Nanostructural Effects of Mitomycin C Applications on Scleral Collagen Fibrils: Atomic Force Microscopy Observation

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ABSTRACT

This study was to investigate the effects of Mitomycin C (MMC) on the scleral collagen surfaces by atomic force microscopy (AFM). Two non-contact mode AFM machines were used to observe changes in the morphological characteristics of human scleral surfaces before and after 0.02% MMC application for 1, 3 and 5 minutes. From AFM topography and deflection images of the collagen fibril, the morphological characteristics of scleral fibrils including the fibril diameter and D-banding were measured using line profile. The sclera collagen fibril treated with 0.02% MMC for 1 min showed no significant increase in mean fibril diameter (155.04 ± 17.46 nm) and the mean D-periodicity (70.02 ± 3.33 nm), compared to the control group. However, the scleral collagen fibrils treated with 0.02% MMC for 3 and 5 min showed a significant increase in mean fibril diameter (182.33 ± 16.33 nm and 199.20 ± 12.40 nm) and mean D-periodicity (70.27±13.66 nm and 72.75 ± 19.32 nm), compared to the control group. This study examined the structural changes in the scleral collagen fibrils before and after MMC application by AFM technique. The results indirectly suggest that long-term MMC application might increase the incidence of complications like a scleromalcia.

Keywords: human sclera, collagen fibrils, Mitomycin C, diameter and D-periodicity, atomic force microscopy

1 INTRODUCTION

Scleral tissue protects the eye from external injury and provides mechanical strength to withstand the intraocular pressure [1]. The strength and resilience of the scleral tissue are achieved by bundles of parallel collagen fibrils. The scleral collagen lamellae branches and interlace are extensive and exhibit a wide-range of dimensions, up to 50 μm in width and 6 μm in thickness [2]. The scleral tissue is traversed by blood vessels and nerves. The anterior ciliary vessels penetrate anterior to the rectus muscles while the posterior ciliary vessels, vortex veins and nerves enter posterior to the muscles [2]. This organization provides the scleral tissue with considerable visco-elastic properties. The scleral visco-elasticity protects the eye from external injury during transient increases in intraocular pressure.

Mitomycin C (MMC) is an alkylating agent that inhibits DNA synthesis, leading to long-term inhibition of tenon's fibroblast proliferation. The use of MMC in pterygium surgery was first reported in 1963. The main effect of MMC is to reduce the recurrence rate of pterygium after excision. One of the main concerns with the use of MMC remains its safety. Although the complication rate associated with a single intraoperative application is low, various anecdotal case reports have created an unfavorable impact regarding the safety of MMC among many ophthalmic surgeons. The complications of MMC described to date are punctate keratitis, chemosis, delayed conjunctival wound healing, conjunctival granuloma, scleral melting, and corneal melting [3].

Morphological observations of the human scleral collagen surface by electron microscopy (EM) have been reported. However, observation of the scleral microstructures using these techniques requires extensive sample preparation and examination conditions. In particular, the samples should be dehydrated, covered with conducting thin films, and placed in a vacuum environment. In contrast, atomic force microscopy (AFM) provides three-dimensional (3D) information of the morphology as well as quantitative information regarding the surface morphology and mechanical properties, with minimal sample preparation [1,4]. The AFM observations of scleral collagen were first reported by Fullwood et al. [5] AFM is commonly used in many research fields including cornea and sclera, monkey eyes, intraoclular lens, etc. To the best of our knowledge, there are no reports examining the nanostructural effects of MMC on human scleral collagen fibrils utilizing AFM. Therefore, this study examined the structural changes in human sclera collagen by MMC application.

2 MATERLANS AND METHODS

2.1 Tissue Preparation

Two human scleral stroma tissues were collected at the Eye Bank of the Kyung Hee University Medical Center, Seoul, Korea. Informed consent for the use of human tissue

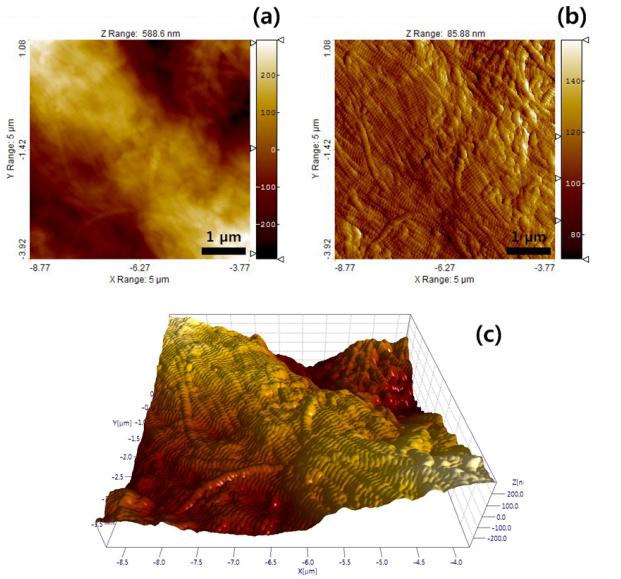

Figure 1: Representative AFM topography (a), deflection (b), and 3D images (c) of the dehydrated scleral collagen fibrils in a scan size of 5 × 5 µm^2.

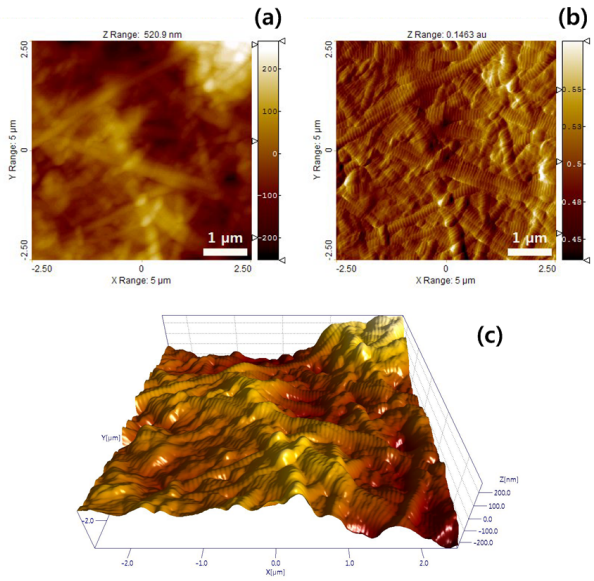

Figure 2: Representative AFM topography (a), deflection (b), and three-dimensional images (c) of the dehydrated scleral collagen fibrils with 0.02% MMC application for 5 minutes in a scan size of 5 × 5 µm^2.

for research was obtained from the Eye Bank. Donor sclera had negative serologic tests for hepatitis, syphilis and human immune deficiency virus. It was preserved for three months in 90% ethanol for sterilization of donor sclera after the donation. The samples were then removed from the ethanol and then irrigated with balanced salt solution (BSS) at room temperature. A sponge soaked in a solution of 0.2 mg/ml MMC was applied for the periods of 1 (group 1), 3 (group 2) and 5 (group 3) minutes. This was followed with the irrigation of BSS. They were prepared by gluteraldehyde fixation and ethanol dehydration for 10 min per step: 60%, 70%, 80%, 90%, 95%, and 100% ethanol (3 changes). The scleral samples were then examined immediately after drying and immobilized on mica with biadhesive tape.

2.2 AFM Measurement

Non-contact mode AFM images were obtained using a NANOS N8 NEOS (Bruker, Herzogenrath, Germany) equipped with a 43 × 43 × 4 µm^3 XYZ scanner and two Zeiss optical microscopes (with Epiplan 200× and 500×, Carl Zeiss Inc. Standort Göttingen-Vertrieb, Germany). External noise was eliminated by placing the AFM machine on an active vibration isolation table inside a passive vibration isolation table. Data acquisition and image processing were performed with SPIP (Scanning Probe Image Processor Version 4.8, Image Metrology, Denmark). The scleral surfaces of each group were scanned in air at a resolution of 512 × 512 pixels and a scan speed of 0.8 line/s.

2.3 Statistics

The quantitative data was expressed as the mean ± standard deviation (SD). Statistical analyses were performed to compare the mean values obtained from each group using a two-tailed Student's t-test. P-values < 0.05 were considered significant.

3 RESULTS AND DISCUSSION

AFM can resolve individual collagen fibrils with a clear axial periodicity. However, it is important to select the appropriate scan size of images in image-based morphological measurements because fringe distortion and measurement error of AFM images are strongly related to the morphological information. Therefore, the morphological characteristics of scleral fibrils were measured experimentally on three scan sizes using line measurements (Figs. 1 and 2). As shown in Table 1, in the 5 × 5 µm^2 scan area, the fibril diameter was 145.22 ± 17.78 nm (n = 178) ranging from 98 to 220 nm with the majority of diameters ranging from 124 to 163 nm. The D-periodicity (Fig. 3b and d) was 69.14 ± 14.15 nm (n = 189) ranging from 40 to 118 nm with the majority of spacings falling between 56-71 nm. In the 1 × 1 µm^2 scan area, the collagen fibril diameter was 106.64 ± 16.87 nm (n = 180) ranging from 65-157 nm. The D-periodicity was 63.38 ± 6.00 nm (n = 300) ranging from 36 to 85 nm with the majority of spacings falling between 46-70 nm. The collagen fibril diameters of groups 1 to 3 were 155.04 ± 17.46 nm in MMC application for 1 min, 182.33 ± 16.33 nm (p < 0.005) in MMC application for 3 min and 199.220 ± 12.40 nm (p < 0.0001) in MMC application for 5 min. D-periodicities of the collagen fibrils showed no significant difference between groups. The

Parameter	Control	0.02% MMC 1 min	0.02% MMC 3 min	0.02% MMC 5 min
Diameter (nm)	145.22 ± 17.78	155.04 ± 17.46	182.33 ± 16.33*	199.20 ± 12.40†
D-banding (nm)	69.14 ± 14.15	70.02 ± 3.33	70.27 ± 13.66	72.75 ± 19.32

*p-value < 0.005 vs. Control group. † p-value < 0.0001 vs. Control group.

Table 1. Morphological changes in the sclera fibrils for control and MMC-treated groups.

diameters and D-periodicities of the collagen fibrils measured from both scan sizes of 5 × 5 μm^2 and 1 × 1 μm^2 were significantly different from each other ($p < 0.0001$). The fibril diameter in the 1 × 1 μm^2 scan area was lower than that in the 5 × 5 μm^2 scan area due to fibrils sandwiched in between the adjacent fibrils. Although the fibrillary structures were measured in images with better resolution, this difference was responsible for the reduced hydration of the samples examined [4]. The D-periodicity of the collagen fibrils was also similar to results of the diameter. However, the majority of spacings in both scan sizes were within the normal 67 nm D-periodicity.

MMC is one of the modalities employed to reduce the recurrence rate in modern pterygium surgery. The intraoperative application of MMC is very effective and the drug is used widely by ophthalmologists. There are abundant data regarding the early postoperative adverse effects of mitomycin C. The possible reasons contributing to the various results of the structures of collagen fibrils in the image-based morphological measurements caused by technical factors during AFM imaging have been investigated [6]. In particular, most images were shown to be tip-dependent. Therefore, the morphological differences between these results and those reported in the literature were compared, as shown in Figs. 1 and 2. Most studies examined the morphological structures of scleral fibrils using EM. Meller et al. [6] reported that AFM showed a greater D-period of fibrils than EM. This difference might be associated with tissue shrinkage during the chemical fixation, dehydration, embedding, and freezing steps in EM sampling. The main advantage of AFM is that it can examine biological samples close to the native state. Therefore, no additional sample preparation process was achieved to minimize the sources of artifacts for further imaging. To our knowledge, there are few reports of the results of AFM observation in morphological observations of human scleral fibrils. Furthermore, previous studies used various scan sizes. Meller et al. [6] reported a somewhat high mean (310.54 nm in diameter and 77.02 nm in D-period) as well as a wide SD (214.59 nm in diameter). The present results showed a diameter half the size (145.22 nm) of that reported by Meller et al. [6] and a 69.14 nm spacing, which is close to the normal 67 nm D-periodicity. Although the convolution effects of the tip are present in all AFM images, their significance depends upon the size of the features being imaged. The AFM topography can potentially reveal the actual dimensions of the surface of the object if the tip is much smaller than the object.13 In this study, the fibril diameter was larger than the radius of the tip, 10 nm.

4 CONCLUSIONS

The present study examined the structural changes in the scleral collagen fibrils before and after 0.02% MMC application according to AFM. The results indirectly suggest that three or more minutes of 0.02% MMC application affects the morphology of scleral collagen[1].

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