THE ROLE OF MITOCHONDRIAL CALCIUM OVERLOAD IN CYCLOSPORINE A-INDUCED GINGIVAL HYPERPLASIA

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ABSTRACT
Aim of the study: Apoptosis (programmed cell death) is a possible mechanism of cyclosporine A (CsA)-induced gingival hyperplasia. The purpose of this paper is to study the effect of ionomycin and ionophore A23187 on normal gingival fibroblasts and also on fibroblasts treated with CsA using flow cytometry methods. Material and methods: All of our experiments were performed on fibroblasts obtained from gingival male rats through explant technique and the opening of the mitochondrial transient permeability pore as a result of calcium cytosol overload was followed using calcein AM and CoCl₂. Results: Our results show a significant difference between normal gingival fibroblasts and those treated with CsA when using ionophore A23187. On the other hand, ionomycin hasn’t significant effects on mitochondrial calcein load in normal or treated fibroblasts. Conclusions: Cytosolic calcium overload is one of the mechanisms involved in drug-induced gingival hyperplasia.

Key words: apoptosis, gingival overgrowth, calcein, flow cytometry, cyclosporine A

INTRODUCTION
Gingival enlargement, also known as gingival hyperplasia or hypertrophy, is an abnormal overgrowth of gingival tissue. There are several causes of gingival enlargement and they can be grouped into four categories: 1) inflammatory gingival enlargement, 2) drug-induced gingival enlargement, 3) hereditary gingival fibromatosis, and 4) systemic causes of gingival enlargement.

Patients under certain medications may develop gingival enlargement. In contrast to inflammatory gingival hyperplasia, the gum tissues in such cases are typically firm, non-tender, pale pink in color, and do not bleed easily. In severe cases, the gingiva may completely cover the crowns of the teeth causing periodontal (gum) disease (due to difficulty in keeping the teeth clean) as well as tooth eruption and alignment disturbance. Medication-induced gingival enlargement may resolve either partially or completely when the medication is discontinued [1]. If the medication cannot cessate, surgical removal of the excessive gingiva (gingivectomy) may be performed, but the condition will likely recur [2]. As this condition is somewhat worsened by the level of plaque accumulation on the teeth, effective oral hygiene measures will reduce the severity.

Clinical and cell culture studies suggest that the mechanism of gingival overgrowth is a result of the interaction between the drug and its metabolites with susceptible gingival fibroblasts [3].

The term apoptosis was introduced in biology by Keer et al. in 1972, in order to designate a particular form of cell death [4]. This term comes from the Greek word apoptosis (apo: far from, marking an end and ptosis: fall), used to define the fall of the leaves or petals. In fact, within a tissue, the cell that dies by apoptosis, detaches itself from its neighbors as a leaf that falls from a tree [5]. Being a fundamental physiological process of pluricellular organ life, apoptosis is equally a
suicidal mode of cellular response to an external chemical or physical aggression nature. Called also programmed cell death (PCD), apoptosis represents an active process, involving a predetermined program of molecular interactions. It has an important role in controlling the normal development of the embryo, in certain diseases such as cancer, degenerative diseases or aging [6]. In addition to the importance of understanding ontogenesis and maintenance of tissue homeostasis, the molecular mechanisms of apoptosis represent major objective of biomedical studies due to the involvement of this phenomenon in various pathologies, such as tumor or degenerative diseases of the nervous system. The first researches on apoptosis were focused on the nucleus, due to the characteristic changes taking place at this level. However, it has quickly come obvious that nuclear changes are far from being a triggering factor of cell death, and they appear late after the cell has been engaged in apoptosis. At the moment, it is known that early events in apoptosis occur at the level of mitochondria and endoplasmic reticulum and the release of cytochrome c and calcium in the cytosol is absolutely necessary for this event to develop. Amplification of apoptosis degree or extension of cell life are processes closely related to the presence of intracellular calcium. Mitochondria play a very important role in the apoptosis condition, providing the effector pathway for apoptosis, with a regulatory site upon Bcl-2 family proteins [7]. As previously described [6], cyclosporine A (CsA) inhibits this permeability pore, thus inhibiting cation transport through mitochondrial permeability transition pore (MTP) and Ca2+ cannot leave mitochondria using this pathway or the outward flow rate is very low. Several data suggest that apoptosis plays an important role in gingival overgrowth controlling and that involves a cascade of biochemical steps that require an increase of intracellular Ca2+. Earlier studies show that fibroblast apoptosis is decreased in gingival overgrowth. One of the best described mechanisms in apoptosis induction is represented by the increases Ca2+ concentration in the cytoplasm of investigated cells [8].

MATERIAL AND METHODS
We aim to investigate the effect of ionomycin and A23187 ionophore upon normal gingival fibroblasts and also on fibroblasts treated with CsA, using flow cytometry methods. Initiation phase of apoptosis, induced by Ca2+ overload is represented by opening of mitochondrial permeability transition pore (MTP). Gingival fibroblasts were achieved from male rats, 150 – 170 g weight, by gingival explants and grown up in specific culture medium consisting of DMEM (Dulbecco’s modified Eagle Medium), supplemented with FBS (fetal bovine serum) and antibiotics. After we obtained fibroblasts we divided them into two groups: one control group that received no treatment, and one group treated with CsA (1μg/ml).

The protocol consisted in normal and treated fibroblasts trypsinization, their flush through centrifugation at 300 x g for 5 minutes, with subsequent resuspension in 1 ml culture medium. Cells were counted (about 1,000,000/ml) and were equally divided in tubes. A tube was depicted as control group, while in other tubes we have added calcein 5μl/ml (2μM concentration) and 5μl/ml of CoCl2 (concentration of 80mM) and allowed for 20 minutes incubation, at 37°C and 5% CO2. In the other two tubes, we added 100μl/ml CaCl2 (1μM) and calcium ionophore-like ionomycin (1 mM) and A23187 (10 μM) for 24 h [9]. After 14 days, the mitochondrial transient permeability pore (MTP) function was monitored by flow cytometry using a Calibur type FACS and related software. The settings used for the acquisition were: FL1 623 V, FL2 505 V, 10,000 events, 488 nm laser. The data was processed with FlowJo 7.6.1 software.
RESULTS AND DISCUSSIONS

Cells were loaded with the acetoxymethyl ester of calcein dye, calcein AM, which passively diffuses into the cells and accumulates in cytosolic compartments, including the mitochondria. Once inside cells, intracellular esterases cleave the acetoxymethyl esters to liberate the very polar fluorescent dye calcein, which does not cross the mitochondrial or plasma membranes in appreciable amounts over relatively short periods of time [10]. The fluorescence from cytosolic calcein is quenched by the addition of CoCl2, while the fluorescence from the mitochondrial calcein is maintained.

By increasing Ca2+ concentration, we have induced the MTP opening by ionomycin and A23187 administration (calcium ionophore). The calcium ionophore response can be blocked with cyclosporine A, a compound reported to prevent mitochondrial transition pore formation by binding cyclophilin D [11]. The purpose of the present research was to establish the sensitivity of normal gingival fibroblasts compared to those treated with CsA in culture medium, to apoptosis induced by Ca2+ overload.

Figure 1. Representative FACS image for the calcein loading of normal gingival fibroblasts: A – FACS image, B and C – fluorescence histograms

Figure 2. Representative FACS image for the calcein loading of normal gingival fibroblasts under ionomycin action: A – FACS image, B and C – fluorescence histograms
**Figure 3:** Representative FACS image for the calcein loading of normal gingival fibroblasts under A23187 ionophore action for 24 h: A – FACS image, B and C – fluorescence histograms

**Figure 4.** FACS analysis for MTP pore opening in mitochondria under ionomycin and A23187 ionophore action in normal fibroblasts

Specific methods developed for measuring Ca2+ concentration in the cytoplasm of living cells lead to major breakthroughs in understanding the control mechanisms of calcium homeostasis and the role of this cation in cell signalling [8].

The existence at the level of the inner mitochondrial membrane of a non-specific pore, whose opening (experimentally confirmed by depolarizing the mitochondria) results in the elimination of accumulated calcium ions, penetration of external solutions into the matrix and swelling of the organelle, provided new data for the understanding of homeostasis mechanisms at this level. The opening of the pore is regulated by a) the level of calcium in the mitochondria, b) pH and c) membrane potential [12].

**Figure 5.** Representative FACS image for the calcein loading of CsA treated gingival fibroblasts: A – FACS image, B and C – fluorescence histograms
Magnesium ions, ATP and some antioxidants, sphingosine, carnitine and CsA block the opening of the mitochondrial pore [13]. The opening of the PTM is blocked by the CsA as a consequence of binding cyclophilin D, a matrix cis trans isomerase that is probably the endogenous modulator of the mitochondrial permeability pore.

Silvestri et al. described that CsA inhibits this transitory permeability pore and consequently, the passing of cations through the mitochondrial permeability transition pore (MTP). This way, Ca2+ cannot exit the mitochondria by this route, or the rate of its efflux is very low.

The chronic treatment with CsA inhibits the transitory mitochondrial permeability, which occurs in the presence of Ca2+ in the mitochondria isolated from rat gingival fibroblasts. It thus becomes obvious that CsA experimentally-induced gingival overgrowth is due to the altering of gingival cell homeostasis rate [14]. By blocking transitory mitochondrial permeability, a fundamental component of apoptosis, cellular life span is extended and further results in hyperplastic fibrosis occurring with the gingival overgrowth, induced by the chronic treatment with CsA [15].

Since the dissipation of the mitochondrial membrane potential could lead to cell death, CsA might prevent this event, being demonstrated in vitro with gingival fibroblasts [16].

The function of the mitochondrial pore is not fully understood but, as long as CsA protects cells against ischemic and oxidative lesions, its opening may be significant for the breakdown of calcium homeostasis.
Figure 8. Calcein loaded fibroblasts and treated by CsA under ionomycin and A23187 ionophore action compared to control group

The hypothesis of Silvestri et al. regarding the role of Ca2+ in CsA induced gingival overgrowth, advocates the involvement of a collagen turnover deregulation which leads to Ca2+ use disorders in the mitochondria of gingival fibroblasts. Although neglected for a long time, the ability of mitochondria to function as a buffer for cytoplasmic calcium is significant, as long as they can take in significant amounts of Ca2+ [12]. Exceeding a certain threshold, however, important changes in the permeability of mitochondria develop, which subsequently result in the release of their Ca2+ content, their swelling and the decoupling of oxidative phosphorylation from electron transport [17,18].

The isolated mitochondrion can suffer a dramatic increase of its permeability to ions and solutions, known as “transient permeability”. The transient permeability is much easier to observe after Ca2+-dependent stimulation in the presence of various “inducing agents”, and can be specifically inhibited by the cyclic immunosuppressive peptide, which is cyclosporine A [18].

CONCLUSIONS

1. Statistical analysis of normal and treated fibroblasts under the action of Ca2+ ionophore A23187 and ionomycin showed a significant difference due to the use of ionophore A23187.
2. Fibroblasts previously treated with CsA in culture medium have a strong dissipation of mitochondrial membrane potential under the action of this ionophore.
3. For ionomycin, statistical analysis did not show significant differences for opening of MTP, in normal fibroblasts and those treated with CsA, as well.
4. Mitochondrial permeability transition pore opening under Ca2+ overload was observed using ionophore A23187, both in normal fibroblasts and especially in those treated with CsA in culture medium.
5. On the other hand, ionomycin, hasn’t significant effects on mitochondrial calcein load in normal or treated fibroblasts.

REFERENCES


