DRUG-INDUCED GINGIVAL OVERGROWTH: AN IN VITRO STUDY ON CYCLOSPORINE AND HUMAN GINGIVAL FIBROBLASTS

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Gingival overgrowth is a serious side-effect that accompanies the use of cyclosporine. Up to 97% of the patients submitted to immunosuppressant drugs have been reported to suffer from this side-effect. Several conflicting theories have been proposed to explain the fibroblast's function in gingival overgrowth. To determine whether cyclosporine alter the inflammatory responses, we investigated its effects on gingival fibroblast gene expression as compared with untreated cells. Fragments of gingival tissue of healthy volunteers (11-year-old man, 68-year-old-woman and 20-year-old-man) were collected during operation. Cells were incubated with cyclosporine and gene expression of 29 was investigated in gingival fibroblasts cell culture, compared with untreated cells. The gene expression level was significantly deregulated only for 10 genes (CCL1, CCR1, CCR4, CCR5, CCR10, IL1A, IL1B, IL5, IL6R and TNFSF10) that were found to be downregulated except for TNFSF10. These results seem to demonstrate that cyclosporine has no inflammatory effect on healthy gingival fibroblast. In the future, it would be interesting understand, the possible effect of the drug on inflammation of patients affected by gingival hyperplasia.

Gingival overgrowth is a serious side effect of the administration of cyclosporine. It is worsened by bacterial plaque accumulation, and may interfere with normal oral functions such as smiling, speaking, eating, resulting in psychological problems (1,2). The incidence of cyclosporine-induced gingival overgrowth (CIGO) is in the range of 20-80% of the patients (3); in addition, up to 90% of the transplant recipient patients, who have been submitted to cyclosporine therapy, present CIGO (4).

Several factors such as age, sex, duration of treatment and dosage of the prescribed cyclosporine are influencing the severity of clinical manifestation of CIGO (5-7). Cyclosporine is a potent immunosuppressive drug widely prescribed for autoimmune diseases therapy, and for treating graft versus host disease in organ transplant patients (8). To prevent CIGO, several approaches have been proposed, such as drug substitution or cyclosporine dose reduction, as well as oral hygiene programs and surgical interventions, but each of these approaches may have contraindications. To prevent CIGO, reducing the dosage or using alternative drugs is not possible in all situations. Other drugs have their own side effects too. However surgical intervention is only proposed for cosmetic cases, whilst oral hygiene protocols have been demonstrated to be efficient in controlling CIGO but could not inhibit its development.

Key words: Gingival overgrowth, gene expression, drugs, cyclosporine

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DISCLOSURE: ALL AUTHORS REPORT NO CONFLICTS OF INTEREST RELEVANT TO THIS ARTICLE.

CIGO and Inflammation

CIGO may produce an inflammation process with increased fibrotic response in the extra-cellular matrix. Inflammation could be necessary to promote the fibrotic process, but not for its progression (9). Several studies stated that pathogens bacteria in gingival tissue as well as mucous membrane trauma may worsen the inflammatory process responsible for the severity CIGO (10).

Immune-inflammatory features associated with CIGO show increased macrophage reparative/ proliferative phenotype, up-regulation of essential growth factors, IL-1β, and IL-6 cytokines and variable lymphocyte proportions (11, 12). Several studies have described a lymphocyte infiltration of plasma cells in CIGO samples of fibroblasts. This would suggest that the humoral immune response replaces the cellular immune response in CIGO fibroblasts, because of immunosuppressive drugs. Moreover, the immunity pattern in patients with CIGO is characterized by the low expression of some types of lymphocytes (natural killer lymphocytes) in contrast to chronic inflammatory periodontal disease. The cellular and tissue features of human gingival overgrowth lesions caused by phenytoin, nifedipine, and cyclosporine, respectively, have different histological characteristics: phenytoin provokes low inflammation and high fibrosis; nifedipine produces more or less inflammation and fibrosis equally, whilst cyclosporine produces high inflammation and low fibrosis. It is possible that CIGO presents a very pronounced activation of immunity system with some moderate antifibrotic effects in the synthesis and deposition of collagen.

A positive balance between the synthesis and degradation of components of the extracellular matrix causes a deposit of the matrix, which has been suggested as one of the most significant events in CIGO (13). Since the pathogenesis of CIGO is not well known, it is supposed that cyclosporine influences the regulation of inflammatory mediators such as chemokines and cytokines in fibroblast (14). Growth factors activity might induce CIGO; in fact, some studies show that cyclosporine modifies the transcription of several chemokines and cytokines such as Chemokine ligand 1 (CCL1), Interleukin 1 alpha (IL1A) and Tumour necrosis factor superfamily member 10 (TNFSM10).

Objective

To determine whether cyclosporine can alter the inflammatory response, we investigated its effects on the gene expression of fibroblast isolated from healthy volunteers.

MATERIALS AND METHODS

Primary Human Fibroblast cells culture

Fragments of gingival tissue of healthy volunteers (11-year-old man, 68-year-old woman and 20-year-old man) were collected during operation. The pieces were transferred in 75 cm^2 culture flasks containing DMEM medium (Sigma Aldrich, Inc., St Louis, Mo, USA) supplemented with 20% fetal calf serum, antibiotics (Penicillin 100U/ml and Streptomycin 100 micrograms/ml-Sigma Aldrich, Inc., St Louis, Mo, USA).

Cells were incubated in a humidified atmosphere of 5% CO_2 at 37°C. The medium was changed the next day and twice a week. After 15 days the pieces of gingival tissue were removed from the culture flask. Cells were harvested after additional 24h of incubation.

Cell viability test

A stock solution of cyclosporine 1mg/mL was prepared. Further dilutions were made with the culture medium to the desired concentrations just before use. Cell lines were seeded into 96-well plates at a density of 10^4 cells per well containing 100µl of cell culture medium and incubated for 24h to allow cell adherence. Serial dilution of cyclosporine (5000 ng/mL, 2000 ng/mL, 1000 ng/mL, 500 ng/mL, 100 ng/mL) were added (three wells for each concentration). The cell culture medium alone was used as negative control.

After 24h incubation, cell viability was measured using PrestoBlue™ Reagent Protocol (Invitrogen) according to the manufacturer's instructions. Briefly, the PrestoBlue™ solution (10µl) was added into each well containing 90µl of treatment solution. Plates were then placed back into the incubator for 1h, after which absorbance was measured at wavelengths of 570 nm excitation and 620 nm emission by an automated microplate reader (Sunrise™, Tecan Trading AG). The percentage of viable cells was

determined by comparing the average absorbance in drug treated wells with average absorbance in control wells exposed to vehicle alone. The results were presented as the mean ± standard deviation of three measures.

Cell treatment

Cell lines were seeded at a density of 1.0 x 10^5 cells/ml into 9cm^2 (3ml) wells and subjected to serum starvation for 16 h at 37°C. Cells were treated with 1000 ng/mL cyclosporine solution for 24h. This solution was obtained in DMEM supplemented with 2% FBS, antibiotics and aminoacids. Cell medium alone was used as negative control. The cells were maintained in a humidified atmosphere of 5% CO_2 at 37°C. After the end of the exposure time cells were trypsinized and processed for RNA extraction.

RNA isolation, reverse transcription and quantitative real-time RT-PCR

Total RNA was isolated from cell lines using GenElute mammalian total RNA purification miniprep kit (Sigma-Aldrich) according to manufacturer's instructions. Pure RNA was quantified at NanoDrop 2000 spectrophotometer (Thermo Scientific).

cDNA synthesis was performed starting from 500 ng of total RNA, using PrimeScript RT Master Mix (Takara Bio Inc.). The reaction was incubated at 37°C for 15 min and inactivated by heating at 70°C for 10 sec. cDNA was amplified by Real Time Quantitative PCR using the VIIA™ 7 System (Applied Biosystems).

All PCR reactions were performed in a 20µl volume. Each reaction contained 10µL of 2x qPCRBIO SYGreen Mix Lo-ROX (Pcrbiosystems), 400nM concentration of each primer, and cDNA.

Custom primers belonging to the "Inflammatory Cytokines and Receptors" pathway were purchased from Sigma Aldrich. All experiments were performed including non-template controls to exclude reagents contamination. PCR was performed including two analytical replicates.

The amplification profile was initiated by 10 min incubation at 95°C, followed by two-step amplification of 15 sec at 95°C and 60 sec at 60°C for 40 cycles. As a final step, a melt curve dissociation analysis was performed.

Statistical analysis

The gene expression levels were normalized to the expression of the reference gene (RPL13) and were expressed as fold changes relative to the expression of the

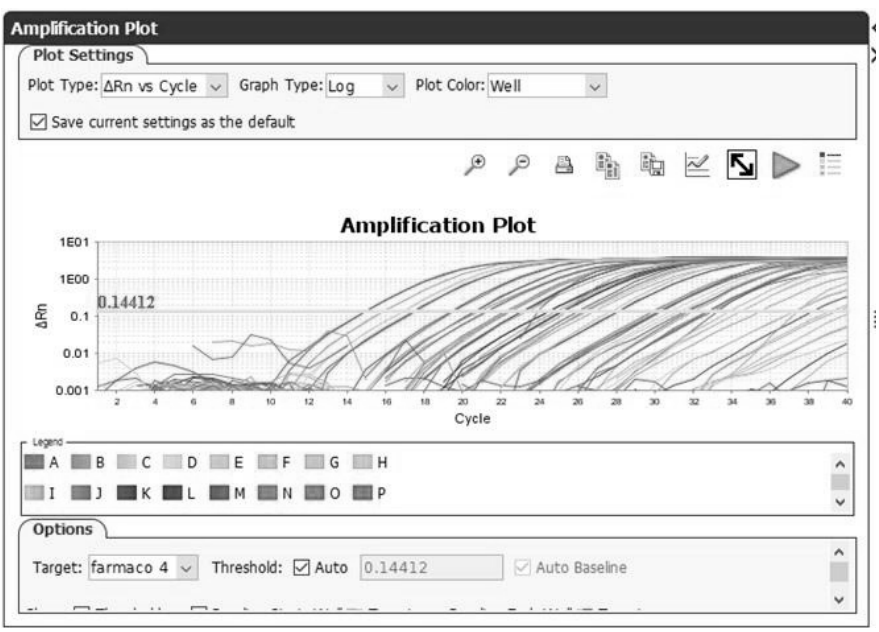

Fig. 1. *Amplification plot curves of the 29 genes belonging to the "Inflammatory Cytokines and Receptors" pathway analyzed using Real time PCR.*

untreated cells. Quantification was done with the delta/delta Ct calculation method (15).

RESULTS

The cell viability test PrestoBlue™ established that the optimal concentration of cyclosporine to carry out the treatment was 1000 ng/ml. At this concentration, about 80% of cells were vital.

After the treatment the gene expression levels of 29 genes belonging to the "Inflammatory Cytokines and Receptors" pathway was studied. The gene name and their fold change measured by Real Time PCR are reported in Table I. Fig. 1 shows the amplification plot profile of the amplifications.

Bold fonts indicate significant variation of gene expression level: fold change ≥ 2 and p value ≤ 0.05 for up-regulated genes, and fold change ≤ 0.5 and p value ≤ 0.05 for significantly down-regulated genes. Table II shows the 10 genes whose expression was significantly deregulated.

All genes (CCL1, CCR1, CCR4, CCR5, CCR10, IL1A, IL1B, IL5, IL6R) were downregulated except TNFSF10 (Table I, Fig. 2)

Table I. *Selected genes used in Real Time PCR belonging to "Inflammatory Cytokines and Receptors" pathway. In bold the Fold change of significant gene expression level.*

Gene	Fold change	Gene function
CCL1	**0,24**	Chemokine
CCL2	0,89	Chemokine
CCL2D	0,80	Chemokine
CCL5	0,92	Chemokine
CCL8	0,59	Chemokine
CXCL5	0,82	Chemokine
CXCL10	0,70	Chemokine
CCR1	**0,10**	Chemokine receptor
CCR4	**0,12**	Chemokine receptor
CCR5	**0,15**	Chemokine receptor
CCR6	1,30	Chemokine receptor
CCR10	**0,46**	Chemokine receptor
CXCR5	0,82	Chemokine receptor
IL1A	**0,30**	Interleukin
IL1B	**0,28**	Interleukin
IL5	**0,10**	Interleukin
IL6	**0,73**	Interleukin
IL7	0,65	Interleukin
IL8	0,72	Interleukin
ILR1	0,70	Interleukin receptor
IL1RN	0,65	Interleukin receptor
IL6R	**0,13**	Interleukin receptor
IL10RB	1,04	Interleukin receptor
BMP2	1,76	Cytokine
SPP1	1,46	Cytokine
TNFRSF	0,96	Cytokine
TNFSF10	**9,39**	Cytokine
VEGFA	1,43	Cytokine
RPL13	1,00	Housekeeping genes

DISCUSSION

CIGO is a pathological expression of the consequences of the assault from both mechanical stimuli and side effects of drugs on the oral mucosa. Gingival mucosa has been developed biological mechanism to combat these noxious stimuli, and in this process, cells of the innate immune system play a major role. CIGO is the response of molecular regulatory pathways that coordinate the host response to the effect of drug administration and other microbiological patterns. Innate immunity also plays a fundamental role in the pathogenesis of CIGO, since it acts as a surveillance mechanism to prevent chronic inflammation. In addiction chronic inflammation is now recognized as a starting mechanism of tumour development. It is thought that upregulated inflammation within the cellular microenvironment, is one of the key elements causing CIGO, however, how immune and inflammatory processes are regulated and how they may result in different clinical manifestations are initial questions that are beginning to be understood in some detail.

CIGO and its chronic inflammation are sustained by oral biofilm including bacteria, viruses and fungi, living in a homeostatic balance with each other and the immunity system (13). The dysbiosis causing CIGO, and the unbalance between oral bacteria and host pro-inflammatory and anti-inflammatory

mediators, are factors influencing the severity of CIGO. Weakened immune system as the result from administration of antisuppresant drugs such as cyclosporine, allows increased dysbiotic oral microflora sustaining the etiopathogenesis of CIGO (14). Although it is known that microbial dysbiosis promotes CIGO, deregulated immune response mechanisms determine the progression and the extent of tissue overgrowth.

Disease progression in CIGO is a complex process that involves the interaction of multiple components of the host immune response and the oral microbiome. Chemokines and cytokines with their receptors releasing inflammatory mediators play a central role in CIGO development, however, the deregulated tissue homeostasis and inflammation process in CIGO, exposes oral mucosa to the product of altered metabolism such as necrotic cells, reactive

Table II. *Significant gene expression levels after 24h treatment with cyclosporine, as compared with untreated cells.*

Gene	Fold change	SD (+/-)	Gene function
CCL1	0,24	0,03	Chemokine
CCR1	0,10	0,01	Chemokine receptor
CCR4	0,12	0,02	Chemokine receptor
CCR5	0,15	0,00	Chemokine receptor
CCR10	0,46	0,05	Chemokine receptor
IL1A	0,30	0,01	Interleukin
IL1B	0,28	0,04	Interleukin
IL5	0,10	0,02	Interleukin
IL6R	0,13	0,00	Interleukin receptor
TNFSF10	9,39	0,27	Cytokine

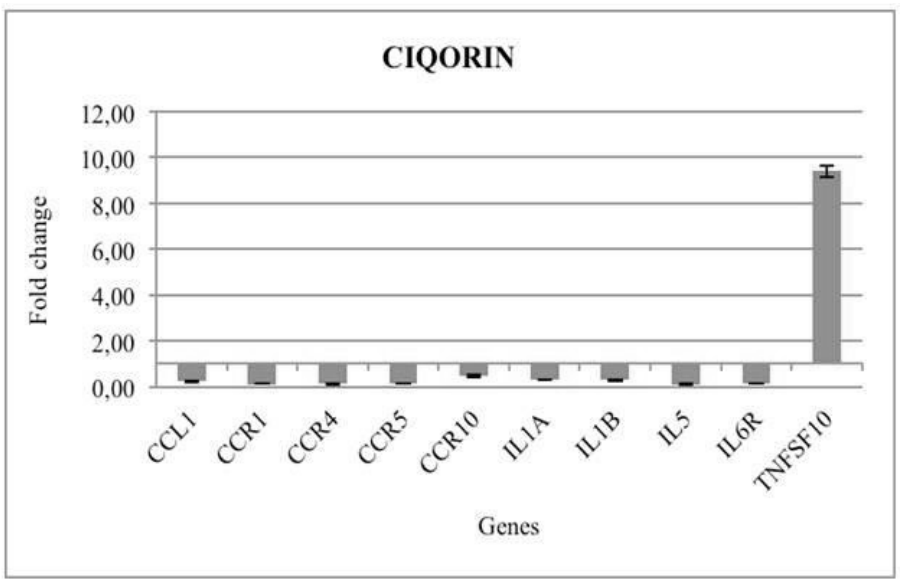

Fig. 2. *Gene expression profile of fibroblast treated with cyclosporine 1000 ng/mL.*

oxygen, free radicals, etc. These host-derived factors may likely alter cells and promote production of more inflammatory mediators, further enhancing inflammation and contributing to CIGO pathogenesis (16).

Chemokine and cytokine

The gingival mucosa is constantly subjected to thermic, chemical, mechanical insults inducing a permanent state of turnover, involving the inflammatory cells, fibroblasts and inflammatory mediators. Many of these mediators are chemokine and cytokines secreted locally by various cells in the gingiva.

In this study, gingival fibroblasts of healthy individuals were treated for 24 h with 1000 ng/ml of cyclosporine. Gene expression analysis showed that only 10 of the analyzed genes were significantly deregulated. Among these there are the chemokine CCL1, CCR1, CCR4, CCR5, CCR10 and the interleukine IL1A, IL1B, IL5, IL6R. All the mentioned genes were down-regulated. The only gene strongly over-expressed was TNFSF10 a cytokine that belongs to the tumor necrosis factor (TNF) ligand family. This protein preferentially induces apoptosis in transformed and tumor cells but does not appear to kill normal cells although it is expressed at a significant level in most normal tissues (17-29).

In this study, all the significantly deregulated genes belonging to the "Inflammatory Cytokines and Receptors" pathway were downregulated, except TNFSF10.

These results seem to indicate that cyclosporine has no effect on the modulation of inflammatory response in gingival fibroblasts.

Probably more explanatory results could be obtained by using fibroblasts isolated from patients affected by gingival hyperplasia in wich the use of cyclosporine seems to aggravate the inflammatory response and the gingival overgrowth

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