Comparative assessment of the cell-surface antigens and gene expression profiles of the gingival tissue biomarkers in subjects with fixed functional and removable functional orthodontic appliances

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\textbf{A B S T R A C T}

Background: This study aimed to examine the cellular components of the gingiva during orthodontic treatment with fixed and removable appliances. The cellular and molecular cues of pathologies of the gingival tissue associated with the use of different orthodontic appliances could be studied.

Materials and methods: Tissue samples of gingiva were received from healthy patients undergoing gingivectomy for aesthetic purpose and from patients with fixed and removable functional orthodontic appliances undergoing gingivectomy for gingival overgrowth. The collected samples were stored in a sterile container with phosphate-buffered saline and to carry out further processes it was transported to the laboratory.

Results: Cells positive for ECAD and NCAD were found to be increased in fixed appliances whereas CD90 and CD105 positive cells showed no significant difference in all the three groups. CD24 and CD146 positive cells were increased significantly in removable and fixed than normal whereas CD133 positive cells were decreased in removable and fixed than normal. CD44 positive cells showed no noticeable change in all three groups. The gene expression levels of KRT5, SOX2, NANOG, and CXCL5 were found to be significantly increased in removable and fixed appliance groups. However, KRT8, CXCL10, and TIMP1 were increased only in fixed appliance group but CXCL10 showed decreased expression in removable appliance group. KRT6A, MYC, and MMP9 were decreased in fixed appliance group whereas MYC and MMP9 were increased in removable appliance group. KRT6A, KRT8, and TIMP1 showed no significant difference in removable appliance group.

Conclusion: This study demonstrated essential roles of various genes, showing their contribution in regulating cell proliferation and migration in both the removable and fixed functional appliances.

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1. Introduction

Occlusal disorders in Class II malocclusion patients have been treated using various orthodontic and surgical treatments. Class II malocclusions with mandibular retrusion are usually treated with functional orthodontic appliances by applying orthopedic forces that are directed over the mandibular structures. These appliances affect the jaws by causing remodeling of glenoid fossa and mandibular condyles. It autorotates the mandible by repositioning the condyles in the glenoid (Pancherz et al., 1998).

One of the greatest challenges during orthodontic treatment is to maintain healthy gingival tissue. The esthetic and functional satisfaction of the patients gets affected due to the gingival enlargements caused due to orthodontic treatment and this can be managed by timely and correct diagnosis of periodontal diseases (Casian Romero et al., 2011). The orthodontic treatment not only improves the esthetics by correcting the deformities of the dentition and jaws, but also providing conditions to maintain improved gingival health (Wu et al., 2003). Orthodontics is predicated on the application of mechanical forces to move teeth through hard tissues. This movement and remodelling of the surrounding tissues occurs through the transduction of force that activates bone and related cells generating an inflammatory response. Orthodontic appliances, whether they be fixed or removable are in contact with oral tissues and remains in the mouth for the duration of treatment which can extend several years. Therefore, a patient’s periodontal health is of concern throughout the treatment period.

Several researchers have analysed the periodontal and oral health of patients undergoing orthodontic corrective treatment and the consequences of treatment to their oral health, their conclusions remain opaque and unclear (Eid et al., 2014; Ghezzi et al., 2008; Kloehn and Pfeifer, 1974; Šurlin et al., 2012; Zachrisson and Zachrisson, 1972; Zanatta et al., 2012). Generally, orthodontic treatment can help improve a patient's oral health status, correct malocclusion and lead to a greatly improved periodontal outcome. Conventional fixed orthodontics can pose a difficulty while cleaning (Marchese et al., 2003). Patients must be cautious and diligent to keep the appliance and their teeth clean to prevent plaque accumulation. Plaque growth can lead to adverse periodontal outcomes and increase patient discomfort. On the other hand, removable orthodontic appliances are less cumbersome and offer an alternative to patients who find it hard to maintain fixed appliances, easier cleaning (Zhou Q, 2014).

There is limited information in the literature regarding the effects of functional appliances on the periodontal health. Hence, this study aimed to examine the cellular components of the gingiva during orthodontic treatment with fixed and removable appliances. Through this study we could understand the cellular and molecular cues of pathologies of the gingival tissue associated with the use of different orthodontic appliances.

2. Materials and methods

2.1. Sample collection

During small surgery maneuvers involving tooth extraction, wisdom molar surgery or gingivectomy, the gingival tissue samples were obtained under local anesthesia. Gingival tissue samples were collected from healthy patients undergoing gingivectomy for aesthetic purpose (n = 8) (age: 16–25 years), from patients with fixed functional orthodontic appliances undergoing gingivectomy for gingival overgrowth (n = 6) (age: 13–17 years), and from patients with removable functional orthodontic appliances who were scheduled for extraction due to orthodontic purposes (n = 8) (age: 15–18 years). Informed consents were signed and collected accordingly by the guidelines of institutional ethical committee. The collected samples were stored in a sterile container which has phosphate-buffered saline (PBS) (Sigma, St. Louis, MO, USA) and to carry further processes it is transported to the laboratory directly.

2.2. Single cell suspension preparation from tissue samples

Sterile PBS with antibiotic antimycotic (Penicillin-Streptomycin-Antihomotericin B) solution was used to rinse the tissues thoroughly. Further, sterile blade was used to mince the tissues and it was directly subjected to enzymatic digestion containing enzyme solution of 0.2% dispase II (Roche Diagnostics GmbH, Mannheim, Germany) and 0.4% collagenase 1 (MP Biomedicals LLC, Santa Ana, CA, USA) and incubated for 20 min at 37 °C. After incubation, to neutralize the enzymatic action fetal bovine serum (FBS) (Gibco, Rockville, MD, USA) was used, the tissue was digested and then passed into a sterile cell strainer with a pore size of 70 μm (Corning, NY, USA) and the mixture was centrifuged for 5 min at 1800 rpm. The pellet of cells thus obtained were directly used for further experimentation.

2.3. Analysis of cells for surface markers by flow cytometry

For flow cytometry analysis, the cells were resuspended from the pellet in PBS from each sample and divided into different tubes. Each tube was individually incubated for 30 min at 4 °C with ECAD-APC, NCAD-PE, CD90-FITC, CD105-APC, CD24-APC, CD44-PE, CD133-FITC, and CD146-PE antibodies (all monoclonal) (Miltenyi Biotec, Bergisch Gladbach, Germany). After incubation, PBS was used to wash the cells and pelleted down. The labelled cells were resuspended in sheath fluid and analyzed with a flow cytometer (Attune NxT, Thermo Fisher Scientific, Waltham, MA, USA). At least for each samples 10,000 events are acquired. Percentage comparison was done with the degree of positive staining and the isotype controls. The median fluorescence intensities for each surface protein were also considered.

2.4. Quantitative analysis of gene expression using real time quantitative polymerase chain reaction (RT-qPCR)

From the cells, the total RNA was extracted using Geneljet purification columns (Invitrogen, Thermo Scientific, Lithuania). Using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA, USA) 1 μg of total RNA was converted into cDNA. Gene expressions were Quantitative analyses with the SYBRGreen PCR master mix (Applied Biosystems, Austin, TX, USA) on QuantStudio 5 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). Appropriate primers (IDT, Coralville, IA, USA) were used for the analysis of KRT5, KRT6A, KRT8, SOX2, NANOG, MYC, CXCL5, CXCL10, MMP9, and TIMP1. The target genes expressions were then normalized to GAPDH by the ΔΔCt technique with the ΔCt method mRNA levels were calculated and were finally quantified by using the 2−ΔΔCt method.

2.5. Statistical analysis of data

The result was presented as the mean ± standard deviation of the values from two independent experimental values. Comparison of each experimental group (Removable and Fixed) was individually done with the Normal group and the data were analysed using unpaired t test (two-tailed) on GraphPad Prism 8 software (GraphPad Software, La Jolla, CA, USA), and p < 0.05
was considered significant and \( p < 0.01 \) was considered highly significant.

3. Results

3.1. Gingival tissue cells from normal removable and fixed showed altered surface antigen expression profiles for epithelial to mesenchymal transition-related cell–cell adhesion proteins (ECAD and NCAD) and mesenchymal stem cell-specific cell surface antigens (CD90 and CD105)

Cells positive for ECAD were found to be significantly decreased in removable and fixed groups than normal (Fig. 1A-C and M). Also, shift in fluorescence intensities of these proteins were also found to be decreased in both the groups than normal (Fig. 1Q). Whereas cells positive for NCAD were found to be increased in the fixed than normal (Fig. 1D-F and N) and the shift in fluorescence intensity was also found to be increased in both removable and fixed than normal (Fig. 1R). CD90 and CD105 positive cells showed no significant difference in all the three groups (Fig. 1G-I, J-L, O, P) but shift in fluorescence intensity of CD90 was significantly increased in fixed than normal (Fig. 1S and T).

3.2. Gingival tissue cells from normal removable and fixed presented distinct surface antigen expression patterns for other stem cell-specific cell surface antigens (CD24, CD44, CD133, and CD146)

CD24 and CD146 positive cells were to be increased significantly in removable and fixed than normal (Fig. 2A-C, J-L, M, and P) and also the shift in fluorescence intensity as well (Fig. 2Q and T). Whereas CD133 positive cells were decreased in removable and fixed than normal (Fig. 2G-I and O) and also the shift in fluorescence intensity as well (Fig. 2S). CD44 positive cells showed no noticeable change in all three groups (Fig. 2D-F and N) but shift in fluorescence intensity of CD44 were observed significantly increased in fixed than normal (Fig. 2R).

3.3. Gingival tissue cells from normal removable and fixed revealed dysregulated gene expressions for epithelial markers (KRT5, KRT6A, and KRT8), stemness-related transcription factors (SOX2, NANOG, and MYC), inflammatory chemokine genes (CXCL5 and CXCL10), and proteins regulating degradation of extracellular matrix proteins (MMP9 and TIMP1)

The gene expression levels of KRT5, SOX2, NANOG, and CXCL5 were found to be significantly increased in removable and fixed than normal (Fig. 3A, D, E, and G). However, KRT8, CXCL10, and
TIMP1 were increased only in fixed than normal (Fig. 3C, H, and J), but CXCL10 showed decreased expression in removable than normal (Fig. 3H). KRT6A, MYC, and MMP9 were decreased in fixed than normal (Fig. 3B, F, and I). Whereas MYC and MMP9 were increased in removable than normal (Fig. 3F and I). KRT6A, KRT8, and TIMP1 showed no significant difference in removable than normal (Fig. 3B, C, and J).

The pictorial illustration of the study is shown in Fig. 4.

4. Discussion

Until today, periodontic-orthodontic interrelationship has been an matter of importance and it still is a controversial issue. The periodontal health gets affected by the malocclusion and one of the objective of the treatment through orthodontic appliances is to promote good oral health that prolongs the life of the dentition (Bollen et al., 2008). Although the dental and skeletal problems are improved by the orthodontic treatment, the placement of the orthodontic appliance in can alteration the habits of oral hygiene and periodontal health the in the patient. The orthodontic appliances and their mechanical procedures can evoke soft tissue responses in the gingiva. The proximity in which orthodontic appliance is placed in relation to gingival sulcus, accumulation of plaque further lead to complications of efficient orthodontic care (Boyd, 1983; Willmot, 2008; Zachrisson and Zachrisson, 1972).

Early interception with functional appliances in children with increased overjet has proven to be very effective in improving self-confidence, reducing the risk of trauma and avoiding negative social experiences. 10%–49% is the reported noncompliance rates
with removable functional appliances reported in the literature (O’Brien et al., 2003; Seehra et al., 2011; Thiruvenkatachari et al., 2013).

The unspecialized cells that differentiate into any type of cell in an organism retaining the self-renewal capacity are the Stem cells. The regeneration and renewal of many vertebrate organs are enabled by adult stem cells (Alvarez-Buylla et al., 2001). The stem cells resides in a microenvironment called as Niche, which even influences the maintenance of these stem cells in various tissue types. The stem cell behavior is determined by the signals that eminate either from or outside the niche (Kai and Spradling, 2003). The fate of stem cells or progenitor cells are also regulated by the growth factors that are secreted from the tissues surrounding the niche (Ciruna and Rossant, 2001).

A study was done by Chengjuan Qu et al on the growth, stemness and angiogenic propereties of dental pulp stem cells where expansion of human dental pulp stem cells in vitro was done using CGMO-defined xeno-free/serum free medium. These cells helped in maintaining their capability for tri-lineage differentiation although very few cells expressed the multipotency marker CD105. The decreased expression of CD105 in the XF medium could be restored when the cells were subsequently cultures in human serum-supplemented medium (Qu et al., 2020).

The transcription factors like Nanog, Sox2, and Oct4 are essential for maintaining the phenotype of pluripotent embryonic stem cell (Rodda et al., 2005). The embryonic stem cells and adult stem cells are maintained by SOX2 in multiple tissues (Arnold et al., 2011). The self renewal of the multiple types of stem cells are regulated by SOX2. It has also been said that SOX2 is oncogenic and plays an active role in forming squamous cell carcinoma in several organs.
The tissue homeostasis is maintained by the progenitor/stem cells and the irregularity in them contributes to the initiations of tumor and progression of cancer (Barker et al., 2009). Sox 2 acts as a signaling molecule and transcription factor and they are important for maintaining stem cells and this needs to be regulated strictly (Bass et al., 2009).

SOX2/OCT4 regulates the target gene NANOG which is expressed in only undifferentiated cells and is another key transcription factor that is a homeobox containing protein. NANOG suppresses or activates OCT4 which is said to maintain the undifferentiated state of the gene (Hepburn et al., 2019; Xu et al., 2020). Furthermore, OCT4/NANOG/SOX2 forms an interconnected regulatory circuit gets established as NANOG acts as a direct target for OCT4/SOX2 binding maintaining ESC pluripotency and self-renewal (Verneri et al., 2020).

A study done by Luchian I et al on Salivary MMP-9 was successful in quantifying periodontal inflammation during orthodontic treatment which stated salivary MMP-9 was strongly involved during orthodontic treatment quantifying inflammation in periodontal tissues. MMP-9 could be used to accurately predict the inflammation level associated with the type of malocclusion, making it viable and real diagnostic instrument during orthodontic treatment to monitor the periodontium evolution (Luchian et al., 2021).

Cells positive for ECAD and NCAD were found to be increased in fixed functional appliances where as CD90 and CD105 positive cells showed no significant difference in all the three groups. CD24 and CD146 positive cells were to be increased significantly in removable and fixed functional appliances than normal group whereas CD133 positive cells were decreased in removable and fixed functional appliances than normal group. CD44 positive cells showed no noticeable change in all three groups. The gene expression levels of KRT5, SOX2, NANOG, and CXCL5 were found to be significantly increased in removable and fixed appliance groups. However, KRT8, CXCL10, and TIMP1 were increased only in fixed appliance group but CXCL10 showed decreased expression in removable appliance group. KRT6A, MYC, and MMP9 were decreased in fixed appliance group whereas MYC and MMP9 were increased in removable appliance group. KRT6A, KRT8, and TIMP1 showed no significant difference in removable appliance group.

Very limited information was available in the literature regarding the cell-surface antigens and their genetic expressions in removable and fixed functional appliances in orthodontics. Through this study, we were able to assess the genetic expressions of the mentioned cells and correlate them with the type of functional appliances used.

5. Conclusion

This study demonstrated essential roles of various genes, showing their contribution in regulating cell proliferation and migration in both the removable and fixed functional appliances.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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