Basic Study / **Temel Araştırma**

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Analysis of tympanosclerotic plaques via atomic force microscope and scanning electron microscope

Atomik kuvvet mikroskobu ve tarayıcı elektron mikroskobuyla timpanosklerotik plakların analizi

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Abstract

Objective: To present new information to the literature by imaging the calcospherule forming tympanosclerotic plaque's structure with atomic force microscope and to analyze the elementary structure of it with energy dispersive X-ray detector adapted to the scanning electron microscope.

Methods: Samples taken from 30 patients who underwent surgery for tympanosclerosis were retrospectively evaluated in our tertiary referral center. The surface topography and three-dimensional images of the hardest plaque was analyzed using atomic force microscopy and examined elemental composition of 5 different calcospherule in 5 different plaques using scanning electron microscopy – energy dispersive X-ray spectroscopy.

Results: Three-dimensional analysis of the tympanosclerotic plaque using atomic force microscopy showed calcium phosphate crystalline structures. Scanning electron microscopy with energy dispersive X-ray spectroscopy was used to quantify the calcium, phosphate, carbon, nitrogen, oxygen, sodium, and magnesium present.

Conclusion: We believe that knowledge of the surface topography and elemental composition of tympanosclerotic plaque will contribute to understanding the etiology of tympanosclerosis and its treatment.

Key words: Tympanosclerosis, plaque, analysis.

Özet

Amaç: Atomik kuvvet mikroskobu ile kalsiyum kürecikleri oluşturan timpanosklerotik plak oluşumunu görüntüleyerek literatüre yeni bilgiler sunmak ve tarayıcı elektron mikroskobuna monte edilmiş enerji yayıcı X-ışını dedektörü ile temel yapısını incelemek.

Yöntem: Timpanoskleroz cerrahisi geçirmiş 30 hastadan alınan örnekler üçüncü basamak sevk merkezimizde geriye dönük olarak incelendi. Atomik kuvvet mikroskobuyla en sert plağın yüzey topografisi ve üç boyutlu görüntüleri analiz edildi ve tarayıcı elektron mikroskobu – enerji yayıcı X-ışını spektroskopisiyle 5 farklı plak içindeki 5 farklı kalsiyum küreciğinin temel bileşimi incelendi.

Bulgular: Atomik kuvvet mikroskobuyla timpanosklerotik plağın üç boyutlu analizi kalsiyum fosfat kristalli oluşumları göstermiştir. Var olan kalsiyum, fosfat, karbon, nitrojen, oksijen, sodyum ve magnezyum miktarının tayini için enerji yayıcı X-ışını spektroskopisiyle tarayıcı elektron mikroskobu kullanılmıştır.

Sonuç: Timpanosklerotik plağın temel bileşimi hakkında bilgi edinmek için yüzey topografisi kullanımının timpanosklerozun etiyolojisi ve tedavisini anlayışımıza katkıda bulunacağına inanmaktayız.

Anahtar sözcükler: Timpanoskleroz, plak, analiz.

Tympanosclerosis is an important sequela of otitis media. Pathologically, tympanosclerotic plaques are localized in the lamina propria between the ciliary cuboidal mucosa of the middle ear and the periosteum of the temporal bone or the squamous epithelium of the tympanic membrane.[1] Isolated calcified structures are also called calcospherules. They consist of calcium phosphate crystals. Many light and electron microscopic studies of the calcospherules in

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tympanosclerotic plaque have been conducted.[2] The plaques occur mostly in the tympanic membrane, around the ossicles and oval window.[3]

The surfaces of materials can be characterized using scanning electron microscopy and atomic force microscopy. These techniques are commonly used in disciplines involving the characterization of materials, including physics, chemistry, biology, architecture, restoration, geology, and dentistry. With scanning electron microscopy, the topographic structure of materials can be visualized on a nanometer or micrometer scale and information on the amount of particles and crystal structure can be obtained. To determine the elemental composition of the surface, scanning electron microscopy with energy dispersive X-ray spectroscopy can be used.[4] With atomic force microscopy, the surface of materials can be seen in three dimensions and surface roughness, phase status, porosity, and size can be analyzed.[5]

In our study, we obtained scanning electron microscopy images of plaques at 50,000x magnification and then determined the percentage elemental composition in its structure using energy dispersive X-ray spectroscopy and obtained surface and three-dimensioned images of the crystal structure using atomic force microscopy.

Materials and Methods

This study was approved by the local ethics committee. We examined tympanosclerotic plaques removed at surgery in 30 patients (13 females, 17 males) who underwent surgery for chronic otitis media. The patients' ages ranged from 11 to 60 years. All of the patients had had chronic otitis media for at least 3 years. Approximately 100 plaques were removed from the 30 patients. The weight of the plaques ranged from 1 to 6 mg. We cleaned the mucosal tissues from around plaques and stored them in sterile tubes at 4 °C. One plaque with most smooth surface was examined for atomic force microscope. One plaque with most large area was examined for scanning electron microscope. Five plaques with the hard and most compact structure were used for the elemental analysis for the scanning electron microscope with energy dispersive X-ray spectroscopy.

Scanning Electron Microscope (Phillips, XL-30S FEG)

A scanning electron microscope can display the structure of materials on micro- or nanometer scales. Using a scanning electron detector, a three-dimensioned image can be obtained, and the elemental composition of the structures can be determined using energy dispersive X-ray spectroscopy qualitatively and quantitatively.

A piece of plaque with a smooth surface was selected, plated with gold/palladium, and then three-dimensioned images of both the outer and a broken surface were taken, and the elemental composition was determined.

Scanning Terminal Microscope (Digital Instruments, MMSPM Nanoscope 4)

This apparatus contains an atomic force microscope and was used to examine the surface properties at the atomic level. The three-dimensioned topography can be determined through the interaction between the surface and probe. For atomic force microscopy, measurements were taken from the smoothest place on the broken surface, because the maximum roughness of the surface in atomic force microscopy should be 5 µm. With both methods, the quantity of the plaque was not important; the microscopic images were independent of the quantity.

Results

In scanning electron microscopic images at 50,000x magnification, the presence of collagenous fibrils in the hardest plaques was noteworthy, although their three-dimensional network could not be determined. They contained oval or spherical lacunae (Fig. 1).

We performed the elementary analysis of 5 different calcospherule in 5 different plaques by energy dispersive X-ray detector (Figs. 2-6). Accordingly, the main elements forming the structure of the plaques were calcium, phosphorus,

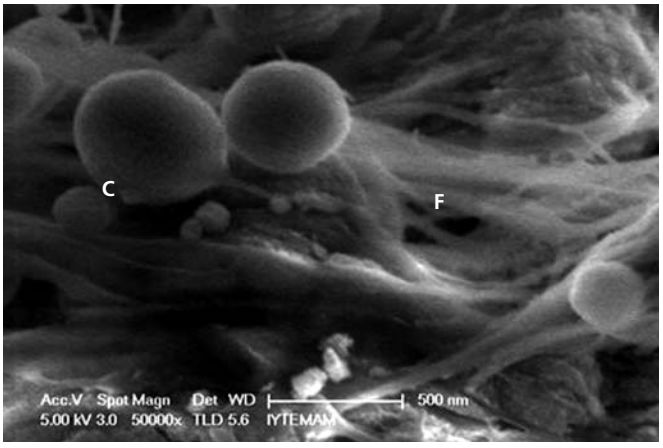

Fig. 1. Cut surface of tympanosclerotic plaque showing calcospherule (C) and irregular collagen fibrilles (F) x50,000.

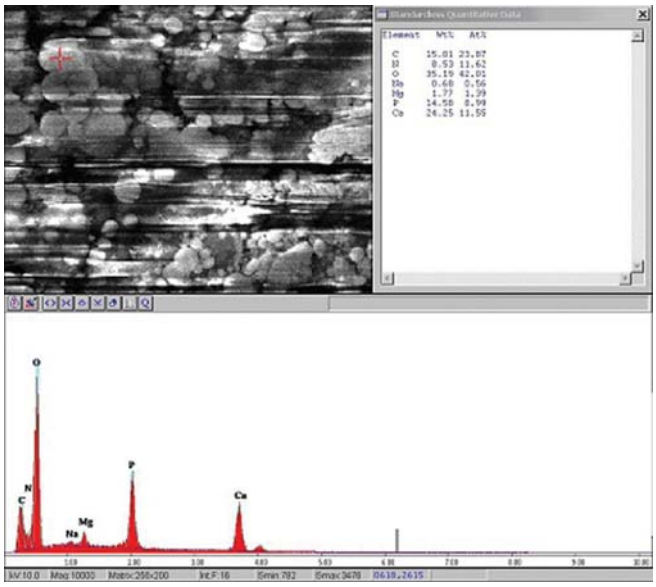

Fig. 2. Analysis of calcospherule using by detector of energy dispersive X-ray spectroscopy in the first plaque (red signed area).

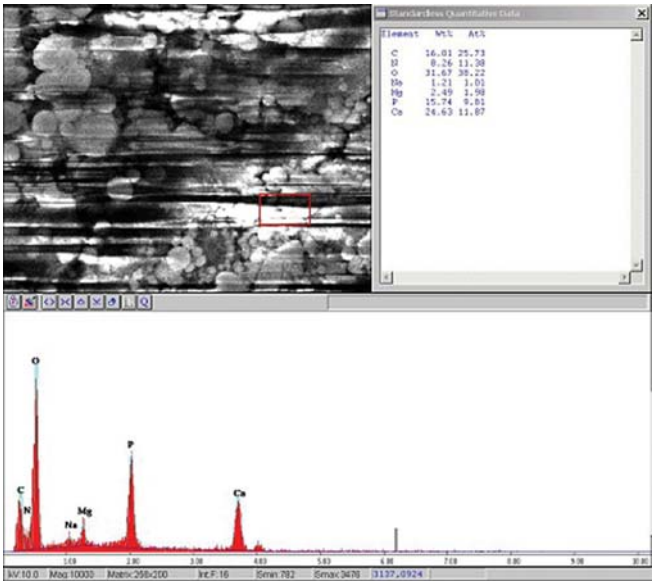

Fig. 3. Analysis of calcospherule using by detector of energy dispersive X-ray spectroscopy in the second plaque (red signed area).

carbon, nitrogen, oxygen, natrium and magnesium. The results were given in percentages and all were similar. Calcium min. 20.09 – max. 24.63, phosphorus min. 13.57 – max. 18.92%, carbon min. 14.54 – max. 24.36, nitrogen min. 8.26 – max. 10.13, oxygen min. 28.80 – max. 37.56, natrium min. 0.57 – max. 1.45, magnesium min. 1.59 – max. 2.52 were determined. Accordingly, mean calcium was found 22.8±2.1%, phosphorus 15.3±2.1%, carbon 17.2±4.02%, nitrogen 8.8±0.7%, oxygen 32.6±3.6%, natrium 0.9±0.3%, and magnesium 2.0±0.4% (Table 1).

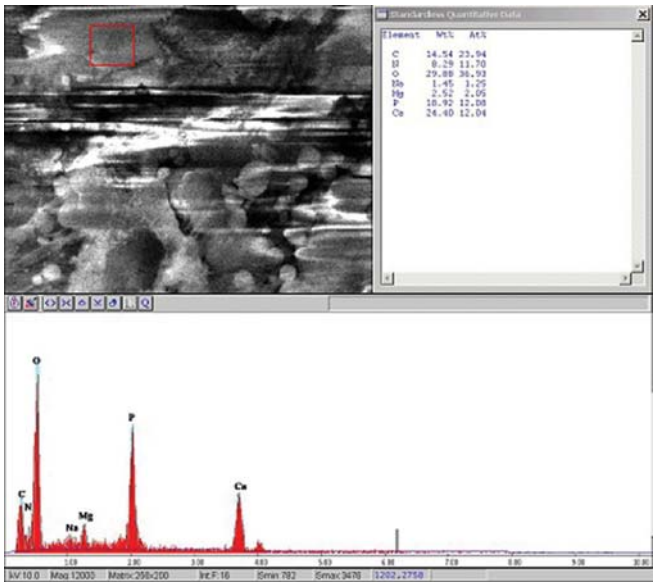

Fig. 4. Analysis of calcospherule using by detector of energy dispersive X-ray spectroscopy in the third plaque (red signed area).

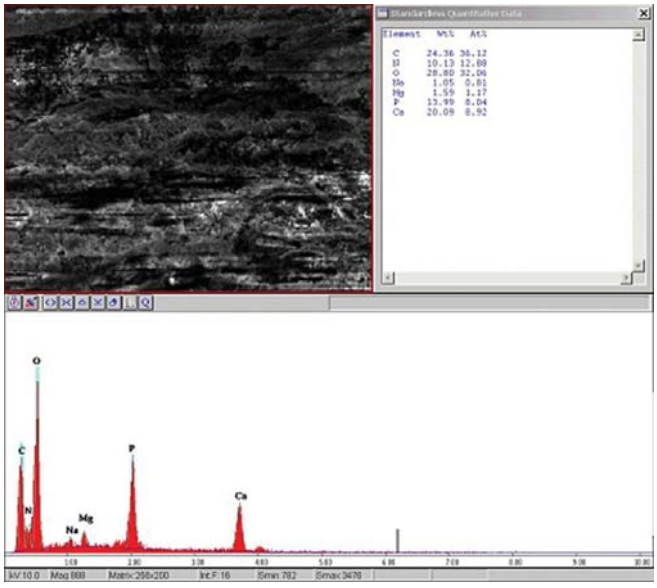

Fig. 5. Analysis of calcospherule using by detector of energy dispersive X-ray spectroscopy in the fourth plaque (red signed area).

For a 5x5 µm area of the plaque surface, we obtained a three-dimensional image of the calcium phosphate crystalline structure and a phase image (Fig. 7). The atomic force microscopy results cannot be quantified.

Discussion

The etiology of tympanosclerosis is unclear, although it is thought to be a disproportionate response of the middle ear to allergy, trauma, infections, or autoimmune reactions.[6] Tympanosclerosis is a dystrophic calcification, because pathological calcification is seen in the degenerate tissues. The mechanism of dystrophic calcification involves calcium uptake in damaged mitochondria and increased hydroxyl ions in the milieu as a result of pH changes, which leads to the formation of hydroxyapatite crystals when calcium ions join together.[7]

The extracellular membranous matrix plays an important role in the mineralization of tympanosclerosis. Anderson et al. showed that the hydroxyapatite is localized in the matrix vesicles, which have calcium-binding activity and accumulate calcium and phosphate.[8,9]

Four different phases in the development of tympanosclerosis can be observed by light microscopy histologically. During the early phase, vesicles 50-300 nm in diameter are formed in the fibroblasts, inflammatory cells, and epithelium cells of the extracellular collagenous matrix. Then, crystalline-like inclusions appear in these vesicles, so-called calcospherules. Calcium and phosphate precipitate in these spherules and mineralization begins. During the last phase, masses that are completely mineralized appear and they are then called plaques.[3]

Transmission electron microscopy images show an extensive collagenous fibrillary network among the calcium phosphate crystals.[7] These crystals form calcospherules, 1-5 µm in diameter, and can be seen in cross-sectional images taken at greater than 2000x magnification. Tympanosclerotic plaques examined using scanning electron microscope at 25,000x magnification showed fibrillar collagen endings and globular aggregates.[10] For the first time, we obtained 50,000x magnification images of a cross-section of a plaque and saw that the three-dimensioned network among the collagenous fibrils was lost, and there were oval or spherical lacunae in the interspaces. This was consistent with literature reports.

Bonnaud was the first to analyze the chemical composition of tympanosclerotic plaques in 1971. He found that calcium averaged 4 g/100 g tissue. Similarly, Buyonover et al. found calcium 1-4 mg/100 mg tissue, using a chemical analy-

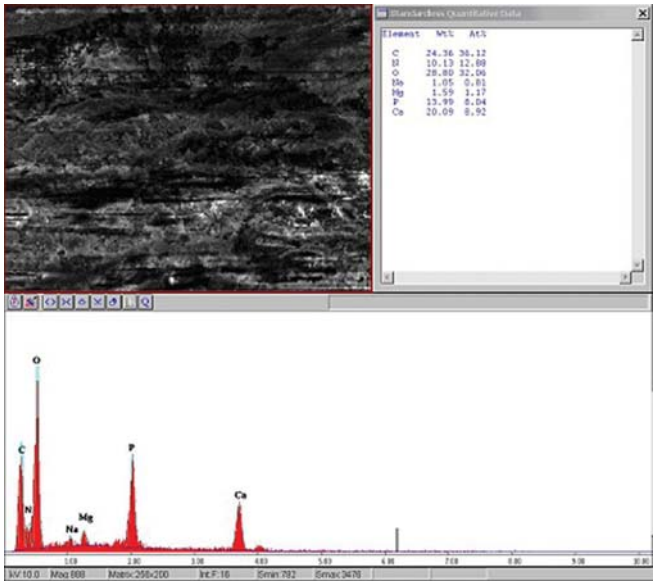

Fig. 6. Analysis of calcospherule using by detector of energy dispersive X-ray spectroscopy in the fifth plaque (red signed area).

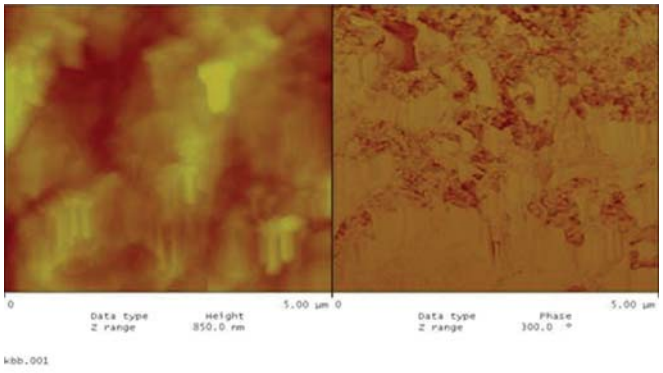

Fig. 7. Three dimensioned images of the crystal structure of plaque by atomic force microscope in 5x5 micrometer.

Table 1. Results of analysis in five different tympanosclerotic plaques.

Elements	Mean±SD (range)
Ca	22.88±2.14 (20.09-24.63)
P	15.36±2.15 (13.57-18.92)
C	17.29±4.02 (14.54-24.36)
N	8.85±0.78 (8.26-10.13)
O	32.62±3.67 (28.80-37.56)
Na	0.99±0.36 (0.57-1.45)
Mg	2.00±0.46 (1.59-2.52)

SD: Standard deviation.

sis method. They attributed the differences to the fact that each tympanosclerotic plaque examined was in a different mineralization phase.[11] Döner et al. reported a biochemical analysis of tympanosclerotic plaques in 2003 and found calcium 2.5±2.6 mg/100 mg tissue, phosphate 0.16±0.11 mg/100 mg tissue, and protein 3.4±3.4 mg/100 mg tissue.[12] Using scanning electron microscopy with energy dispersive X-ray spectroscopy for the first time, we scanned five calcospherule from five plaque and determined that the elemental composition, in percentages, mean calcium was found 22.8±2.1%, phosphorus 15.3±2.1%, carbon 17.2±4.02%, nitrogen 8.8±0.7%, oxygen 32.6±3.6%, natrium 0.9±0.3% and magnesium 2.0±0.4% (Table 1). We found no other information on calcospherules in the literature.

Our use of atomic force microscopy was also an innovation and there is no report on the three-dimensional images of the crystals in a calcospherule.

Conclusions

Our results provide new information that increases our understanding of the structure of calcospherules and consequently tympanosclerotic plaques using scanning electron microscopy with energy dispersive X-ray spectroscopy and atomic force microscopy.

Acknowledgments

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Conflict of Interest: No conflicts declared.

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