 3.1) or 48 h (13.9 ± 2.9) had intracellular mucin stores identical ($P > 0.8$) to those grown in CsA for the entire 14 days (13.8 ± 3.8).

CsA does not act as a prolactin antagonist to enhance differentiation

Because CsA can act as a prolactin receptor antagonist (Russell et al., 1987) and prolactin has been reported to inhibit goblet cell differentiation in vivo (Gona, 1981), we investigated the possibility that CsA was acting via an inhibition of an autocrine release of prolactin (Fig. 7). Human prolactin (100 ng/ml) had no direct effect, however, on intracellular mucin stores (108% of control, $P > 0.1$) nor was it able to antagonize the response to CsA. CsA alone evoked a 180% increase in mucin stores, which was not significantly different ($P > 0.1$) from the 176% seen in monolayers exposed simultaneously to prolactin + CsA.

CsA does not act as a calmodulin antagonist to enhance differentiation

CsA can also act by inhibiting calmodulin, a calcium signal transducing protein (Colombani et al., 1985). Since calmodulin inhibitors promote partial differentiation of enterocyte clones of the HT29 cell line (Rochette-Egly et al., 1988), the ability of trifluoperazine (TFP), a classic calmodulin antagonist, to mimic the effect of CsA on goblet cell differentiation was tested. A 2-week exposure to 1 μM TFP had no significant effect on intracellular mucin stores (89% of control; $P > 0.1$). Exposure to 10 μM TFP caused massive cell death within 3 days.

CsA does not alter cyclooxygenase function to enhance differentiation

CsA can both stimulate and inhibit prostaglandin formation (Whisler et al., 1984; Zoja et al., 1986; McCauley et al., 1991). The ability of the cyclooxygenase inhibitor indomethacin to either mimic or block CsA's effect was tested. In two experiments, 1 μM indomethacin by itself had no effect on intracellular mucin stores (97 and 107%, respectively; $P > 0.1$) but significantly reduced the response to 1 μM CsA (58 and 47%; $P < 0.01$). Higher concentrations of indomethacin were toxic and, at a concentration of 10 μM, inhibited differentiation in the absence of CsA, and at 100 μM killed the cells within 3 days. The response to 1 μM indomethacin, however, was variable. In two subsequent experiments, 1 μM indomethacin had no effect on CsA-induced differentiation. We were also unable to inhibit CsA's effect with other cyclooxygenase inhibitors (naproxen, NS-398, nimesulide; data not shown). The instability of these inhibitors in aqueous buffers (Cayman Chemical product sheets) and their tendency to induce cytotoxic side effects and apoptosis at doses close to their effective concentrations (Shiff et al., 1996) made interpretation of these results difficult. As an alternative approach, we used an ELISA to measure total prostaglandin E_2 concentrations (intracellular stores + secretions) in the final 24 h of monolayers grown in PFHM ± CsA for 14 days. There was no significant difference between the PGE_2 levels of control (788 ± 50 pg/ml) and CsA-treated monolayers (775 ± 16 pg/ml). Exogenous prostaglandins were also unable to either mimic or enhance CsA's effect on differentiation. Treatment of HT29-18N2 cells with exogenous prostaglandins (0.01–10 μM PGD_2, PGE_1, PGE_2, PGF_{2a}, carbaprostacyclin, or the stable PGH_2 agonist U-46619) for 2 weeks had no significant ($P > 0.1$) effect on intracellular mucin stores (data not shown). In two experiments, the response to simultaneous addition of 1 μM PGE_2 + 1 μM CsA was not significantly different from that of CsA only (107 and 103% of CsA response, $P > 0.1$).

CsA does not alter baseline secretion of glycoproteins

The possibility that CsA increased intracellular mucin stores by inhibiting the slow baseline secretion of mucin glycoproteins—and not by increasing differentiation and mucin synthesis—was investigated. HT29-18N2 cells were grown in the presence or absence of 1 μM CsA for 2 weeks and then pulse-radiolabeled with

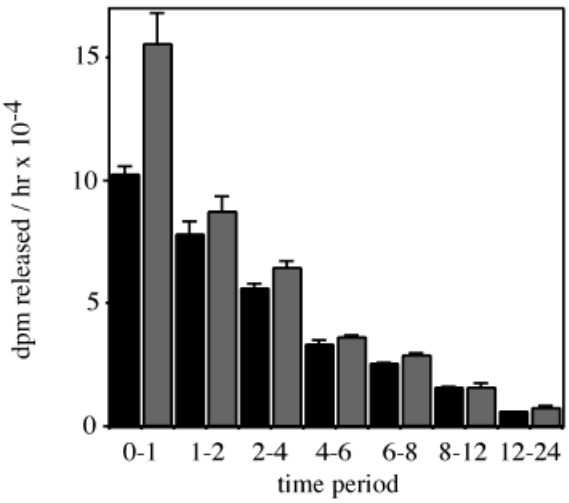

Fig. 8. CsA does not block glycoprotein secretion in HT29-18N2 cells. Mock-treated control (black bars) and CsA-treated (gray bars) cells were pulse-radiolabeled with ^{3}H-glucosamine for 1 h. Following rinsing, the rate of radiolabeled glycoproteins released into the culture medium was measured. The data are expressed as the dpm of radiolabeled glycoprotein released/h during the time period indicated. Consistent with their higher degree of differentiation, CsA-treated cells release slightly higher levels of radiolabeled glycoproteins than mock-treated controls at all time points. Both groups of cells show a similar rate of decline in their release of pulse-radiolabeled glycoproteins between the 2 and 24 h time points.

45 μCi of ^{3}H-glucosamine for 1 h. Secreted radiolabeled glycoproteins were collected from the culture medium at 1, 2, 4, 6, 8, 12, and 24 h after the pulse; at each time point, the culture medium was replaced with fresh medium (Fig. 8). Earlier autoradiographic studies have shown that in the initial 12 h of chase, the recovered radiolabeled glycoproteins are a mixture of nonmucin glycoproteins being secreted constitutively by a pathway not involving transient in mucin secretory granules and mucins arising from goblet cells with a limited number of secretory granules. After 12 h, most of the radiolabeled glycoproteins are coming from mucin secretory granules that have slowly migrated to the apical pole of well-differentiated goblet cells (Phillips et al., 1988). This results in a continual release of radiolabeled glycoprotein over the course of approximately 24 h in both control and CsA-treated cells. CsA-treated cells consistently released about 10% more radiolabel than the mock-treated controls between 2 and 24 h after the radiolabel pulse. It is clear that the time required for pulse-radiolabeled mucins and other glycoproteins to pass through the cells is similar in both control and CsA-treated cells.

DISCUSSION

CsA caused a dramatic increase in the level of differentiation of the HT29-18N2 human colonic goblet cell line that was independent of any effect on the proliferation rate. It caused cells to accumulate mucins at a faster rate and to a greater degree than possible in the absence of treatment. The increase was the result of a combination of a higher percentage of cells with mucin-filled secretory granules and an increase in the volume of mucin within a typical goblet cell. The release of pulse-radiolabeled glycoproteins, which would include high molecular weight mucins as well as lower molecular weight glycoproteins, followed similar time courses. This would support the hypothesis the increases in intracellular mucin stores found in CsA-treated monolayers were a result of increased synthesis and not simply a slowing of the baseline rate of mucin discharge.

Although CsA is best known as a T-cell immunosuppressant, numerous earlier studies have established its ability to act directly on nonlymphoid cells. A direct, nonimmunosuppressant contribution to CsA's therapeutic effect in psoriasis is suggested by the drug's ability to inhibit DNA synthesis and cellular proliferation of normal murine keratinocytes and transformed murine and human keratinocytes in vitro (Furue et al., 1988) and decrease bromo-2′-deoxyuridine labeling of not only human skin xenografts but also in endogenous epidermal and tongue tissues in nude mice lacking T-cells (Ramirez-Bosca et al., 1990). Consistent with two of the major adverse side effects of prolonged CsA therapy (glomerulosclerosis and diffuse interstitial fibrosis of the kidney), CsA increased the synthesis of extracellular matrix proteins of rat and human tubular epithelial primary cultures (Ghiggeri et al., 1994). CsA has also been previously shown to increase the doubling time and decrease the survival of the MC-26 mouse colon adenocarcinoma cell line in vitro (Saydjari et al., 1987).

CsA's inhibition of T-helper lymphocytes and their production of interleukin-2 has a well-characterized mechanism of action. CsA binds to a ubiquitous cytoplasmic protein called cyclophilin and the resulting CsA–cyclophilin complex binds and inactivates calcineurin, a calcium and calmodulin-sensitive serine, threonine phosphatase. Inactivation of calcineurin interferes with the nuclear entry of transcription factors, such as NF-AT, which are essential for induction of interleukin-2 and other cytokines. This cyclophilin-mediated pathway cannot be responsible for the direct action of CsA on the goblet cell line, since the nonimmunosuppressive analog PSC-833, which does not bind cyclophilin (Gaveriaux et al., 1991), was able to mimic CsA's effect.

In addition to its immunosuppressive action, CsA (and PSC-833) can reverse the multidrug resistance (MDR) phenotype. This reversal is believed to be a result of both an inhibition of MDR modulators binding to the target P-glycoprotein and a prevention of the upregulation of the *MDR-1* gene, which encodes P-glycoprotein (Hu et al., 1996). The P-glycoprotein is an ATP-dependent efflux pump, which excretes small hydrophobic drugs, such as *Vinca* alkaloids or anthracyclines from the cytoplasm (Endicott and Ling, 1989). It is unlikely the promotion of goblet cell differentiation by CsA and PSC-833 is mediated via a reversal of MDR, since the HT29-18N2 cell line would not be expected to express functional levels of P-glycoprotein. Neither P-glycoprotein expression nor P-glycoprotein–specific RNA was detected in the HT29 parent cell line from which the HT29-18N2 goblet cell clone was derived (Gaveriaux et al., 1991; Breuer et al., 1993). The inability of PSC-833, at any concentration, to fully mimic the effect of CsA also argues against a role for P-glycoprotein, since PSC-833 is known to be more

efficacious than CSA in reversing the MDR phenotype (Gaveriaux et al., 1991).

CsA can also act as a calmodulin antagonist (Colombani et al., 1985). This seemed a potential explanation for CsA's effects on goblet cell differentiation, since calmodulin inhibitors have previously been shown to increase the level of differentiation, as assessed by morphological evaluation of apical membrane microvilli and sucrase-isomaltase expression, of the parent HT29 cell line (Rochette-Egly et al., 1988). The inability of the classical calmodulin inhibitor TFP to promote goblet cell differentiation in the present study suggests, however, that CsA and PSC-833 are not acting as calmodulin antagonists at sublethal doses. This is consistent with an earlier report demonstrating that, although CsA decreased the proliferation of keratinocytes in vitro, it did not alter the bioactivity or immunological reactivity of the keratinocyte calmodulin (Fairley et al., 1989).

Another potential mechanism for CsA to modulate goblet cell differentiation would be via a blockade of autocrine growth factors binding to their receptors. It is known that CsA interferes with the binding of prolactin to its receptor (Russell et al., 1987). Prolactin has been reported to decrease the number of goblet cells in the rat ileum following daily injections over the course of 2 weeks, whereas treatment with ergocryptine, which blocks prolactin release in vivo, increased the number of goblet cells (Gona, 1981). In the present study, prolactin had no effect on the baseline differentiation of HT29-18N2 goblet cells nor was it able to interfere with CsA-evoked increases in mucin production.

CsA has both inhibitory and stimulatory effects on prostaglandin production in vivo and in vitro. CsA stimulates prostaglandin synthesis in peripheral blood monocytes (Whisler et al., 1984), bovine endothelial cells (Zoja et al., 1986), and smooth muscle cells (Lindsey et al., 1983). On the other hand, CsA decreases prostaglandin E_2 production in renal mesangial cells (McCauley et al., 1991). In the present study, it is unlikely that CsA's effect on goblet cell differentiation is mediated by inhibition of endogenous prostaglandin formation, since the cyclooxygenase inhibitor indomethacin had no direct effect on baseline differentiation in the absence of the drug. The inability of 1 μM indomethacin to directly alter differentiation of the HT29-18N2 implies that endogenous prostaglandin formation is not essential for differentiation in the absence of CsA. Indomethacin was able to inhibit CsA-enhanced differentiation in two experiments but this response could not be consistently replicated nor could it be duplicated by other cyclooxygenase inhibitors. The inability of exogenous prostaglandins to enhance differentiation, combined with absence of change in prostaglandin concentration following CsA exposure, suggests that the apparent inhibition of CsA in some experiments may have resulted from the well-known cytotoxic effects of indomethacin on HT29 and other cell lines, and not from a specific inhibition of cyclooxygenase activity (Shiff et al., 1996).

Decreased mucin production is linked to the dryness of the eye associated with KCS. Interestingly, although CsA is effective in reversing the symptoms of KCS in both dogs and humans, it does so without an obligatory increase in the aqueous component of tears (Kaswan et al., 1989; Gunduz and Ozdemir, 1994). In one of these studies, the authors concluded that CsA must modulate goblet cell function is some undefined fashion (Gunduz and Ozdemir, 1994). In the other study, the investigators suggested three hypotheses to explain CsA's therapeutic action including (1) prolactin regulation, (2) a direct epitheliotrophic effect, or (3) a direct secretory effect on lacrimal glands (Kaswan et al., 1989). The present study is consistent with both of these earlier studies inasmuch as CsA has now been shown to have a direct epitheliotrophic effect on goblet cells.

Our findings have an important implication in the treatment of ulcerative colitis. Decreased intracellular mucin stores are a widely accepted hallmark of ulcerative colitis. It is more controversial whether the decrease in mucins precipitate the disease or are simply a nonspecific response to inflammation. Chromatographic analysis of mucins from ulcerative colitis tissues (Podolsky and Isselbacher, 1984) or a cotton top tamarin model of the disease (Podolsky et al., 1985) were both found to be missing a similar mucin glycoform. Furthermore, retrospective epidemiological studies identified 10 sets of homozygous twins in which only one twin had ever suffered from inflammatory bowel disease, even though both twins were missing the suspect mucin glycoform (Tysk et al., 1991). This suggests that the absence of a particular mucin glycoform can lead to a predisposition to the disease but is not the direct cause. Although CsA can be effective in severe cases of ulcerative colitis that are unresponsive to conventional treatments, the complications arising from its immunosuppressive properties and toxic effects on the kidney limit its use to a bridging therapy (Sandborn, 1995). If some or all of the therapeutic benefit of CsA does, in fact, derive from its direct effect on goblet cells, the present study opens the possibility that nonimmunosuppressive analogs such as PSC-833 could be used as alternative treatments with fewer side effects.

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