THE LACUNO-CANALICULAR SYSTEM (LCS) AND OSTEOCYTE NETWORK OF ALVEOLAR BONE BY CONFOCAL LASER SCANNING MICROSCOPY (CLSM)

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ABSTRACT

The osteocyte lacuno-canalicular system (OLCS) is a large network intercommunicating the lacunae and canaliculi which contain the osteocytes and their cytoplasmic processes within the mineralized bone matrix. The vitality and functioning of the osteocytes and cytoplasmic processes depend upon this intercommunication. To date, the 3-dimensional features of OLCS in the alveolar bone have not been studied; therefore the aim of this study was to use confocal scanning microscopy to do so. Samples of alveolar bone from male Wistar rats were fixed in buffer formalin and stained with basic fuchsin to visualize the lacuno-canalicular system. In decalcified samples of the same bone, the actin was labeled using fluorescent phallotoxin to visualize the osteocyte network. The samples were observed at the level of the mesial root of the first upper molar in bucco-palatal direction using a confocal laser scanning microscope. The results showed that in the area near the inner aspect (bundle bone) of the buccal plate, the osteocyte lacunae are oval-shaped and relatively uniform in size, aligned parallel to each other and with their major axes parallel to the periodontal bone surface, and the osteocytes are oval-shaped, with their main axes perpendicular to the periodontal bone surface, and the cytoplasmic processes irradiate in all directions. In the area near the inner aspect (bundle bone) of the palatal plate, the osteocyte lacunae are rounded, have different sizes and their orientation does not follow any specific pattern, and the osteocyte bodies have major axes parallel to the periodontal surface, a larger number of cytoplasmic processes, and run in a straighter direction than in the buccal plate. These results will contribute to the understanding of the changes that may occur in OLCS microarchitecture as a result of a pathological process, surgical technique or force applied to the alveolar bone.

Keywords: bone, histology; osteocytes; confocal microscopy

CARACTERÍSTICAS DEL SISTEMA LACUNO-CANALICULAR (LCS) Y LA RED OSTEOCITARIA DEL HUESO ALVEOLAR OBSERVADAS POR MICROSCOPÍA LÁSER DE BARRIDO CONFOCAL (CLSM)

RESUMEN

El sistema lacuno-canalicular osteocitario (OLCS) comprende una amplia red de intercomunicación entre las lagunas y los canalicullos que contienen a los osteocitos y sus procesos citoplasmáticos dentro de la matriz ósea mineralizada, de lo que depende su vitalidad y funcionamiento. Hasta el momento no se han estudiado las características tridimensionales del OLCS en el hueso alveolar por lo que el objetivo del presente trabajo fue determinarla por microscopía de barrido confocal. Muestras de hueso alveolar de ratas Wistar machos, luego de fijadas en buffer formalin y teñidas con fucsina básica para visualizar el sistema lacuno-canalicular y a muestras descalcificadas del mismo hueso mediante una falotoxina fluorescente se les marcó la actina para visualizar la red osteocitaria. Las muestras se observaron a nivel de la raíz mesial del primer molar superior en sentido buco-palatino con un microscopio láser de barrido confocal. Los resultados mostraron que en la zona aledaña a la cortical periodontal de la tabla ósea vestibular las lagunas osteocitarias presentan forma ovalada y tamaño relativamente uniforme, alineándose paralelas entre sí y con su eje mayor paralelo a la superficial ósea periodontal en tanto que los osteocitos, de forma ovalada, presentan su eje mayor orientado perpendicularmente a dicha superficie y con los procesos citoplasmáticos irradiándolose en todos los sentidos del espacio. En la zona aledaña a la cortical periodontal de la tabla ósea palatina las lagunas osteocitarias se presentan de forma redondeada y tienen distintos tamaños, sin seguir un patrón de orientación específico, y los cuerpos osteocitarios presentan su eje mayor paralelo a la superficial periodontal presentando mayor número de procesos citoplasmáticos y adoptando una dirección más recta que en la tabla vestibular. Estos resultados contribuirán a comprender los cambios que pudieran ocurrir en la microarquitectura del OLCS como consecuencia de algún proceso patológico, la aplicación de alguna técnica quirúrgica o luego de la aplicación de fuerzas en el hueso alveolar.

Palabras clave: hueso, histología; osteocitos; microscopía
INTRODUCTION

Osteocytes, the most abundant cells in the bone tissue, are non-proliferating cells that are embedded in the mineralized bone matrix, in lacunae. They have long cytoplasmic processes that project through the canaliculi arising from the lacunae, through which they make contact and communicate with cells of the osteoblast lineage present at the surface and with neighboring osteocytes, forming a syncytial intercommunication network: the osteocyte lacuno-canalicular system (OLCS). This system enables communication among cells through gap junctions between the cytoplasmic processes, forming the connected cellular network (CCN), and extracellular communication by means of the fluid that passes through the lacunar-canalicular system (LCS). The osteocytes are anchored to the surrounding bone matrix by integrin-dependent focal adhesions. Within the cells, integrins attach to the actin filaments of the cell cytoskeleton, which is particularly abundant in the cytoplasmic processes. Among other functions, osteocytes are able to sense the forces received by the bone and transmit the signal to neighboring osteocytes and to the bone cells at its surface, which are capable of initiating the adaptive remodeling process. The movement of interstitial fluid through the periosteocytic space may generate the stress which, through the tension of the integrins, may activate the osteocytes metabolically.

Histomorphometry applied to bright field microscopy images has enabled the study of parameters such as lacunar density, osteocyte density, presence of empty lacunae, distribution of lacunae, lacunar volume and canalicular density. However, parameters such as canalicular diameter are difficult to define and evaluate because of the rough, irregular canalicular walls and the fact that canaliculi thicken towards the osteocyte lacuna. In recent years, confocal laser scanning microscopy (CLSM) combined with fluorescent labels has been used successfully to study the osteocyte network in samples of decalcified calvarial bone from chicken embryos. Gorustovich and Guglielmotti successfully combined block staining of non-decalcified bone samples with CLSM to study newly formed bone on the surface of bioactive glass bone substitutes, observing clearly the lacunae and osteocyte canaliculi. In alveolar bone, the study of osteocytes by CLSM has only been reported with relation to apoptotic changes after applying compressive forces, showing the presence of chromatin condensation, nuclear fragmentation, contraction of the cell body and disruption of the cytoplasmic processes.

To study the LCS, Ciani et al. developed a technique enabling visualization of the bone’s interstitial fluid space in the LCS by using the fluorescent properties of fluorescein isothiocyanate (FITC) in conjunction with CLSM. However, the three-dimensional characteristics of this system have not been studied to date, and no information is available on the three-dimensional characteristics of the osteocyte network in alveolar bone. Therefore, the AIM of this study was to determine the morphological three-dimensional structure of the LCS and the osteocyte network in alveolar bone of Wistar rats by CLSM. To do so, we studied non-decalcified alveolar bone samples stained with basic fuchsin and decalcified alveolar bone samples with fluorescent phallotoxin-labeled actin.

MATERIALS AND METHODS

Animals

Male Wistar rats with an average body weight of 200-220g were used. They were fed rat chow and given water ad libitum, housed in steel-cages, and maintained on a 12:12 h light dark cycle. The animals were euthanized by an intraperitoneally administered overdose of sodium pentobarbital. The Guidelines of the National Institutes of Health for the care and use of laboratory animals (NIH Publication No. 85-23, Rev. 1985) were observed. The protocol was examined and approved by the institutional ethics committee of the School of Dentistry, University of Buenos Aires.

Histological processing

The upper maxillae were resected, cleaned of any soft tissue and fixed in 4% formalin in 0.2 M sodium phosphate buffer (SPB) for 48 h at 4°C. After fixing, the samples underwent two protocols:

1. To visualize the lacuno-canalicular system

   Block staining with basic fuchsin

After fixing, and without decalcification, the upper maxillae were block stained with basic fuchsin. The samples were immersed in 20 ml of 1% basic fuchsin in methanol for 24 h, changing the fuchsin solution after 8 h to dehydrate it. After the 24 h, the samples were placed in a glass container for 48 h until the solution had evaporated completely. Then the samples were rehydrated for 4 days in demineralized water.
Embedding in methyl methacrylate and sectioning by grinding
After block staining, the samples were processed for embedding in methyl methacrylate as follows: 1) equal parts of acetone and water for 24 h, 2) acetone, 3) equal parts of acetone and acrylic for 24 h, 4) acetone for 24 h, 5) the samples were left in a stove at 32°C for 4 days. After embedding the samples, they were placed in bucco-palatal direction at the level of the mesial root of the first upper molars with the help of a diamond disk and micro-motor to obtain sections which were subsequently wet-sanded with decreasing coarseness (220, 360, 600) until a section approximately 100 µm thick was obtained. The sections were mounted with glycerin for visualization by CLSM.

II. To visualize the osteocyte network
Incubation with phallotoxin
To visualize the actin in the osteocytes, fluorochrome-conjugated phallotoxin was used following the protocol described by Kamioka et al., using fragments of chick embryo calvariae as a positive control (Fig. 1). The following procedure was used: the fragments were fixed in 5% formalin buffer for 12 h at 4°C, after which they were permeabilized with 0.3% Triton X-100 in PBS for 10 minutes. After washing them with PBS, they were incubated for 48 h at 4°C with the phallotoxin Alexa Fluor 594 (excitation wavelength = 581 nm; emission wavelength = 609 nm, Invitrogen, Molecular Probes Inc., Eugene, OR) in a 1:200 dilution in PBS containing 1% albumin. After incubation, they were washed with PBS and mounted with glycerin and PBS to be observed immediately by CLSM.

Then we introduced the changes needed to adapt the protocol to alveolar bone, as follows: after fixing the upper maxillae, they were decalcified in 5% EDTA at 4°C, changing the EDTA solution weekly until decalcification was complete, which was achieved at 45 days. Sections 100 to 150 µm thick were cut in bucco-palatal direction at the level of the mesial root of the first upper molar. These sections underwent the same protocol of incubation with phallotoxin as the calvaria fragments. After incubation and washing, the fragments were mounted on grooved slides with glycerin and observed immediately with the confocal microscope. The results thus obtained allowed us to show that decalcification in EDTA under the conditions described had enabled a positive response of incubation with phallotoxin Alexa Fluor 594.

Confocal laser scanning images
Samples were observed and photographed using an Olympus FV300 confocal laser scanning microscope (Olympus, Tokyo, Japan) belonging to the IFIByNE Laboratory (UBA-CONICET) at the School of Exact and Natural Science, University of Buenos Aires. Fluoview version 3.3 acquisition software provided by the microscope manufacturer was used. The histological and histomorphometric analyses were performed on zones near the bundle bone of the buccal plate (BP) and palatal plate (PP) corresponding to the alveolus of the mesial root of the first upper molar, in the areas indicated in Fig. 2. A green HeNe laser (wavelength: 543 nm) was used to excite both fluorochromes. The emission was collected with a 60X PlaApo Olympus oil immersion objective (numerical aperture 1.4) applying a 2.5X digital zoom, providing a final magnification equivalent to a 150X objective. This emission of fluorescence was finally filtered with a 605/55 band-pass filter BP (Chroma, Rockingham, VT). A Kalman filter was applied to each image (n = 3) to reduce background noise. The images were obtained with a resolution of 8 bits per channel and a size of 512 X 512 pixels. Each selected area measures 100 µm X 100 µm. The series of optical sections in the samples with basic resolution...
fuchsin were obtained at 0.5 µm thicknesses, scanning an average thickness of 35 µm (70 sections, on average). The series of optical sections in the phallo-toxin-labeled samples were obtained at 1 µm thicknesses, scanning an average thickness of 70 µm (70 sections, on average).

**Three-dimensional reconstruction of the images obtained by confocal microscopy**

The 3-dimensional structure of the LCS and the osteocyte network was reconstructed with the software 3D constructor, which is accessory software to the Image Pro Plus 5.1 (UTHSCSA), from the images of the optical sections obtained by confocal microscopy.

**Histomorphometric measurements**

The histomorphometric measurements of the images were taken with the software Image Pro Plus 5.1 (UTHSCSA). The following measurements were taken for all the lacunae and osteocytes present in each selected area:

**In the basic fuchsin samples:**
- Diameter of canaliculi (µm), using the length feature provided in the software

**In the phallo-toxin samples:**
- Number of cytoplasmic processes emerging from each osteocyte
- Length of cytoplasmic processes (µm), determined using the length feature provided in the software
- Diameter of cytoplasmic processes (µm), determined using the length feature provided in the software

The 3-dimensional reconstructions were used to evaluate lacunar volume (µm) using the area feature provided in the software and expressed as mean ± SD.

**RESULTS**

**Morphological analysis of the LCS in the alveolar bone**

In the area near the bundle bone of the buccal plate, osteocyte lacunae are oval-shaped and relatively uniform in size, aligned parallel to each other, with their major axes parallel to the periodontal bone surface. In some lacunae closer to the periodontal ligament, the osteocyte canaliculi arise at right angles from the lacunar wall, running perpendicular to it, while the lacunae located towards the interior of the bone plate have canaliculi that radiate from the lacunar wall (Fig. 3A).

In the area near the bundle bone of the palatal plate, the osteocyte lacunae are rounded and of different sizes. The orientation of the lacunae does not follow a specific pattern with regard either to each other or to the periodontal bone surface. In this plate, the canaliculi arise in all directions from each lacuna and run without alignment towards the periodontal surface. There are even areas of bone in which the network of canaliculi appears to be interrupted (Fig. 3B). The pictures also showed the relationship between canaliculi belonging to neighboring lacunae (Fig.
as well as the interrelationship between canaliculi and vascular structures (Fig. 3D), and the branches arising from the canaliculi (Fig. 3E). Moreover, the 3-dimensional reconstructions of the LCS in each plate allowed us to confirm more clearly the location and spatial orientation of the osteocyte lacunae and their arrangement with regard to the periodontal surface (Figs. 3F and 3G).

**Morphological analysis of the alveolar bone osteocyte network**

In the buccal plate, the osteocyte bodies adopt an oval shape with their major axis perpendicular to the periodontal surface. The cytoplasmic processes radiate in all directions, establishing connections with the cytoplasmic processes of neighboring osteocytes (Fig. 4A).

In the palatal plate, the osteocyte bodies are also oval shaped but their major axes run parallel to the periodontal surface. The cytoplasmic processes, which are more abundant than in the buccal plate, are straighter and perpendicular to the periodontal surface, with plentiful branches (Fig. 4B).

The actin (Fig. 4C) is distributed as a peripheral band of homogeneous appearance underneath the plasmatic membrane forming the cell cortex and also all along the cytoplasmic processes. The expression of actin showed the proximity between...
the cytoplasmic processes, both between neighboring osteocytes (Fig 4D) and between osteocytes and the cells present on the bundle bone (Fig. 4E).

The 3-dimensional reconstructions of the osteocyte bodies showed the ovoid shape with the major axis oriented perpendicular to the periodontal surface in the buccal plate (Fig 4F) and parallel to the periodontal surface in the palatal plate (Fig. 4G), volumetrically confirming the observations in the two-dimensional images.

**Histomorphometric analysis**

The lacunar volumes were 247.74 ± 54.78 µm³ in BP and 277.65 ± 51.97 µm³ in PP. Cell volumes were 239.07 ± 78.67 µm³ in BP and 227.94 ± 101.82 µm³ in PP. The periosteocyte space at the level of the lacunae was determined by calculating the difference between the lacunar and cell volumes, which was 8.67 µm³ and 49.71 µm³ respectively for BP and PP.

The number of cytoplasmic processes in the osteocytes was 10.75 ± 2.89 in BP and 12.32 ± 3.04 in PP. The length of these processes was 10.44 ± 2.53 µm for osteocytes in BP and 8.75 ± 1.52 µm for osteocytes in PP. The diameter of the canaliculi was 0.93 ± 0.18 µm in BP and 0.96 ± 0.1 µm in PP. The diameter of the cytoplasmic processes was 0.85 ± 0.15 µm for osteocytes in BP and 0.75 ± 0.13 µm for osteocytes in PP. The periosteocytic space at the level of the canaliculi was determined by calculating the difference between the diameters of the canaliculi and the diameters of the processes, and was found to be 0.08 µm in BP and 0.21 µm in PP.
DISCUSSION
The use of confocal laser scanning microscopy (CLSM) in combination with fluorescent labels enabled, for the first time, a 3-dimensional study of the osteocytic lacunar canalicular system in the area near the bundle bone of the palatal plate and buccal plate of the alveolar bone. To date, there was no evidence of the use of CLSM for the joint 3-dimensional study of the LCS (lacunae and canaliculi) and the osteocyte network (osteocyte bodies and cytoplasmic processes) in the alveolar bone. Our work has shown how the use of two complementary techniques enabled the study of the 3-dimensional arrangement of the lacunar canalicular interconnection network and the osteocyte network in the same zones of the alveolar bone.

Block staining with basic fuchsin is a relatively simple and inexpensive technique that was described by Frost in 1960 for detecting microscopic cracks in bone. The ability of basic fuchsin to penetrate and diffuse in spaces and breaks in continuity enables it to diffuse and penetrate within the LCS, making it an ideal candidate for studying the LCS by CLSM.

The combination of the block staining technique with basic fuchsin, observation by CLSM and 3-dimensional reconstruction of images enabled us to determine morphologically, for the first time, the structure of the LCS of the alveolar bone. Our results showed that the osteocyte lacunae have different shapes and 3-dimensional orientation in the buccal and palatal plates of the alveolus. In the buccal plate towards which the physiological movement of rat molars occurs, the oval-shaped osteocyte lacunae are aligned parallel to each other with their major axes parallel to the periodontal bone surface, whereas in the palatal plate, the osteocyte lacunae are rounded and arranged without a specific pattern regarding each other or the periodontal bone surface. This suggests that the 3-dimensional alignment of the lacunae may be closely related to the direction of the physiological movement of the molars, which in rats is towards buccal. The 3-dimensional evaluation of the LCS under conditions of physiological loads is of great interest for subsequent analysis of a possible alteration after applying external loads such as orthodontic forces, which involve the generation of forces with different behavior (tensile and compressive forces) on opposite walls of the same dental alveolus.

One of the main obstacles for studies of the cell structure of osteocyte bodies is that they are located within a mineralized matrix. This is why there are few studies reporting on the 3-dimensional morphology of osteocytes in vivo. Even though the use of transmission electron microscopy (TEM) and more recently, tomography applied to TEM (TEM tomography) have enabled in-depth studies of some subcellular aspects of the osteocyte network, both these techniques require very small sample sizes. In this regard, the main advantage of CLSM is that larger samples can be used for more complete evaluations of the osteocytic lacunar canalicular system.

In contrast to other kinds of microscopy, CLSM enables the study of other parameters in the osteocyte network, such as quantity and length of cytoplasmic processes and connections between cells. It is one of the best tools for evaluating the osteocyte canaliculi and the entire length and diameter of osteocyte cytoplasmic processes. The combination of the two protocols used in this research to study alveolar bone - block staining with basic fuchsin and phalloidin staining of cell actin, also enabled us to calculate the peri-osteocytic and peri-canicular space through which the bone fluid passes. The results of our study, which provide knowledge of the microarchitecture of the osteocytic lacunar canalicular system in physiological conditions, may contribute to estimating the changes that may occur in this architecture, whether due to a pathological process, the application of a surgical technique or after applying force on the alveolar bone, among other conditions.

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