Fibrous Long Spacing Collagen Ultrastructure Elucidated by Atomic Force Microscopy

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ABSTRACT Fibrous long spacing collagen (FLS) fibrils are collagen fibrils in which the periodicity is clearly greater than the 67-nm periodicity of native collagen. FLS fibrils were formed in vitro by the addition of α_1-acid glycoprotein to an acidified solution of monomeric collagen and were imaged with atomic force microscopy. The fibrils formed were typically ~150 nm in diameter and had a distinct banding pattern with a 250-nm periodicity. At higher resolution, the mature FLS fibrils showed ultrastructure, both on the bands and in the interband region, which appears as protofibrils aligned along the main fibril axis. The alignment of protofibrils produced grooves along the main fibril, which were 2 nm deep and 20 nm in width. Examination of the tips of FLS fibrils suggests that they grow via the merging of protofibrils to the tip, followed by the entanglement and, ultimately, the tight packing of protofibrils. A comparison is made with native collagen in terms of structure and mechanism of assembly.

INTRODUCTION

Collagen is the most abundant structural protein found in animal connective tissues. It exists in a variety of morphological forms, ranging from long fibrils to complex, interwoven network-like structures (Nimni, 1988). Individual molecules of collagen, which are semiflexible rods ~280 nm in length and ~1 nm in diameter, undergo self-assembly to form these structures. The most well-studied of the assembly products is the native fibril, which is characterized by a banding pattern with a ~67-nm period. Fibrillogenesis of type I collagen in particular has been examined for over 40 years by a wide variety of techniques (Kadler et al, 1996). In vitro, fibrils with the characteristic 67-nm pattern similar to that of the native fibril (and henceforth called *normal fibrils*) have been produced, enabling the investigation of the effects of factors such as temperature, concentration, and ionic strength on the assembly process (Veis and George, 1994).

Altering the conditions under which the assembly takes place can lead to the formation of different end products. For type I collagen, a fibrillar variant, which was named fibrous long spacing collagen (FLS), was first observed during in vitro experiments carried out on acidified connective tissue extracts (Highberger et al., 1950). These fibrils are characterized by a periodicity in the banding pattern much larger than that in normal collagen, typically ~200–300 nm. These early observations gave rise to a postulated model of FLS fibril structure with the collagen monomers lined up in register (0D stagger), so that the 200–300-nm banding is caused by the separation between rows of individual monomer units (Gross et al., 1954). Further investigations led to the conclusion that α_1-acid glycoprotein was somehow promoting the formation of FLS fibrils in such extracts (Highberger et al., 1951). Much later, analysis of FLS amino acid composition in conjunction with radioactivity studies (Franzblau et al., 1976) led to the suggestion that the glycoprotein is incorporated into the fibril in some manner. However, the exact nature of the α_1-acid glycoprotein interaction with collagen, and its role in the structure and assembly of FLS collagen, remain unclear.

Subsequently, FLS collagen has been found in both pathological and normal tissues (Ghadially, 1988). The first in vivo detection of FLS collagen was in ocular tissue (Jakus, 1956). Soon after it was detected in nervous tissue, both normal (Cauna and Ross, 1960) and neoplastic (Luse, 1960; Naumann and Wolfe, 1963). Ensuing investigations showed the existence of FLS collagen associated with various pathological conditions such as athlerosclerotic plaques (Morris et al., 1978), Hodgkin's disease (Nakanishi et al., 1981), myeloproliferative disorder (Kamiyama and Shimamine, 1977; Kamiyama, 1982), and silicosis (Slavin et al., 1985). These fibrils found in vivo typically had periodicities of ~100–150 nm, resulting in a postulated model with the monomers at half-stagger (Ghadially, 1988). However, periodicities of ~250 nm have also been observed (Ghadially and Mierau, 1985).

Two major hypotheses of the origin of in vivo FLS fibrils have been proposed (Kajikawa et al., 1980; Park and Ohno, 1985). The first suggests that FLS fibrils are the degradation product formed by the activity of endogenous collagenases upon reticular collagen fibers; the second suggests that FLS forms as a result of interactions between immature collagen microfibrils and acid mucopolysaccharides present in tumor tissues. Evidence exists for both views (Kobayasi et al., 1985; Miki et al., 1993; Park and Ohno, 1985), indicating that FLS fibril formation may have a significant dependence upon physiological circumstances. Other researchers have proposed that glycoproteins or polysaccharides play a role

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during in vivo FLS fibril formation (Dingemans and Teeling, 1994; Ghadially, 1988; Hashimoto and Ohyama, 1974), and glycoproteins have been shown to be present in at least one form of in vivo FLS fibrils (Dingemans and Teeling, 1994).

Because the pathological significance of FLS collagen in vivo has yet to be determined, clarification of the exact structure of FLS collagen and the precise manner in which it assembles are very important issues. Analysis of the in vitro assembly process should provide important insights into the nature of the in vivo assembly and potentially into the nature of diseases associated with the existence of FLS.

A technique suitable for the study of collagen fibrils produced by in vitro assembly is atomic force microscopy (AFM). AFM is a technique by which surface topography can be measured to sub-angstrom precision in some cases (Binnig et al., 1986). As AFM involves both minimal sample preparation and perturbation, it is a useful tool for analyzing biological systems at and below the ultrastructural level, and has been applied in many cases (Hansma and Hoh, 1994). This technique has been successfully utilized for the examination of normal collagen (Baselt et al., 1993; Revenko et al., 1994) and of its in vitro assembly (Gale et al., 1995; Goh et al., 1997).

In this report we utilize AFM imaging for a detailed examination of the ultrastructure of FLS collagen fibrils formed in vitro in the presence of α_1-acid glycoprotein. We compare the ultrastructure with that of normal collagen. The images of FLS collagen obtained provide insight into the mechanism by which the self-assembly of these fibrils occurs. In particular, our results are not consistent with the proposed alignment of monomers; rather, we postulate the existence of protofibrils as assembly intermediates.

MATERIALS AND METHODS

In vitro FLS collagen assembly

Type I calf skin collagen (Sigma) was dissolved over ice in 0.05% acetic acid, with occasional sonicating to facilitate the breakdown of collagen aggregates. The mixture was centrifuged at 5000 rpm for 60 min at 4°C. After centrifugation, the supernatant was filtered through 0.45-μm Millipore filters (Sigma) and mixed with the α_1-acid glycoprotein solution. Solutions of α_1-acid glycoprotein were prepared by dissolving the solid protein (Sigma) in 0.05% acetic acid. Collagen and glycoprotein solutions were combined to yield a mixture with a final collagen concentration of ~0.5 mg/ml, an α_1-acid glycoprotein concentration of ~0.0375%, and a final pH of 3.5. The protein mixture was transferred into dialysis tubing (molecular weight cutoff 12,000–14,000) and dialyzed at 21°C against Millipore-filtered water overnight (~17 h). Dialysis produced a white, turbid solution with a final pH of 7. A series of dilutions ranging from 10- to 1000-fold were prepared by mixing the dialysis product with an appropriate volume of Millipore water. The diluted samples were deposited onto freshly cleaved sheets of mica in 10-μl aliquots and allowed to dry for 1 h before imaging in the AFM.

Atomic force microscopy

Samples were imaged with a Nanoscope III instrument (Digital Instruments, Santa Barbara, CA), typically using square pyramidal silicon nitride tips of spring constant 0.58 N/m. To check for tip artifacts, and in an attempt to improve resolution, other tips, such as silicon oxide, were utilized periodically. Both height and deflection mode images were taken simultaneously; in many instances, the deflection mode images highlighted features not easily seen in the height mode. However, quantitative measurements were taken from the height mode images only. To facilitate relocating the same samples for repeated inspection, thin mica substrates were placed on top of copper locator grids as described by Markiewicz and Goh (1997).

RESULTS AND DISCUSSION

The method described above produced a large number of FLS fibrils, which were easy to locate in the AFM, and which were identifiable because of their distinct banding pattern (~245-nm periodicity). The measured periodicity of 245 nm agrees with the results of Highberger et al. (1951), which were obtained using electron microscopy (EM). However, the AFM measurements show differences in morphology between normal and FLS fibrils that are not readily apparent in the EM results. In addition, the high vertical resolution of the AFM enabled us to examine minute ultrastructural details found on the surface of the fibrils.

Figs. 1 *a*, 2, and 3 show examples of FLS fibrils. Longitudinal and cross-sectional measurements of different mature fibrils were taken, showing them to be typically ~100–200 nm in diameter and ~10 μm in length. A typical fibril and its sections are shown in Fig. 1 *a–c*. For comparison, a mature normal collagen fibril, assembled as described by Gale et al. (1995), is shown in Fig. 1 *d–f*. Aside from the different periodicities, FLS fibrils are much thicker in diameter than normal collagen fibrils, whereas their lengths are roughly the same.

As can be seen in Fig. 1 *b*, the banding pattern in FLS is very periodic, much more so than in the normal collagen fibril (Fig. 1 *e*). In addition, it is clear that the banding pattern in Fig. 1, *a* and *b*, consists of a series of evenly spaced ridges. These ridges are ~30 nm high and ~100 nm wide, and are separated by a relatively flat region that spans ~150 nm. This is in stark contrast with the observations for normal collagen fibrils, in which the fibril surface consists of a continuous series of bumps. This can be seen in the section (Fig. 1 *e*). The banding pattern seen in Fig. 1 *d* in this case is due to relatively sharp depressions or grooves instead of protrusions.

Because the bands are well separated, the AFM tip can access both the interband regions and the bands themselves for closer inspection. Within these regions, fine ultrastructural features can be resolved. These features, shown in Fig. 2 *a*, appear as small ridges and grooves oriented roughly along the main axis of the fibril. The grooves were typically 2 nm deep and 20 nm in width, and were continuous along the bands and the interband regions for all mature fibrils examined. It is important to note that the finite tip width means that the depth of these ridges may be an underestimate, whereas tip convolution effects mean that the width is an overestimate (Murray et al., 1993; Hansma et al., 1992).

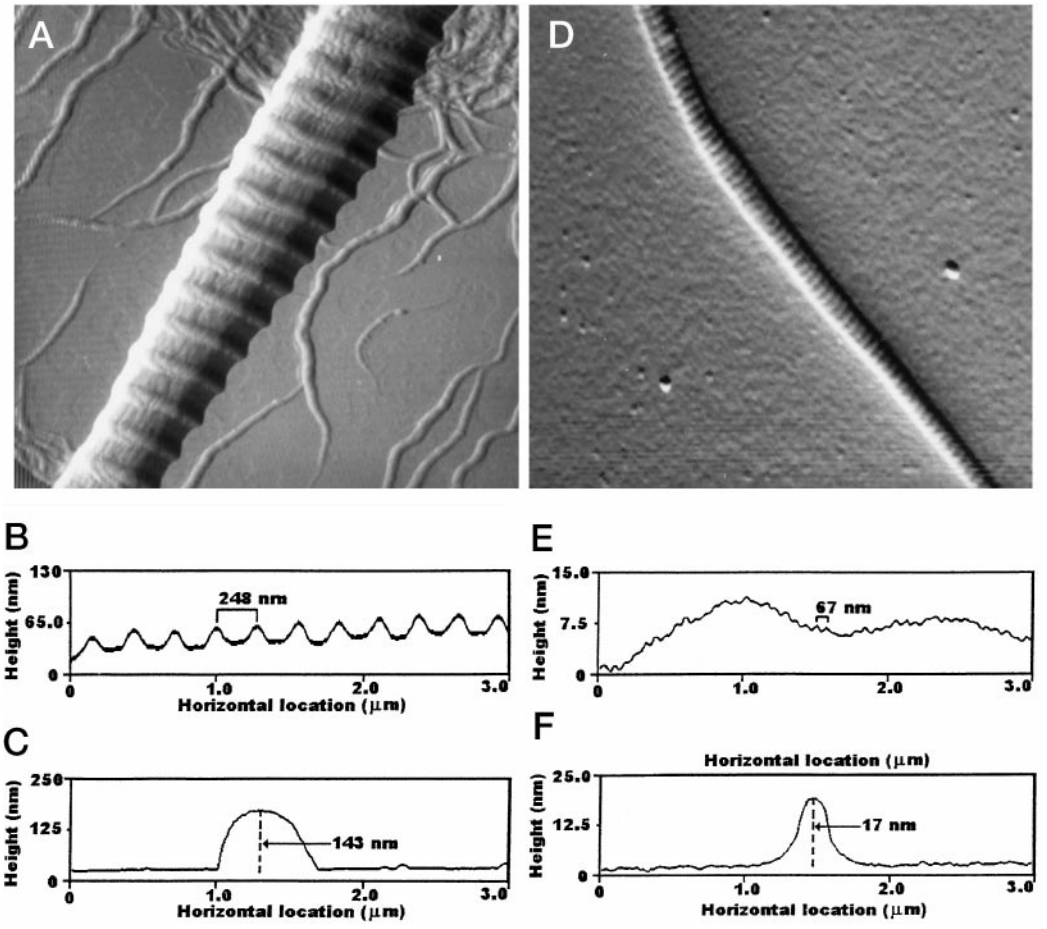

FIGURE 1 (*a–c*) FLS collagen fibril. (*d–f*) Normal collagen fibril. (*a* and *d*) AFM image in deflection mode (scan sizes: 3.5 μm × 3.5 μm and 3 μm × 3 μm). (*b* and *e*) Longitudinal section, showing the banding pattern. (*c* and *f*) Cross section, showing the diameter. Note the differences in vertical scale.

Several different AFM tips, including standard silicon nitride tips, sharpened oxide tips, and tips produced by electron beam deposition, were used to confirm that the observed features were not due simply to tip effects. The same ultrastructural features could be observed using all of the different tips. A better measurement of the dimensions of these features can be obtained by processing the tip geometry out of the AFM image (Keller, 1991; Markiewicz and Goh, 1994). However, it is simpler to estimate their dimensions by noticing that their apparent widths are about the same as those of the filamentous structures that are on the mica substrate. The real diameters (free of tip convolution effects) of these filamentous structures can then be obtained by measuring their height above the mica surface (Hansma et al., 1992); this was found to be ~5–7 nm.

In their EM study of in vitro FLS fibrils, Highberger et al. (1951) also mentioned the presence of some interband region ultrastructure in FLS, but because of the inability of electron microscopy to characterize depths, they were unable to obtain much information about these features. AFM has provided information about this ultrastructure, and has revealed the presence of ultrastructure on the bands themselves. Other studies of in vitro FLS fibrils note the presence of striations or minor bands that were oriented parallel to the main bands (Chapman and Armitage, 1972). In our investigations, we did not observe any indications of the presence of minor bands that are parallel to the main bands. However, on a longitudinal section of a mature fibril, one can sometimes discern the presence of small lumps, which may possibly appear as minor bands when stained for EM. Another possibility is that the minor bands evident in EM images arise from morphology within the core region of the fibril, and are not mirrored in the surface topography, to which the AFM is sensitive.

This ultrastructure does not remain the same throughout the mature fibril. Consider the fibril shown in Fig. 1 *a*, which is narrower and compact at one section and grows wider in the other direction. On the narrower end, as highlighted in Fig. 2 *a*, the corrugations are less visible and appear more orderly and parallel to the main fibril axis. As the fibril becomes wider (shown in Fig. 2 *b*), these corrugations become more clearly defined and less ordered, showing mostly as lumps instead of clear filaments. We can examine the full length of a fibril, and locate its tip. Examination of several such FLS fibrils, some of which are shown in Fig. 3 *a*, indicates that their tips do not have the

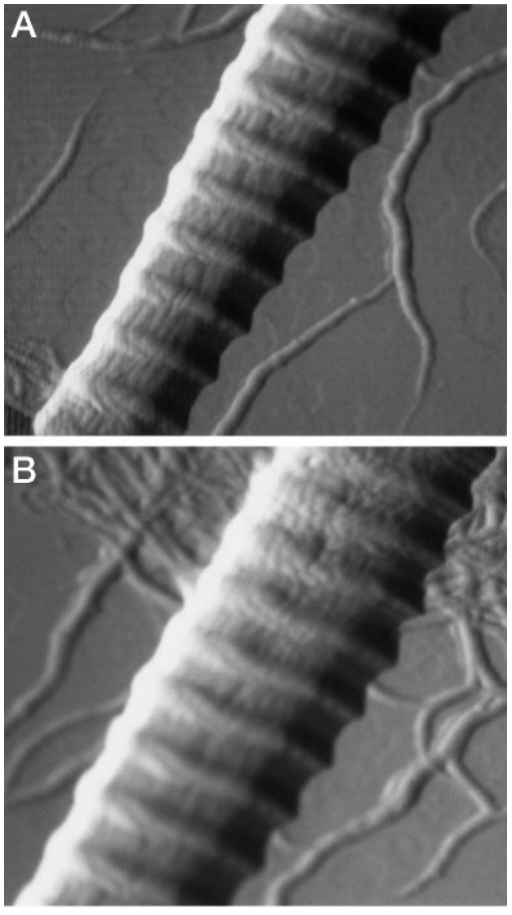

FIGURE 2 AFM deflection mode image of the same FLS fibril, at different regions. (*a*) Region along the fibril appears more ordered with protofibrils tightly packed and aligned along fibril axis (scan size: 1.75 μm $\times$ 1.75 μm). (*b*) Region with clear ultrastructure, but disordered and loose. Notice that the ridges also have the same ultrastructural pattern as the flatter regions (scan size: 2 μm $\times$ 2 μm).

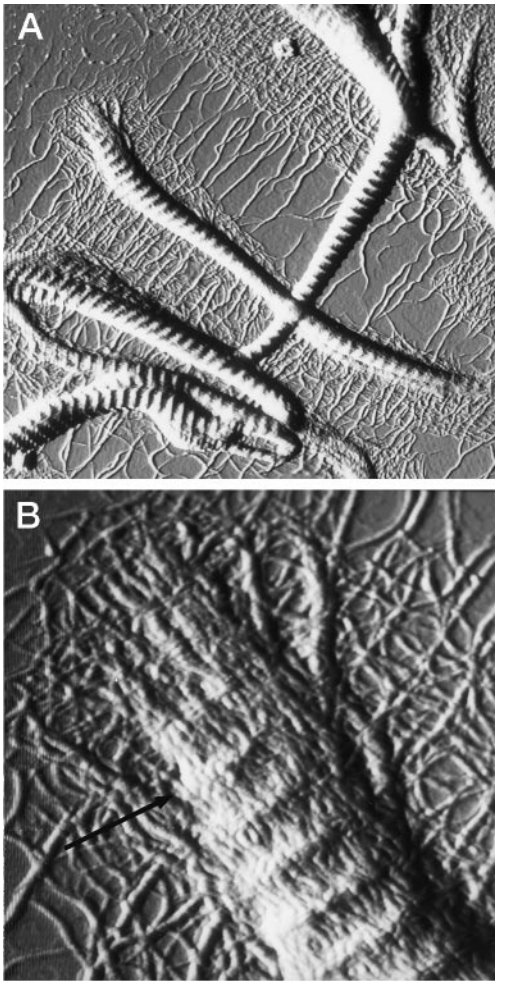

FIGURE 3 AFM deflection mode image of FLS fibril showing tips and details. (*a*) Several fibrils (scan size: 12 μm $\times$ 12 μm). (*b*) A close-up view of a fibril tip. Note the precursor to ridges spaced at ~245 nm (*arrow*) and the intertwining of protofibrils (scan size: 2.25 μm $\times$ 2.25 μm).

same parabolic shape as found in normal collagen (Holmes et al., 1992). Instead, they are wide and somewhat blunted, as can be seen in Fig. 3.

In these images and in other similar samples, the wide tip is always surrounded by a large number of the ~5–7-nm-diameter filamentous structures, similar to those that were mentioned previously. The fine, disordered ultrastructure discussed previously is also evident and is much more pronounced. Based on these results, we postulate that the mature FLS fibril is composed of these finer filamentous structures, which we shall henceforth call *protofibrils*. With this assumption, we can ascribe the ultrastructural pattern in Fig. 1 *a* to the existence of a tighter packing of the protofibrils at the narrower section of the fibril, and a looser packing at the wider section. As one nears the wide tip of the mature fibril, the protofibrils become even more loosely packed, as can be seen in Fig. 3 *b*. This implies that the tip is the region of growth, and that growth occurs by addition of protofibrils to the mature fibril. This view is further supported by a closer inspection of the tip in the AFM deflection mode, which is more sensitive to changes in topography. In Fig. 3 *b* we can see a region of protofibrils (indicated by an *arrow*) that appears to be giving rise to a partially formed band, which is axially located ~245 nm from a complete band. This is further evidence suggesting the importance of protofibrils in FLS structure.

Although it is difficult to get a precise value for the length of the protofibrils because of their high degree of entanglement, we estimate this to be ~1–2 μm. These protofibrils are much larger than collagen monomers, which are ~280 nm in length and 1 nm in diameter. Their relative uniformity in size suggests that they are stable intermediates in the assembly of FLS fibrils.

The mechanism of FLS fibrillogenesis is poorly elucidated and not well studied. Because the banding pattern of in vitro FLS fibril is close to the length of the collagen monomer, it is generally held that FLS fibrils consist of monomers polymerized side by side in register (Gross et al., 1954). We have seen no evidence of such monomers, and the observation of protofibril components in the body and in the growing tip of the mature fibril is contradictory to this

view. The presence of stable protofibril intermediates argues that there is a modular component to the assembly. Our data suggest that, at least at this late stage of fibrillogenesis, the mature fibril grows by some entanglement of protofibrils at the tip, which anneals to a more orderly and tighter packing.

The role of α_1-acid glycoprotein is not discussed in any of this, or in any other literature, although it is believed that glycoproteins or proteoglycans are necessary components of both in vitro and in vivo FLS. It has been speculated that FLS fibrils form in tissue by disassembly of normal collagen and subsequent reassembly into FLS in the presence of glycoproteins or proteoglycans developed (Kajikawa et al., 1980; Park and Ohno, 1985). In our in vitro studies, this is unlikely to be the case. We did not find normal collagen fibrils in our preparations. This is consistent with observation of a large temperature effect on the rate of fibrillogenesis of normal collagen (Gelman et al., 1979). At the temperature at which we conduct FLS assembly, we have not been able to form normal collagen fibrils within any reasonable time period with our standard procedure. However, we had previously identified intermediates for normal collagen fibrillogenesis (Gale et al., 1995), and if it were simply a matter of kinetics, we should have seen some of these. We found no evidence for such, implying that FLS fibrillogenesis proceeds along a completely different route, perhaps intimately tied to the role of α_1-acid glycoprotein. We are currently investigating this issue by examination of the early stages of FLS assembly.

SUMMARY

FLS collagen fibrils formed in vitro have been studied by AFM. Our measurements yield fibril diameters and periodicities of ~100–200 nm and ~245 nm, respectively, which are in good agreement with electron microscopy results. The banding pattern consists of evenly spaced ridges separated by large, relatively flat interband regions. We have probed at higher resolution the surface of mature FLS fibrils to reveal details about their ultrastructure. A fine structure consisting of side-by-side protofibrils aligned roughly along the axis of the fibril was observed in both the interband spaces and on the bands. The tips of FLS fibrils were found to be blunt and flattened, in contrast to the parabolic shape found in native type I collagen fibrils. We observed evidence for the addition of protofibrils, fibrous collagen species of approximate dimensions ~5–7-nm diameter and ~1–2-μm length, to blunted FLS fibril ends. The AFM images obtained suggest that the formation of FLS in vitro is related to entanglement of protofibrils at the blunted tip, followed by the eventual tightening of this entanglement, and a final close packing of the protofibrils. Further study of the intermediate stages in the assembly process may provide greater insight into the nature of the FLS growth mechanism and the role of α_1-acid glycoprotein in FLS formation.

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