SHORT COMMUNICATION

O-25. CHARACTERIZATION OF NEMOTIC DENTAL FIBROBLASTS

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Key words
Nemosis, pulp fibroblasts, inflammation, cyclooxygenase, growth factors

Introduction
Up to now, fibroblasts were known to be implied in the production and the maintenance of the extracellular matrix. These cells have also been described in inflammatory processes. But, their activation is at present not described. Finn researchers have found a new way of fibroblast activation, called nemosis, using 3-dimensional cultures (spheroids). In this study, we cultured spheroids with two fibroblasts cell types from lung and from dental pulp. Then, we followed their growth and observed their cell surface by using electron microscopy. We also focused on the expression of various molecules by using RT-PCR and ELISA test and analysed the cytotoxicity throughout the spheroids.

Materials and Methods
Cell culture:
Dental fibroblasts were obtained from the dental pulp of sound human third molar germ extracted for orthodontic reasons. Cell cultures between the second and eighth passage were used in this study. MRC5 are embryonic sound fibroblasts from lung tissues. Cells were cultured with DMEM supplemented with 10% fetal calf serum, 100U/mL penicillin, 100µg/mL streptomycin, glutamine (2mM) and HEPES (20mM) and were maintained in an humidified atmosphere containing 5% CO2 at 37°C. Experiments were performed at 24h, 48h, 7 2h and 96h.
Initiation and growth of spheroids:
96-well plates were treated with 1% agarose prepared in PBS with Ca2+ and Mg2+ to form a non adhesive surface. Fibroblasts were detached from culture dishes by trypsin/EDTA and a single cell suspension was prepared at concentrations 5.104cells/mL. To initiate spheroid formation, 200µL were seeded into individual wells and incubated at 37°C during 4 days.
Measurement of cell viability:
Acid phosphatase (APH) assay based on quantification of cytosolic acid phosphatase activity was
performed to determine cells viability in all spheroids. This activity was quantified by measurement of optical density at 405nm using a microplate reader.
RT-PCR analysis:
Total RNA (1µg) was extracted from corresponding monolayer and spheroid cultures at 72 hours and was reverse-transcribed with the M-MulV reverse-transcriptase and random primer. Subsequent amplification for detection of cyclooxygenase2 was carried out for 26 cycles.
Spheroids morphology:
- Growth kinetics: Spheroids diameters were measured on phase-contrast images with Photoshop software.
- Spheroids surface analysis by SEM (scanning electron microscopy): Spheroids were fixed
with 2,5% glutaraldehyde for 4 hours, dehydrated and recovered with a thin film of gold palladium before SEM observation.
Enzyme-Linked Immunosorbent Assay (ELISA):
Supernatants of 3-Dimensional and monolayer cultures were collected at 24h, 48h, 72h and 96h. Concentrations of HGF and VEGF in the supernatants were determined by an ELISA kit according to manufacturer's instructions.

Results
In this study, we demonstrate, using the APH assay, an important loss of cells in our spheroids of
about 55% to 72% after 24 hours in the cell types studied. At the same time, spheroids diameters decreased with values of 255µm for dental fibroblasts and 310µm for MRC5 after 96 hours. SEM helped us to analyse spheroids surface and we could observe an altered viability with dead cells and disrupted intercellular jonctions. We also demonstrated an important expression of the enzyme cyclooxygenase-2 at 72 hours (7 to 23-fold) in spheroids compared to monolayer cultures. Finally, thanks to ELISA test, we showed a high secretion of VEGF in all 3-dimensional cultures and a significant production of HGF/SF in MRC5 spheroids.

Discussion
Fibroblasts are heterogeneous mesenchymal cells and form a major part of cells in dental pulp. To characterize nemosis process, we cultured dental fibroblasts and MRC5 in a 3-dimensional way. Multicellular aggregation is dependent on the interaction integrin-fibronectin necessary for fibroblasts activation. Compact spheroids are formed within 24 hours and no apoptosis markers are found. In this study, we presented for the first time this nemosis process in dental and lung fibroblasts. We displayed that, during nemosis, spheroids produced different molecules mostly implied in inflammation. We observed a large induction in the expression of Cox-2 mRNA within 72 hours and a high production of VEGF in the two cell types. At the same time, spheroids began to decompose and displayed a “necrosis-like” morphology. Indeed, we demonstrated an important loss of cells for 4 days, an altered cell surface and a decrease of spheroids diameters.
Activated fibroblasts also produce proteolytic enzymes such as matrix metalloproteinases and chemokines able to recruit specific populations of leukocytes...Moreover, nemosis seems to be different in the cell types studied. However, this process may be an interesting model to study interactions between mesenchymal cells, surrounding cells and microenvironment.

Conclusion
For the first time, this work shows nemosis process in 3-dimensional cultures of dental pulp fibroblasts and MRC5. This model of fibroblast activation opens perspectives to understand mechanisms implied in pulpitis and test, for example, different pulp capping products.

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