Introduction:

Collagen, the most abundant protein in the human body, is essential for the structural integrity of tissues. In order to tissue engineer collagen and collagen-related structures, it is important to understand collagen fibril assembly. Study of in vitro collagen fibril assembly is useful because it allows for better control of variables than in vivo and thus easier deduction of assembly factors. Currently, a major problem encountered with in vitro collagen assembly is irregular growth. This study sought to illuminate further, using atomic force microscopy (AFM) and dark-field microscopy, the mechanism by which collagen molecules self-assemble into their ultrastructure.

Materials & Methods:

Type I collagen molecules from calf skin (United States Biological, Swampscott, MA) were used for fibril formation. The collagen was mixed with phosphate buffer saline (PBS), creating a 0.01% (w/v) collagen solution. The pH of the solution was adjusted to 7.4 using a 1N NaOH solution. Different solution preparations were then incubated at 37 °C for various lengths of time. Each solution was later examined by dark-field microscopy (Axioplan2, Carl Zeiss, Gottingen, Germany).

For AFM investigation (Synergy ESPM, Novascan Technologies, Ames, IA), the fibril solution was diluted to a 1/10X concentration in distilled water. 10 µl of fibril solution was then dropped on a freshly cleaved mica surface and left to dry for one minute. The mica was then carefully washed in distilled water. After air-drying, the specimen was examined by AFM in tapping mode using a silicon nitride tip (NSC12, Mikromasch USA, Portland OR). Each tip had a cantilever length of 130 µm and a resonance frequency of 145 kHz. The images were recorded with 512 X 512 resolution using a 1 Hz scan rate. Measurements were taken using image analysis software. To measure a fibril’s diameter, the height of the fibril image was measured rather than the lateral image width because of the convolution effect due to the interaction between the AFM tip and the topography of the sample.

Results:

A series of dark-field and AFM images were prepared for incubation times of 0.5 hr to 2 days. There were long fiber structures already at 0.5h of incubation as exhibited through dark-field imaging (Figure 1a). A dark-field image for 17.5h of incubation is shown in Figure 1b. AFM imaging further revealed the detailed structure during the early stages of assembly (Figure 2a) and later stages of assembly (Figure 2b). Analysis of the AFM images showed that the diameters of the fibrils reached 15-20 nm, but that there was no significant disparity in visible diameter for the different incubation times.

Discussion:

After collagen were mixed with PBS, longitudinal assembly progressed quickly, while the lateral assembly was simultaneous but at a lower pace. Fibrils with diameters around 20 nm started to form after 1.5 hr and the D-period was visible after 3 hr. After 24 hr, however, the growing process stalled. The solutions were very inhomogeneous. Even in the final stage of assembly, a large portion of the collagen still existed in protofibrillar form.

At the beginning of assembly, the collagen grew end-to-end at a quick pace. Because the length to diameter ratio was over 10000, the collagen curved into loops and the fibrils tangled with each other. Up to 1.5 hr, some fibrils had grown to 20 nm; however, the D-period was not obvious until after 3 hr. The fibrils with perceptible D-periods occupied only a small portion of the whole solution, most of the solution consisting of either protofibrils or long collagen too thin to distinguish a D-period. Dark-field microscopy images displayed apparently homogenous fibrils in solution after 18 hr, but AFM revealed plenty of small collagen or subfibril in the solution. We suspect the high rate of longitudinal assembly at the beginning causes the extreme curvation and intertwining of the protofibrils, producing a morphology that is unfavorable for lateral assembly. To form uniform and thick fibrils, it might be necessary to impede the longitudinal assembly at the beginning in order to reduce disordered entangling.

Although dark-field microscopy is a simple and efficient tool to investigate fibril assembly, it can only reveal fibrils with large enough diameter. Its resolution is not sufficient to distinguish between actual fibrils and a mere alignment of protofibrils in one direction. In the images from 0.5 hr and 1 hr of assembly, most of the area was occupied by what appeared to be up to 100 µm long fibril-like structures. However, the average length of the fibrils during a later stage of assembly, 18 hr, was about 20 µm. Detailed fibril assembly analysis requires higher resolution microscopy like AFM and EM.

In summary, aggregation of collagen molecules occurred rapidly in the longitudinal direction very early in the growing process, but their entanglement possibly hindered lateral assembly. In addition, dark-field microscopy was unable to resolve the heterogeneity of the solutions.

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Fig 1a – Dark-field: 1 hr (100 µm × 75 µm)

Fig 1b – Dark-field: 17.5 hr (100 µm × 75 µm)

Fig 2 (A) AFM image at 1.5 hr incubation time. (B) AFM image at 22 hr incubation time.

53rd Annual Meeting of the Orthopaedic Research Society

Poster No: 0812