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Induction of Apoptosis to Control Drug-Induced Gingival Overgrowth: An *In Vitro* Study

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ABSTRACT

Gingival overgrowth is an adverse effect of several classes of drugs including anticonvulsants, calcium channel blockers and the immunosuppressant cyclosporine A (CsA). Current treatment options of drug-induced gingival hyperplasia include both nonsurgical and surgical interventions. Surgical interventions have a high rate of recurrence and are not the most appropriate treatment options in immunocompromised patients. The preferred nonsurgical interventions are symptomatic and do not resolve the condition, and as yet, there is no effective, nonsurgical option for its treatment. Gingival tissue is constantly involved in cycles of tissue resorption, remodeling and replacement by apoptotic pathways. Apoptosis and cell clearance are necessary for constant tissue remodeling, and a lack of these processes plays a critical role in gingival overgrowth. We hypothesized that CsA-induced gingival overgrowth can be controlled by the use of specific agents to induce apoptosis. An *in vitro* cell culture model of gingival cells was overproliferated using CsA to mimic gingival overgrowth, following which, the cells were exposed to either 100ng/ml or 500ng/ml of Cytochrome C to induce apoptosis at 3, 6 and 9 day time points. Cell densities were calculated both pre and post Cyt C treatment. Cells were also immunostained with DAPI to visualize the nuclei and laser scanning confocal microscopy was used to image and record the features of apoptotic nuclei. Statistical analyses were carried out. Our data indicate that following treatment with Cyt C, cell densities at 3, 6 and 9 day time points showed statistically significant decreases. This study is an important first step in determining if inducing apoptosis could be a viable, nonsurgical method of managing cellular proliferative disorders like drug-induced gingival overgrowth.

INTRODUCTION

Gingival overgrowth is an undesirable and well recognized side-effect of oral, intramuscular, or intravenous use of various drugs, including phenytoin, phenobarbital, valproate, nifedipine, verapamil and cyclosporine (Beveridge et al., 1981). Cyclosporine A (CsA) is a lipophilic, cyclic endecapeptide, isolated as an antifungal and used as an immunosuppressant. It functions to greatly reduce T-helper cell proliferation during organ transplants, so that the body will accept the foreign tissue successfully (Britton et al., 1982). It has been estimated that 25-80% of patients on a regimen of CsA experience gingival hyperplasia (Lawrence et al., 1994), an overgrowth of gingival tissue resulting from an inhibition of normal apoptotic pathways. Apoptosis and cell clearance are necessary for constant tissue remodeling, and a lack of these processes plays a critical role in gingival overgrowth. Drug-induced gingival overgrowth begins as an enlargement of the papillary gingiva, which is more pronounced on the labial surfaces and less on the palatal and lingual surfaces (Tyldesley et al., 1984). Although the overgrowth is initially restricted to the width of the gingiva, in extremely severe cases, the overgrowth can completely extend over and cover the crowns of the teeth. In such extreme cases, the gingival overgrowth interferes with occlusion, mastication and speech in affected patients (Lawrence et al., 1994). Hyperplastic gingival tissue readily bleeds on probing, and is much more susceptible to infections (Seymour and Jacobs, 1992). Current management of drug-induced gingival overgrowth typically is carried out through surgical procedures like gingivectomies. Surgical procedures are associated with inherent risks, including but not limited to, complications of anesthesia, severe post-operative bleeding, prolonged healing periods in immunocompromised patients and increased risk of infection. Other methods to reduce gingival overgrowth include the use of electrocautery or CO_2 lasers, but these procedures can be costly and have similar adverse consequences (Hegde et al., 2012).

The purpose of the current study was to explore a nonsurgical method to manage CsA-induced gingival overgrowth. We hypothesized that CsA-induced gingival overgrowth can be controlled by the use of specific agents to induce apoptosis. Apoptotic cell death is preferred over necrotic cell death, since necrosis is associated with sustained inflammatory cell damage caused when necrosed cells swell and undergo lysis to spew their cytoplasmic contents. When a cell commits "cell suicide" by apoptosis, it undergoes cell shrinkage, chromatic condensation, nuclear fragmentation and cytoplasmic budding, with a resultant noninflammatory clearance from the tissue (Potten et al., 2004). The current study used an *in vitro* cell culture model to test our hypothesis.

MATERIALS AND METHODS

A commercially available Human Gingival Epithelial Progenitor cell line (HGEP, ZenBio, Research Triangle Park, NC) obtained from a single donor was expanded using routine cell culture techniques. Specifically, HGEP cells were aseptically cultured in 12-well plates (Corning Incorporated, Corning, NY) using a specialized, progenitor cell targeted, culture medium (CnT-24 media, ZenBio, Research Triangle Park, NC) in a 5% CO_2 environment at 37°C. Supplements provided by the manufacturer were added to the media as per manufacturer's directions. Cells were grown until they reached 80% confluency and then subcultured as shown in Figure 1.

The subcultured HGEP cells were grown either directly on the bottom of the 12-well plates or onto glass coverslips placed into the 12-well plates till they reached 80% confluency. Cells were then exposed

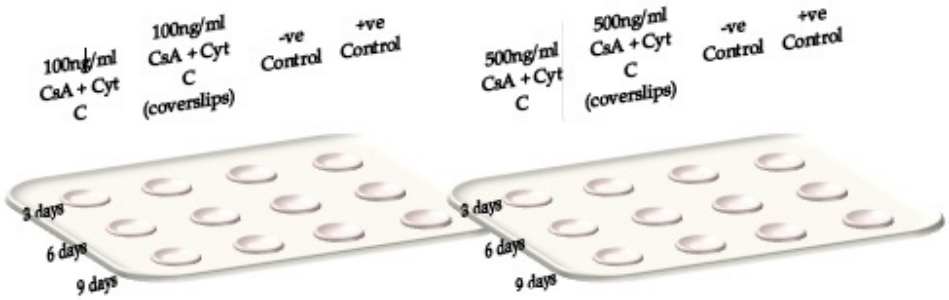


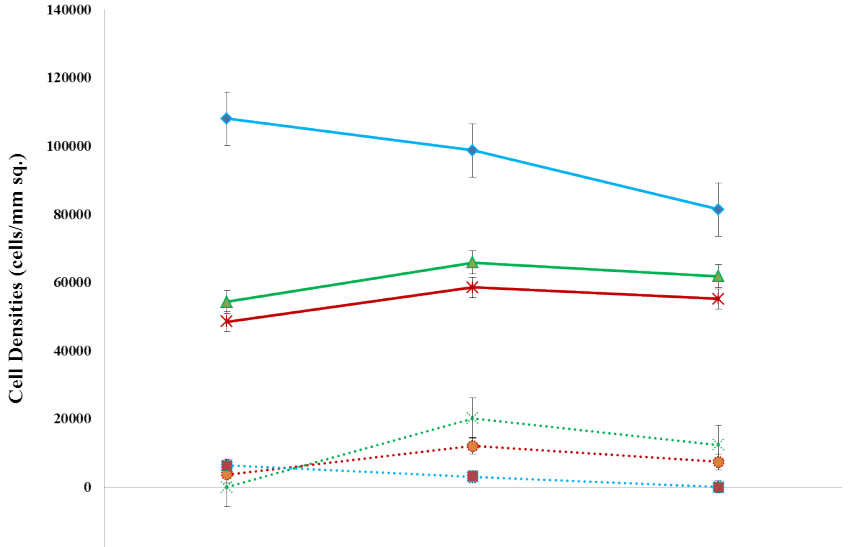
Figure 1. Summary of experimental design. Cells were subcultured into 12-well plates, with or without glass coverslips, exposed to 100ng/ml or 500 ng/ml CsA for 3, 6 and 9 days. At the three time-points, cells were exposed to exogenous Cyt C for 30 minutes. Cell counts were obtained from wells without cover slips using Trypan Blue technique, while cells on cover slips were immunoreacted with DAPI to visualize nuclear apoptotic changes.

to either 100ng/ml or 500ng/ml CsA (Sigma-Aldrich, USA) and allowed to overproliferate for 3, 6, and 9 days. At the three time-points, cells in the experimental groups were incubated in exogenous Cytochrome C (Cyt C from bovine heart, Sigma-Aldrich, USA) for 30 minutes, following which, cell counts were obtained from wells which did not contain the glass cover slips using the Trypan Blue dye exclusion technique in a hemocytometer. In order to assess apoptotic changes, HGEP cells grown on cover slips were fixed in 4% paraformaldehyde, nuclei stained with DAPI, cover slips mounted on glass slides and imaged using a laser scanning confocal microscope (Fluoview FV1200, Olympus, USA). Corresponding control cultures were maintained at all three time points; the negative control was incubated only in Cyt C treatment and the positive control was exposed to CsA but did not receive the Cyt C treatment. All cell counts were expressed as cell density in cells/ml and the experiment was carried out in triplicate.

Data were analyzed with multivariate repeated measures analysis of variance (MANOVA) with two within-subject (repeated-measures) factors [(1) day (3, 6, or 9) and (2) pre or post] and one between group factor (control arm versus treatment arm (100 or 500 ng/ml CsA). An advantage of MANOVA is that it does not require that the assumptions of compound symmetry and sphericity to be fulfilled. As part of this analysis, summary statistics and plots were created. The Tukey honestly significant difference test was used for post-hoc testing. Statistical testing was performed with Statistica Release 10 (StatSoft, Inc., Tulsa, OK).

RESULTS

Following treatment with Cyt C, cell densities at 3, 6 and 9 day time points showed statistically significant decreases (Figure 2). Mean cell densities on day 3, prior to Cyt C treatment was 54333, 65833 and 61833 for controls, 100ng/ml CsA and 500ng/ml CsA groups respectively. Following Cyt C treatment, these cell densities dropped significantly to 0, 20166 and 12333 respectively. On day 6, prior to Cyt C treatment, mean cell densities were 48500, 58500 and 55166 for control, 100ng/ml and 500ng/ml groups respectively. Following Cyt C treatment, mean cell densities decreased significantly to 3666, 12000 and 7333 respectively. The day 9 time point followed a similar trend with pre Cyt C treatment levels at 108000, 98666 and 81333 and post Cyt C levels at 6333, 3000 and 0 mean cells for control, 100ng/ml and 500ng/ml groups respectively (Data shown in tabular form within Figure 2). Percentage decreases in mean cell densities of post Cyt C treatment



	Control	100ng/ml CsA	500 ng/ml CsA
3 days Pre-CytC	54333.33333	65833.33333	61833.33333
3 days Post-CytC	0	20166.66667	12333.33333
6 days Pre-CytC	48500	58500	55166.66667
6 days Post-CytC	3666.666667	12000	7333.333333
9 days Pre-CytC	108000	98666.66667	81333.33333
9 days Post-CytC	6333.333333	3000	0

Figure 2. Overproliferated gingival (HGEP) cells show significant decrease in cell density post Cytochrome C treatment. Statistically significant decreases in cell densities were seen at all three time points between pre Cyt C treatment and post Cyt C treatment groups ($p \leq 0.01$). Significant differences were also seen within each group at the three time points ($p \leq 0.01$). Line plots at 3, 6 and 9 days are shown for pre (solid lines) and post (dotted lines) Cyt C treatment. Mean values of cell densities are shown in the included table for control, 100ng/ml CsA and 500ng/ml CsA exposed cells. Data are shown as mean ±SE.

Table 1. Significant percentage decreases in mean cell densities at the three time points post Cytochrome C treatment (values are calculated compared to pre Cyt C levels).

	Control	100ng/ml CsA	500ng/ml CsA
3 days Post-CytC	100%	69.36%	80.05%
6 days Post-CytC	92.43%	79.48%	86.70%
9 days Post-CytC	94.13%	96.95%	100%

as compared to pre Cyt C treatment were also calculated (Table 1). Cell densities post Cyt C treatment decreased within a range of 69.36% to 100% compared to pre Cyt C treatment at the various time points.

DAPI staining of nuclei with concomitant confocal imaging showed cells in various stages of apoptosis, with characteristic apoptotic features. The apoptotic features noted included, chromatin condensation (white arrows-Figure 3), nuclear pyknosis (white arrowheads-Figure 3) and nuclear fragmentation (bottom panels-Figure 3).

DISCUSSION

The current study investigated if cyclosporine A (CsA) can be used to control drug-induced gingival overgrowth by inducing apoptosis in gingival epithelial cells, using an *in vitro* cell culture model. Two experimental conditions using specific CsA doses, 100ng/ml and 500ng/ml, were chosen to reflect the serum-levels of CsA that are typically maintained in patients over time (Chaudary et al., 2008).

Cytochrome C (Cyt C) is an enzyme that is stored in the mitochondria and is involved in the apoptotic protease activating factor 1/"Apaf-1" pathway of programmed cell death or apoptosis. When the mitochondrial membrane potential is disturbed by a large calcium uptake or other triggers such as physiological stress, the mitochondrial permeability transition pore/MPTP opens up and releases Cyt C. Upon release, Cyt C binds to Apaf-1, forming apoptosomes. The apoptosomes bind to and cleave caspase-9, releasing the mature activated form of the pre-proteins. Subsequent caspases are then stimulated which initiate a cascade committed to the process of apoptosis. When gingival cells are exposed to Cyclosporine A, the drug binds to the cyclophillin-D proteins of the mitochondrial MPTP and results in a conformational change, thus blocking the MPTP. The blockage of the MPTPs prevents the release of Cyt C into the cytosol in response to pro-apoptotic stimuli. Cyt C cannot therefore combine with Apaf-1 in the cytosol to activate caspase-9, which in turn cannot activate caspase-3 and caspase-7 to initiate the apoptotic cascade (Walter et al., 1998).

We overproliferated human gingival cells (HGEP cells) by exposure to CsA and then induced apoptosis by the introduction of exogenous Cyt C into CsA-treated gingival cells. Our results indicate a significant reduction in cell numbers at 3, 6 and 9 days post-Cyt C treatment. Data show that incubation with 100ng/ml CsA and 500ng/ml CsA caused a statistically significant overproliferation of HGEP cells as compared to control cells at all three time points. Within each of the three groups (control, 100ng/ml CsA, 500ng/ml CsA), mean cell density at day 6 was the lowest, and as expected, mean cell density at day 9 was the highest. Decreased cell proliferation of pre Cyt C cells at day 6 compared to day 3 resulted in a consequent decrease in the mean cell density post Cyt C treatment at day 6 as well. Since this phenomenon was encountered across all experimental repetitions at the day 6 time point, we speculate that experimental/cell culture conditions alone might not have caused it. The HGEP cells could have potentially exhibited a transient growth in-

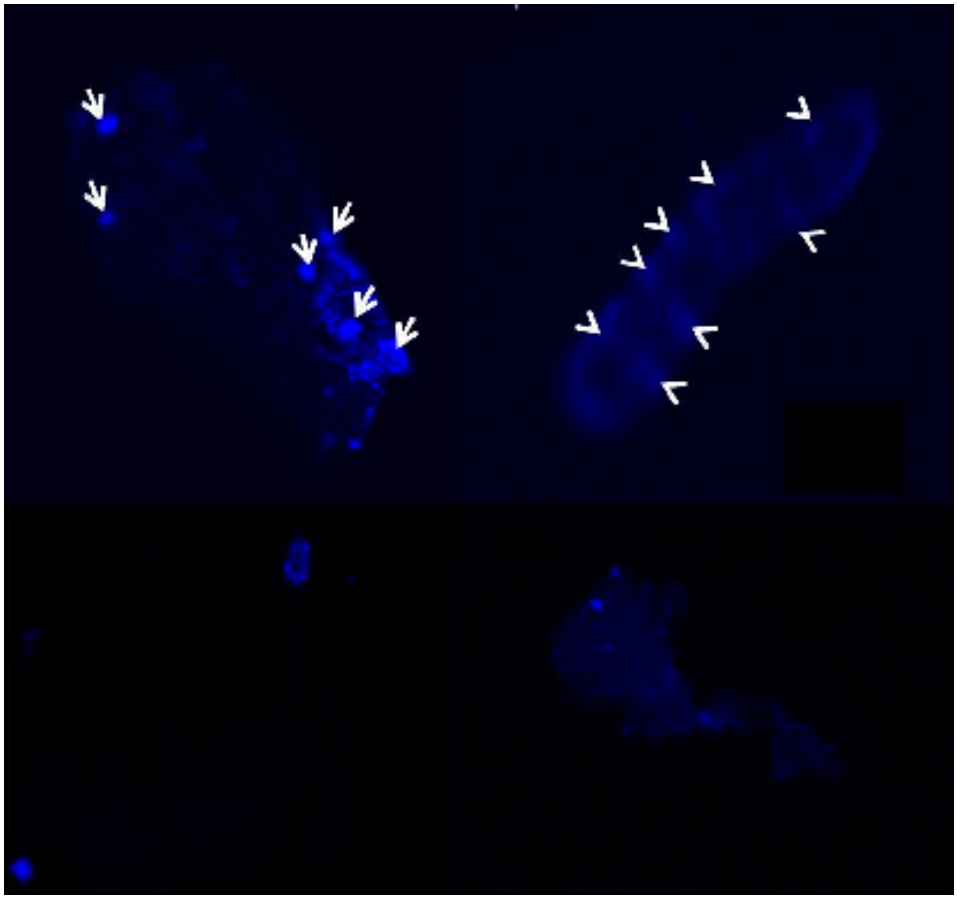


Figure 3. Montage of laser scanning confocal images of gingival epithelial cells in various stages of apoptosis, post-CytC treatment. HGEP cells cultured on glass coverslips, were immunostained with DAPI after treatment with Cyt C to visualize nuclei. Apoptotic nuclear features seen include, chromatin condensation (white arrows), pyknotic nuclei (white arrowheads) and scattered nuclear fragments/karyorrhexis of varying sizes (bottom panels).

hibition induced by CsA at this time point (Lauer G et al., 2006). This phenomenon was transient since cells at day 9 exhibited a dramatic increase in cell numbers on exposure to CsA. It is also possible, that after the immediate overproliferation caused by CsA up to 3 days, the cells potentially activated internal pathways to regulate the cell cycle as a temporary compensatory mechanism. By day 9, the balance tipped back in favor of cell overproliferation. Future investigation into specific cellular pathways that could have been triggered is warranted.

Cells incubated in 100ng/ml and 500 ng/ml CsA showed statistically significant increases in cell density when compared to control cells at day 3 and day 6 time points. At day 9, pre Cyt C cell density was less than control group, and could be attributed to the potential transient growth inhibition. Cells incubated in 500ng/ml CsA also showed statistically significant decrease in cell densities as compared to cells incubated in 100ng/ml at all three time points. These data corroborate the previously documented evidence that higher concentrations of CsA (500ng/ml) results in growth inhibition (Lauer et al., 2006; Tyldesley et al., 1984, Walter et al., 1998). With both the experimental groups, we noted that long term exposure coupled with the high doses of CsA tipped the balance in favor of cell overproliferation, mimicking the condition seen *in vivo*.

Following treatment with Cyt C, decrease in cell numbers was seen as expected. Data clearly show a statistically significant decrease in cell densities at 3, 6 and 9 days post Cyt C treatment in the controls, and both experimental conditions (100ng/ml and 500ng/ml). Cell densities were also significantly decreased when compared between groups (i.e. 100ng/ml CsA vs. controls and 500ng/ml CsA vs. controls). The rapid and dramatic decrease at day 3 could be attributed to the sudden intracellular influx of Cyt C. Around day 6, the cells seem to have stabilized to the Cyt C treatment and show less decrease in cell densities when compared to the day 3 time point. Independent of the control cell density, at the day 9 time point, there was significant cell density decrease from pre Cyt C to post Cyt C treatment following incubation in both 100ng/ml and 500ng/ml CsA. This indicates that Cyt C treatment could be effective in inducing apoptosis and decreasing cell densities in this *in vitro* model.

Apoptosis is characterized by a series of morphological changes of the cell and nuclei (Kerr et al., 1972; Kerr et al., 1994). Onset of apoptosis is indicated by cell shrinkage and condensation of chromatin. The process continues with eventual formation of pyknotic nuclei. Ultimately, the nuclei fragments and breaks down, a process known as karyorrhexis. We stained gingival cells grown on cover slips with DAPI to visualize the nuclei. Our data clearly show the condensation of chromatin (white arrows-Figure 3) and the formation of pyknotic nuclei along the margins of the nuclear membrane (white arrowheads-Figure 3). As expected, DAPI staining of gingival nuclei also showed nuclear fragmentation and blebbing, consistent with apoptotic cell death (bottom panels-Figure 3).

Taken together, the data from these experiments constitute an important first step in determining if inducing apoptosis could be a viable, nonsurgical method of managing cellular proliferative disorders like drug-induced gingival overgrowth. Future studies in our laboratory will elaborate the current study to correlate apoptotic features with apoptotic pathways. We will also test the hypothesis using gingival tissue models that will more closely simulate the clinical condition.

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