REVIEW ARTICLE

Collagen fibril formation

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Collagen is most abundant in animal tissues as very long fibrils with a characteristic axial periodic structure. The fibrils provide the major biomechanical scaffold for cell attachment and anchorage of macromolecules, allowing the shape and form of tissues to be defined and maintained. How the fibrils are formed from their monomeric precursors is the primary concern of this review. Collagen fibril formation is basically a self-assembly process (i.e. one which is to a large extent determined by the intrinsic properties of the collagen molecules themselves) but it is also sensitive to cell-mediated regulation, particularly in young or healing tissues. Recent attention has been focused on 'early fibrils' or 'fibril segments' of ~ 10 μm in length which appear to be intermediates in the formation of mature fibrils that can grow to be hundreds of micrometres in length. Data from several laboratories indicate that these early fibrils can be unipolar (with all molecules pointing in the same direction) or bipolar (in which the orientation of collagen molecules reverses at a single location along the fibril). The occurrence of such early fibrils has major implications for tissue morphogenesis and repair. In this article we review the current understanding of the origin of unipolar and bipolar fibrils, and how mature fibrils are assembled from early fibrils. We include preliminary evidence from invertebrates which suggests that the principles for bipolar fibril assembly were established at least 500 million years ago.

INTRODUCTION

Collagen is distinct from other proteins in that the molecule comprises three polypeptide chains (α-chains) which form a unique triple-helical structure. For the three chains to wind into a triple helix they must have the smallest amino acid, glycine, at every third residue along each chain. Each of the three chains therefore has the repeating structure Gly-Xaa-Yaa, in which Xaa and Yaa can be any amino acid but are frequently the imino acids proline and hydroxyproline. More than 20 genetically distinct collagens exist in animal tissues. Collagen types I, II, III, V and XI self-assemble into *D*-periodic cross-striated fibrils [1–4] (Figure 1) (where $D = 67$ nm, the characteristic axial periodicity of collagen) and collectively are the most abundant collagens in vertebrates. The fibril-forming collagen molecules consist of an uninterrupted triple helix of approx. 300 nm in length and 1.5 nm in diameter flanked by short extrahelical telopeptides. The telopeptides, which do not have a repeating Gly-Xaa-Yaa structure and do not adopt a triple-helical conformation, account for 2% of the molecule and are critical for fibril formation (see below).

Type I collagen $\{[\alpha1(I)]_2\alpha2(I)\}$ is found throughout the body except in cartilaginous tissues. It is also synthesized in response to injury and in the fibrous nodules formed in the sequelae of fibrotic disease. Type II collagen $\{[\alpha1(II)]_3\}$ is found in cartilage, developing cornea and vitreous humour. These major collagen fibrils are almost certainly not formed from just one collagen type but instead are co-polymers of two or more fibril-forming collagens. Type III collagen $\{[\alpha1(III)]_3\}$ is found in the walls of arteries and other hollow organs and usually occurs in the same fibril with type I collagen. Type V collagen [$\alpha1$(V), $\alpha2$(V), $\alpha3$(V)] and type XI collagen [$\alpha1$(XI), $\alpha2$(XI), $\alpha3$(XI)] are minor components of tissue and occur as heterotypic fibrils with type I and type II collagen respectively (for a review of collagen distribution, see [5]).

Much of what is known about collagen fibril assembly has resulted from studies of the type-I-collagen-containing fibrils in tendon and skin and from studies in which fibrils are reconstituted *in vitro* from purified type I collagen. Therefore, out of necessity, the present review is concerned primarily with the assembly of

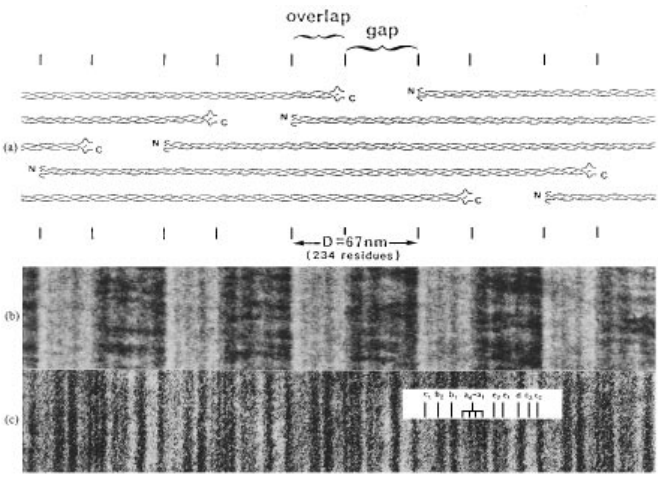

Figure 1 Axial structure of *D*-periodic collagen fibrils

(**a**) Schematic representation of the axial packing arrangement of triple-helical collagen molecules in a fibril, as derived from analysis of the positive (**c**) and negative (**b**) staining patterns. (**b**) Collagen fibril negatively stained with sodium phosphotungstic acid (1%, pH 7). The fibril is from a gel of fibrils reconstituted from acetic-acid-soluble calf-skin collagen. The repeating broad dark and light zones are produced by preferential stain penetration into regions of lowest packing (the gap regions). (**c**) Similar fibril positively stained with phosphotungstic acid (1%, pH 3.4) and then uranyl acetate (1%, pH 4.2). The darkly staining transverse bands are the result of uptake of electron-dense heavy-metal ions from the staining solutions on to charged residue side groups of collagen. For a detailed explanation of the band assignments and analysis, see [1].

Abbreviations used: *D*, the axial periodicity of collagen fibrils (= 67 nm); pNcollagen, procollagen containing the N-propeptides and lacking the C-propeptides; pCcollagen, procollagen containing the C-propeptides and lacking the N-propeptides; DPS III, *D*-periodic symmetrical banding type III.

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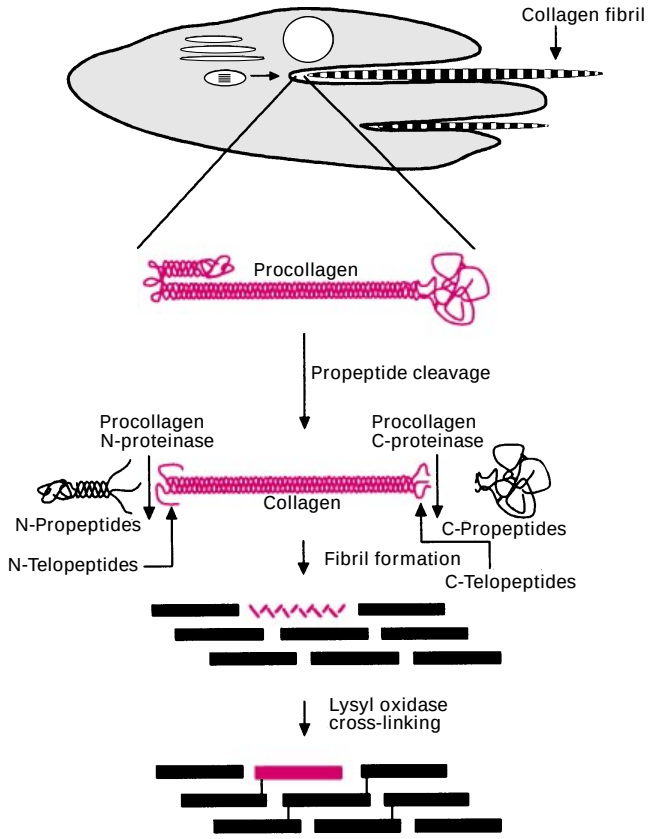

Figure 2 Extracellular events in the synthesis of fibrillar collagens

Procollagen consists of a 300-nm-long triple-helical domain (comprised of three α-chains each of approx. 1000 residues) flanked by a trimeric globular C-propeptide domain (the right-hand side of the diagram) and a trimeric N-propeptide domain (the left-hand side of the diagram). Procollagen is secreted from cells and is converted into collagen by the removal of the N- and C-propeptides by procollagen N-proteinase and procollagen C-proteinase respectively. The collagen generated in the reaction spontaneously self-assembles into cross-striated fibrils that occur in the extracellular matrix of connective tissues. The fibrils are stabilized by covalent cross-linking, which is initiated by oxidative deamination of specific lysine and hydroxylysine residues in collagen by lysyl oxidase. The process is shown occurring in cell-surface crypts according to the model generated by Birk and co-workers (see text for references).

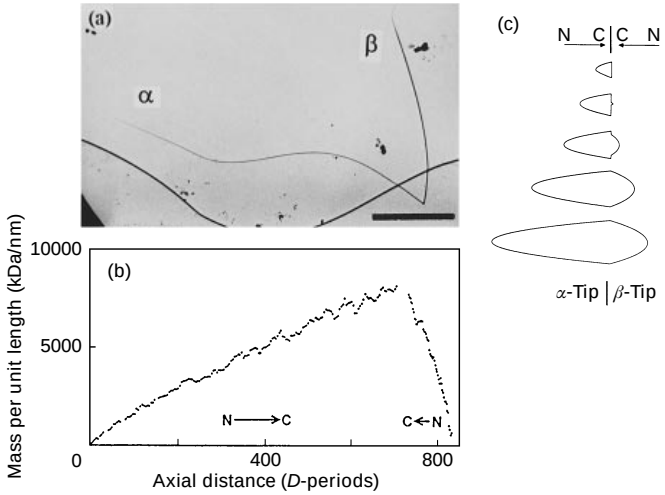

Figure 3 Bipolar fibrils formed *in vitro* by cleavage of purified pCcollagen with procollagen C-proteinase

(**a**) Transmission electron micrograph of a positively stained collagen fibril generated by cleavage of pCcollagen (50 μg/ml) with the C-proteinase (50 units/ml) at 37 °C. The fibril displays fine (α-tip) and coarse (β-tip) ends. Scale bar = 1 μm. (**b**) Axial mass distribution of an entire unstained fibril similar to the one shown in (**a**). The fibril shows a near-linear axial mass distribution of the two tips, with no evidence of a limiting diameter. Arrows show orientations of collagen molecules within the fibril; E/S [enzyme units/substrate mass (μg)] = 50:50. (**c**) Schematic representation of the growth of a bipolar fibril in the cell-free system. The model shows a two-stage model, as indicated by light-microscope observations [8], in which growth occurs first from a pointed tip (the α-tip) and additional growth occurs from the blunt end after the formation of a second pointed tip (β-tip) for growth in the opposite direction. The tip profiles are shown as sections of parabolas, consistent with the linear axial mass distributions of a fibril with a circular cross-section.

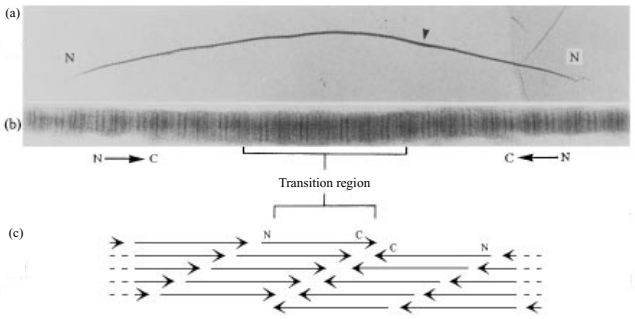

Figure 4 Transmission electron micrograph of a positively stained bipolar fibril from 18-day chick embryo metatarsal tendon

(**a**) The fibril is 10.5 μm long and shows a polarity reversal 3.5 μm from one end (arrowhead). (**b**) Enlargement of the banding pattern at the polarity reversal region (brace). The braced region shows four *D*-periods where the staining pattern illustrates molecules in anti-parallel arrangement. The centre two *D*-periods show a symmetrical pattern with two axial planes of mirror symmetry. These mirror planes occur between the **d** and **c2** staining bands and in the vicinity of the **a3** band. (**c**) Analysis of the staining pattern in the transition region indicates an anti-parallel arrangement of molecules. The schematic representation shows that the axial extent of the transition region is about four *D*-periods. This corresponds to the minimal distance possible to achieve polarity reversal and to maintain the *D*-periodicity concomitantly.

type I collagen into fibrils. Other collagens will be mentioned only with regard to how their assembly into fibrils differs from that of type I collagen and how they influence or participate in fibril formation.

The assembly of collagen molecules into fibrils is an entropy-driven process, similar to that occurring in other protein self-assembly systems, such as microtubules, actin filaments and flagella (for a review, see [6]). These processes are driven by the loss of solvent molecules from the surface of protein molecules and result in assemblies with a circular cross-section, which minimizes the surface area/volume ratio of the final assembly. Although the broad principles of collagen fibril self-assembly are generally accepted, less is known about the molecular mechanisms of the assembly process.

A fundamental feature of fibril-forming collagens is that they are synthesized as soluble procollagens (Figure 2), which are converted into collagens by specific enzymic cleavage of terminal propeptides by the procollagen metalloproteinases. Without these proteinases the synthesis of collagen fibrils would not occur. A suitable cell-free system of assembling fibrils has been developed in which procollagen is sequentially cleaved with the purified procollagen metalloproteinases to generate collagen *de novo* [6–9]. Fibrils generated in the system initially have a near-paraboloidal pointed tip [10] and a blunt end, and growth is exclusively from the pointed tip [11]. As growth proceeds, the blunt end becomes a new pointed tip for growth in the other

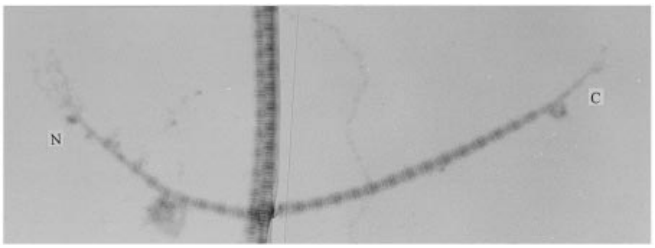

Figure 5 Transmission electron micrograph of a positively stained unipolar collagen fibril from 18-day chick embryo metatarsal tendon

The unipolar fibril is positioned from left to right and is seen crossing a larger cross-banded fibril (running from top to bottom). The unipolar fibril is 2 μm in length and shows N- and C-terminal tips with no polarity reversal.

direction [12]. Furthermore, the two pointed tips each have collagen molecules oriented with N-termini closest to the fibril end [11] (Figure 3). Thus the fibrils are N–N bipolar, in which a switch in molecular orientation occurs at a region along the fibril. These features had previously not been seen in fibrils formed *in vivo* and initially appeared to be artefacts arising from contaminants in the preparations of procollagen or the procollagen metalloproteinases. However, subsequent work has shown that bipolar fibrils having two N-terminal paraboloidal tips occur in developing chick tendon [13] (Figure 4). Thus the cell-free system had accurately predicted several fundamental features of the assembly of collagen fibrils, including the paraboloidal shape of the tip and the occurrence of N–N bipolars. Recent data from our laboratories have shown that fibrils in developing chick tendon have features additional to those of fibrils formed in the cell-free system. For example, some fibrils are unipolar, having molecules pointing exclusively in one direction, in which case fibrils have a C-terminal and an N-terminal end [13] (Figure 5).

UNIPOLAR FIBRIL FORMATION FROM ACID-SOLUBLE COLLAGEN

Collagen may be extracted from several tissues into neutral salt buffers or, with greater yield, into dilute acidic solutions [14]. Typical acetic acid extracts of skin and tendon yield milligram quantities of type I collagen, mainly in the form of monomers but also including variable amounts of cross-linked components (dimers, trimers and some higher components). Preparations may also vary in respect of the intactness of the proteinase-susceptible, non-helical, telopeptide regions of the molecule. Such preparations, when neutralized and warmed to temperatures between 20 and 34 °C, produce a gel of *D*-periodic fibrils over the course of several hours (Figure 6). At 34 °C fibril diameters are typically in the range 20–70 nm. Lower temperatures generally result in broader fibrils, with diameters of up to 200 nm found at 20 °C [15,16]. Samples of these final gels show a meshwork of very long fibrils in which ends are not observed (Figure 6). The rate of assembly of fibrils can be monitored by measuring turbidity which, to a close approximation, is proportional to the amount of fibrillar material formed [14–17]. A typical near-sigmoidal plot shows three regions: a lag region, a growth region and a plateau. Diameter measurements on fibrils obtained during the time course of assembly have demonstrated that a limiting fibril diameter distribution occurs when about 20 % of the collagen molecules have assembled into fibrils, suggesting that the latter stages of assembly must be at the ends of existing fibrils [17].

Fibrils formed from acid-soluble collagen are unipolar, *D*-periodic and have two smoothly tapered ends. Early fibrils, ranging in length from 1 to 20 μm, are observed at the end of the lag phase and in the early growth phase (Figure 6). Such early fibrils showed a well defined shape under particular solution conditions, with the occurrence of a 'limiting early fibril' of about 90 *D*-periods (6 μm) in length and with a maximal cross-section containing about 160 molecules (Figure 6) [18,19]. Such observations imply a greater level of growth control in the self-assembly of these fibrils than is indicated from observations on the final fibril gel.

Other workers [20], however, have reported the occurrence of non-banded filaments (of diameter in the range 10–20 nm) during this early phase of fibril assembly, and concluded that the final banded fibrils are formed by lateral fusion of the first formed filaments. These apparently conflicting observations of the assembly pathways were found to be due to differences in the method used to initiate fibril formation, rather than to differences in collagen preparation or solution conditions [19]. The same sample of collagen could show different aggregation states depending on the order of warming and neutralizing of the solution. The occurrence of non-banded filaments required the solution to go through a cold neutral step. The molecular mechanism leading to these different assembly routes is likely to involve changes in the conformation of the telopeptides that accompany fibril assembly (see below).

Initial oligomer formation in fibril assembly has been studied by photon correlation spectroscopy of solutions [21–26] or by electron microscopy of rotary-shadowed samples adsorbed to support films [27,28]. Studies have included experiments starting from either near-monomeric preparations of lathyritic collagen or monomer fractions of rat-tail tendon collagen. (Lathyritic collagen is obtained from animals fed 2-aminopropionitrile, which inhibits the enzyme lysyl oxidase, and consequently the animals have collagen with a much decreased cross-linking capacity.) In both solution and electron microscope studies the formation of a 4*D*-staggered dimer has been identified as a preferred initial aggregation step. Some solution studies have indicated a second stage of assembly involving the lateral aggregation of dimers and trimers into oligomers [25,26]. These species cannot be definitively assigned to a specific part of the early fibril assembly pathway such as nucleation or propagation. Some key intermediates may not accumulate in solution, whereas other abundant species may not be true intermediates contributing to the final fibril. The difficulty in experimentally determining a nucleating and accreting species is pronounced if the acid-extracted collagen is warmed prior to being neutralized to initiate fibril growth. In this method of initiating fibril formation there is a rapid onset of heterogeneity of aggregate size, with both dimers and early fibrils (containing 10^3–10^4 molecules) present at the end of the lag phase [19].

Fourier-transform IR spectroscopy suggests that conformational changes occur in the collagen molecule during assembly into fibrils [29]. Changes in the carbonyl group spectrum (amide I; 1700 to 1600 cm^{-1}) were evident in the 22–26 °C temperature range, under fibril-forming conditions, which led to the hypothesis that the triple helix of the semi-flexible collagen molecule is actually perfected during the lag phase, facilitating nucleation and intermolecular interaction. Spectra were also obtained in the amide II and III regions. Further spectral changes after fibrils had formed showed that the molecules are once again distorted as they are bent to fit within the fibrils.

Partial loss of the telopeptides of the collagen molecule has major effects on fibril growth [30,31]. These include loss of diameter uniformity, loss of unidirectional packing and changes

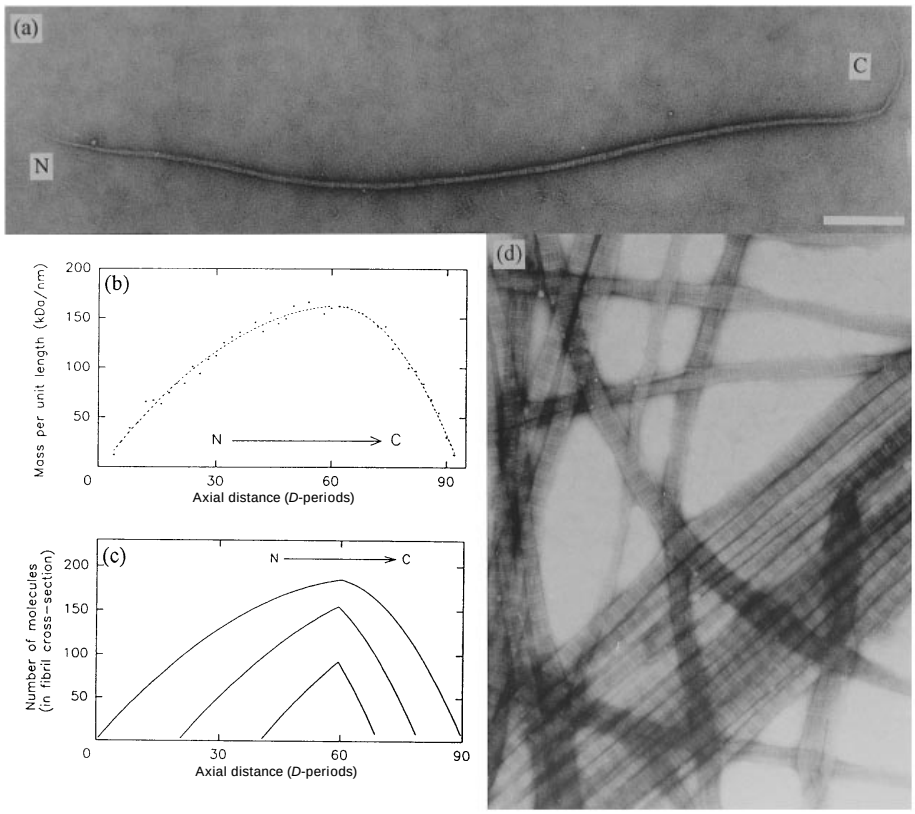

Figure 6 Unipolar fibrils formed *in vitro* by reconstitution from acetic-acid-soluble calf-skin collagen

(**a**) Transmission electron micrograph of a negatively stained unipolar fibril displaying an N- and a C-terminal tip. The fibril was sampled when approx. 1% of the collagen had assembled into fibrils. Bar = 300 nm. (**b**) Axial mass distribution of an unstained early fibril. Mass determination was by quantitative scanning transmission electron microscopy. (**c**) Set of growth curves obtained by averaging axial mass distributions similar to that shown in (**b**). The slopes of the N- and C-ends of the fibril remain constant with fibril elongation. Analysis of the slopes of the axial mass distributions indicates an increase of five collagen molecules per *D*-period at the N-tip and 10 molecules per *D*-period at the C-tip. (**d**) Negatively stained sample of the final fibril gel shown at the same magnification as (**a**). Note that the final fibrils are larger in diameter than early fibrils and that the ends of the fibrils are not observed.

in the fibril assembly pathway, depending on the extent of removal of each of the N- and C-telopeptides. Experimental approaches include exposure of the collagen solution to pepsin with partial removal of both telopeptides [32,33], or selective degradation of the N-telopeptide or of the C-telopeptide with, respectively, leucine aminopeptidase and carboxypeptidase [30,31]. Loss of the N-telopeptides has been linked with the formation of *D*-periodic symmetrical fibrils with molecules in anti-parallel contact, while loss of part of the C-telopeptides has been associated with the formation of *D*-periodic tactoids. Complete removal of both telopeptides prevents the formation of fibrils, assembly being limited to the formation of small non-banded fibrous aggregates. The experimental data have been interpreted in terms of a simple model where the N-telopeptide is critical for the formation of the polarized 4*D*-staggered dimers that occurs as an early stage of assembly, and the C-telopeptide has a dual role, promoting a lateral accretion of linear aggregates as well as participating in the formation of the early linear assemblies [31].

Electron optical data [34] and X-ray data [35] both indicate that the N-telopeptides are axially contracted when the collagen molecules are assembled into fibrils. The X-ray data predict a mean residue spacing of 0.7 h, where h is the axial spacing of residues (= 0.286 nm) in the triple helix. Other experimental evidence suggests that the condensed structure is explained by a hairpin conformation of the telopeptides. Thus NMR studies on N-telopeptides in solution indicate the occurrence of β-folds and flexible hinge regions [36,37], and sequence information points to a hairpin loop conformation for N-telopeptides [32]. Rotary shadowing of individual procollagen molecules and mass mapping of assembled pNcollagen (i.e. procollagen containing the N-propeptides) molecules confirms that the N-terminal ends are in a bent-back conformation [38–41].

NMR studies of synthetic peptides show no preferred secondary structure of the C-telopeptides [42]. However, X-ray data do indicate that the C-telopeptides are axially contracted with a mean residue spacing of 0.5 h when the collagen molecules are assembled into fibrils. Sequence analysis suggests that the telopeptides form a hydrophobic cluster [31]. The absence of structure when the telopeptides are free in solution suggests that the contracted conformation of the C-telopeptides may only occur when molecules are in close association with neighbouring collagen molecules in a fibril.

BIPOLAR FIBRIL FORMATION BY CLEAVAGE OF PROCOLLAGEN CONTAINING THE C-PROPEPTIDES (PCCOLLAGEN) WITH PROCOLLAGEN C-PROTEINASE

Fibrils formed by neutralizing and warming of solutions of extracted collagen do not usually have the same diameters as the fibrils from which the collagen was extracted. Thus cell-mediated control must be exerted over the self-assembly process *in vivo*.

Evidence suggests that N- and C-propeptides of procollagen may play a role in such diameter regulation (see below for references). For these and other reasons, a cell-free system of fibril assembly was developed whereby procollagen is sequentially cleaved to collagen by the procollagen metalloproteinases. Fibrils formed by cleavage of purified type I pCcollagen with procollagen C-proteinase are exclusively bipolar. Early studies showed that fibrils formed by cleavage of pCcollagen with C-proteinase at low temperatures (29–32 °C) have sufficiently large diameters to be visualized by dark-field light microscopy. The earliest seen aggregates are needle-like, with a pointed end and a blunt end. Growth, observed by time-lapse photography, occurs exclusively at pointed tips. Growth at a blunt end only occurs at high pCcollagen concentrations and, at temperatures above 34 °C, by the appearance of a spear-like projection from the blunt end (as shown schematically in Figure 3), with the projection becoming a new pointed tip for growth in the opposite direction. Smaller-diameter fibrils formed at 37 °C have been shown by electron microscopy to have two smoothly pointed tips. Each tip has collagen molecules oriented with their N-termini directed towards the tip, indicating that a reversal in molecular polarity must exist within the central body of the fibril. Furthermore, these bipolar fibrils are usually shape-asymmetrical and have a fine (α-) tip and a coarse (β-) tip. The tips have linear axial mass distributions, consistent with a paraboloidal shape (Figure 3). In addition, the fineness of a tip (slope of the axial mass distribution) appears to be set at an early stage of growth, such that subsequent fibril growth preserves the shape of the tips. Recent work has shown that the level of C-proteinase has a major effect on the shape of the fibrils in this cell-free system (D. F. Holmes, R. B. Watson, J. A. Chapman and K. E. Kadler, unpublished work). At a constant initial concentration of pCcollagen, increased levels of C-proteinase led to increased slopes of the axial mass distributions of α-tips. Increasing the C-proteinase levels also resulted in a reduced shape asymmetry, yielding fibrils that were nearly shape-symmetrical. It cannot be assumed that all the features of fibril growth *in vitro* are applicable to the process *in vivo*. However, the recent observation of both unipolar and bipolar early fibrils occurring *in vivo* has given encouragement to the simple working model that some aspects of fibril assembly in tissues can be understood as an intrinsic self-assembly process governed by the interaction properties of the collagen monomer. Since early fibrils formed by reconstitution from acid-extracted collagen are exclusively unipolar [43] and those formed by cleavage of pCcollagen with C-proteinase are exclusively bipolar, it seems possible that the occurrence of bipolar fibrils is, in part, dependent on the initial concentration of pCcollagen at the onset of fibril assembly.

These studies raise a number of questions. First, do type II, III, V and XI collagens exhibit similar assembly properties? Limited studies *in vitro* suggest that recombinant type II collagen [44] assembles into thin fibrils arranged in network-like structures [45]; the rate of assembly is lower than that observed for type I collagen under the same experimental conditions [45], and the fibrils formed are C–C bipolar fibrils in which most of the central shaft consists of molecules in an anti-parallel orientation [4,45a]. Fibrils formed from type III and V collagen appear to have the usual parallel orientation of collagen molecules seen in type I collagen fibrils, but no information is available on the occurrence of unipolar or bipolar fibrils of these collagen types.

Secondly, what factors determine the shape of fibrils? Do fibrils grow at equilibrium with a shape of minimum free energy or is, as seems more likely, their shape determined by kinetic factors? Computer-generated simulations of fibril growth have been used to seek answers to these questions. Two models have been proposed for a kinetically determined growth process. One is an interface-controlled growth model where the rate of accretion on to the fibril surface is limited by transition processes on that surface; the shape of the fibril would be determined by the existence of different types of binding site with different accretion rates [46]. In another model, fibrils form by diffusion-limited aggregation [47,48]. Both models can account for tips with linear axial mass distributions. The model suggested by Silver et al. [46] adopts a set of growth rules for the formation of a nucleus and the subsequent addition of monomers to the fibril surface. Several growth steps can be defined involving axial growth of microfibril units and lateral growth which generates additional microfibrils. The model proposed by Parkinson et al. [47,48] involves a general scheme of assembly of rod-shaped molecules where the rate of fibril assembly is limited by the rate of diffusion of monomer to the fibril surface. Such a process was shown to generate fibrils with the same structural features as fibrils formed by cleavage of pCcollagen with C-proteinase *in vitro*, in that they had no constant diameter and the tips showed linear axial mass distributions. The combination of these advanced computer modelling methods together with the experimental capability of obtaining precise size and shape data on fibrils (by quantitative scanning transmission electron microscopy) provides a new route to understanding the underlying principles of self-assembly of collagen fibrillar structures.

FIBRIL ASSEMBLY AND POLARITY *IN VIVO*

A remarkable feature of fibrils formed *in vivo* (see Figures 4 and 5) is that not all are unipolar (i.e. with the molecular polarity unchanged throughout the entire length of the fibril). In 18-day chick embryos about half of the early fibrils are bipolar and half are unipolar. Analysis of the positive stain pattern throughout the entire length of the bipolar fibrils reveals the axial zone of molecular polarity reversal to be highly localized [13]. The transition region contains molecules in anti-parallel contact, of little more than 4 *D*-periods in extent. Analysis of the staining pattern in the transition region demonstrated that the anti-parallel arrangement of the molecules is such that the C-termini of oppositely directed molecules are in axial register. A similar anti-parallel contact is seen in a *D*-periodic symmetrical fibril (where molecules are in anti-parallel arrangement throughout the length of the fibril) formed *in vitro* from type I collagen (*D*-periodic symmetrical banding type III; DPS-III) [49,50]. Furthermore, this arrangement allows for axial continuity of the intermolecular cross-links in fibrils. As described below, fibrils from echinodermis are also N–N bipolar, with a centrally located molecular switch region in which the transition region is restricted to approx. 10 *D*-periods [51].

The mechanical and physical properties of a tissue depend on a hierarchical spatial arrangement of collagen fibrils, whether unipolar or bipolar. Thus narrow fibrils ($\sim$ 20 nm) in precise orthogonal array occur in the cornea, where optical transparency is important, whereas mature tendon, where high tensile strength is paramount, displays a high density ($\sim 10^8$ fibrils/mm^3) of large-diameter fibrils ($\sim$ 500 nm) arranged in parallel bundles. Experiments by Birk and co-workers using electron microscopy of serial transverse sections of embryonic chick tendon have shown that cells can have considerable control over the shape of the pericellular space where fibrils occur. Early fibrils are deposited in extracellular compartments formed between cellular protrusions close to the cell body [52]. Further away from the cell, such compartments (containing a few fibrils) anastomose to generate larger spaces containing bundles of fibrils. In 14-day chick tendon, entire fibrils showing smoothly tapered ends can be

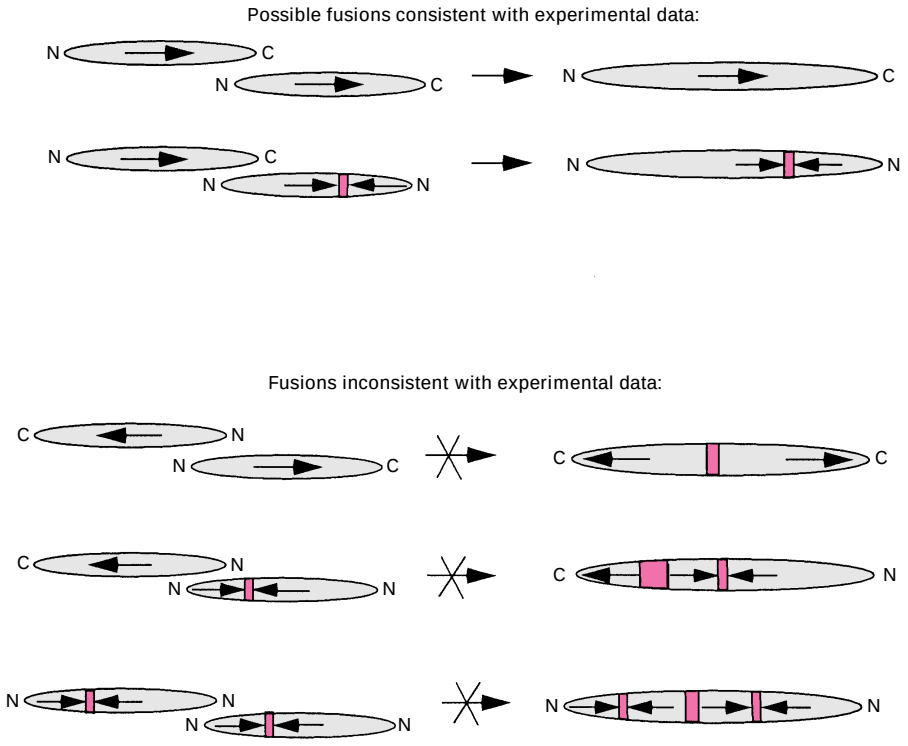

Figure 7 Models of fibril fusion

The fusion of fibrils is modelled using the assumption that fibrils can only have either two N-ends or one N-end and one C-end. Extended or multiple regions of asymmetrical orientation of collagen molecules were not allowed. There is the possibility that two unipolar fibrils pointing in opposite directions and with limited overlap could fuse to generate a bipolar fibril with a molecular switch region limited to approx. four *D*-periods (not shown). Arrows indicate molecular polarity within a fibril. Pink boxes indicate regions of polarity reversal.

found within such bundles [52,53]. Moreover, the tips of fibrils are of different taper, giving an overall shape asymmetry to the fibril. The fibrils are bipolar, and it has been proposed by Birk and co-workers that end-to-end fusion of these early bipolar fibrils could contribute to fibril elongation, and that their side-by-side fusion may account for the observed increase in fibril diameter during tissue development [54]. Fibril fusion, it is suggested, may also be influenced by the presence of surface-bound molecules of decorin [54,55].

End-to-end fusion of bipolar fibrils of type I collagen would seem, however, to be inconsistent with existing experimental data (Figure 7). Bipolar fibrils of type I collagen observed hitherto have invariably displayed two N-ends, i.e. none with two C-ends have been seen, either *in vivo* or *in vitro*. Moreover, the switch region (the polarity transition region in which molecules occur in anti-parallel array) is of limited axial extent and fibrils with multiple switch regions have not been found. Unipolar fibrils can fuse in a number of ways that would accord with the experimental data. For example, two unipolar fibrils with molecules oriented N- to C- from left to right could fuse in side-by-side register (to result in a thicker fibril), or in end-on-end register (giving a longer fibril). End-on-end fusion of two oppositely directed unipolar fibrils (i.e. one fibril oriented N- to C- from left to right and the other from right to left) would also seem to be permitted (yielding a bipolar fibril). Side-by-side fusion of two oppositely directed unipolar fibrils is, however, disallowed because this would result in axially extended regions of anti-parallel axial packing of collagen molecules, which is not seen *in vivo* or *in vitro*.

The need to satisfy these criteria introduces severe limitations on the participation of bipolar fibrils in fibril fusion. In only one special case, where the molecular switch regions of two neighbouring bipolar fibrils are perfectly aligned, is the fusion of two bipolar fibrils allowed. In all other combinations, the fusion of two bipolar fibrils would result in extended or multiple regions of anti-parallel molecular packing within the resultant fibril. In a mixed population of unipolar and bipolar fibrils, end-to-end fusion is allowed but would have the effect of decreasing the unipolar population, resulting in a population enriched in bipolar fibrils, unable to fuse further. Could a possible function for bipolar fibrils be to limit the extent of fibril fusion?

DIAMETER MEASUREMENTS OF FIBRILS FORMED *IN VIVO*

Extensive diameter measurements have been made on fibrils in a large range of tissues, mainly from electron micrographs of fibril transverse sections. Diameter distributions are typically narrow and unimodal during early stages of development, but the mean diameters and spread of diameters increase with maturation of the tissue. Parry and Craig [56] have reported the occurrence of preferred diameters of multiples of 8 nm. This conclusion was based mainly on the mean diameters from unimodal distributions obtained from different tissues at various stages of development in a large number of species. In addition, occasional observations have been made of fibril diameters showing a multi-modal distribution with peaks at multiples of 8 nm [57]. Such diameter measurements have been made on dehydrated, plastic-embedded samples where shrinkage is typically 27%, as indicated by comparative electron microscopy and X-ray diffraction data [57–59]. The corrected value for the preferred diameter increment

is then 11 nm, resulting in fibrils of diameter 22, 33, 44, 55 nm, etc. [60,60a].

Studies have located partially processed type I collagen molecules retaining N-propeptides at the surface of fibrils [61–64], and this has led to the suggestion that uncleaved N-propeptides at the fibril surface limit further accretion of collagen molecules on to growing fibrils [65]. A quantitative model of diameter limitation that predicts the reported quantification of diameters has been proposed by Chapman [60,60a]. The model is based on the transient retention of N-propeptides. In this model the N-propeptides are constrained to the surface of the growing fibril, leading to an initial limiting diameter, when N-propeptides become close-packed, of about 22 nm (based on a lateral separation of between 1.8 and 2.0 nm between N-propeptide units). Subsequent cleavage of surface-located N-propeptides and accretion of a further round of pNcollagen molecules leads to a second limiting diameter of about 33 nm. Repetition of this cycle of deposition and cleavage yields fibrils with preferred diameters of multiples of 11 nm. Variants of this type of diameter-limitation process could involve other surface-bound macromolecules. An alternative mechanism of diameter limitation involving surface-associated small proteoglycans has been proposed [66–72]. Here the glycosaminoglycan chains are extended around the circumference in complexes and act as a molecular ruler to define the lateral extent of the fibril.

Evidence from immunoelectron microscopy and biochemical studies suggests that collagen fibrils *in vivo* are composed of several collagen types, partially processed collagens and other macromolecules, including proteoglycans. For example, it has been suggested that dermal collagen fibrils are hybrids of type I and type III collagens and that the type III collagen is located at the periphery of the fibril. The surface location of type III collagen implies that this collagen may have a different role than type I collagen and may have a regulatory function in fibrillogenesis [73]. Double immunoelectron microscopy of foetal skin using an antibody directed against the N-propeptide of type III procollagen and another directed against the N-propeptide of type I procollagen revealed labelling of type I and III pNcollagens on the same thin (20–30 nm) fibrils. Larger fibrils (90–100 nm) were coated with type III collagen and type III pNcollagen but not with type I pNcollagen. The type I N-propeptide was present on thin fibrils only at restricted locations in adult skin. Immunoblotting of skin extracts revealed the presence of both type III pNcollagen and type I pNcollagen in foetal skin, but only type III pNcollagen in adult skin. These data were interpreted to suggest that type I and type III collagens form hybrid fibrils and that type III pNcollagen is added to mature fibrils [74]. The data did not, however, exclude the possibility that type III pNcollagen can be deposited on preformed type I collagen fibrils after the fibrils are assembled.

Experiments *in vitro* in which mixtures of type III pNcollagen and type I collagen were generated simultaneously by enzymic cleavage of precursor forms of the proteins suggested that type III pNcollagen forms true co-polymers with type I collagen [75]. In addition, the results demonstrated that co-polymerization of type III pNcollagen with type I collagen generated fibrils that were thinner than fibrils generated under the same conditions from type I collagen alone. Of particular interest is the finding that co-polymers of type III pNcollagen and type I collagen are apparently circular in outline and are not like the hieroglyphic fibrils formed from mixtures of type I pNcollagen and type I collagen. A further important difference was that the co-polymerization increased the concentration in solution at equilibrium of type I pNcollagen, whereas co-polymerization with type I collagen decreases the concentration in solution of type III pNcollagen [76]. These differences imply important biological roles for types I and III pNcollagen.

As a further example of the occurrence of hybrid fibrils, type V collagen has been immunolocalized to the type-I-collagen-containing fibrils and as fine-diameter fibrils in the corneal stroma [77,78]. Corneal stroma has a higher concentration of type V collagen than do other type-I-collagen-containing tissues with large-diameter fibrils. This has led to the suggestion that co-polymerization of type V and type I collagens may limit the diameter of the fibrils formed. It is noteworthy that type V collagen contains a small pepsin-sensitive N-terminal domain, presumably part or whole of the N-propeptide of the molecule. When type I collagen was mixed with type V collagen, the mean fibril diameter decreased with increasing type V/I collagen ratio [78]. Moreover, the N-terminal domain of type V collagen was needed for this regulatory effect, and without the domain little diameter-regulating ability was observed.

Type IX, XII, XIV and XIX collagens are members of a subfamily of fibril-associated collagens with interrupted triple helices (FACITs) which are found at the surfaces of, and may participate in the formation of, collagen fibrils. Type IX collagen associates with the surfaces of the narrow-diameter fibrils in cartilage, vitreous and developing cornea, where it is covalently bound to chains of type II and XI collagens [79]. It has been proposed, based on immunolocalization using monospecific antibodies, that type XI collagen forms a small cylindrical core around which molecules of type II collagen are arranged. Type IX collagen appears to be located exclusively at the fibril surface, where it may act as a molecular linker between collagen fibrils and macromolecules in the extracellular matrix. Type XII and XIV collagens have very large N-terminal domains. These molecules have been implicated in modulating the deformability of the extracellular matrix, as they are localized near the surface of banded collagen fibrils and they can mediate interactions between fibrils *in vitro* [80,81].

FIBRILS IN INVERTEBRATES

Collagen fibrils have been major constituents of the connective tissues of animals since the emergence of multicellularity. Because invertebrates comprise approximately 95 % of animal species, it might be expected that their collagens would form a great variety of fibrillar aggregates. Indeed, a number of different aggregate forms of fibrillar collagens have been described, and the subject has been reviewed [82–85]. However, the aggregation of triple-helical collagen molecules into unbranched, quasi-crystalline fibrils has been the dominant mode of fibril organization since the sponges first evolved.

As in vertebrates, the fibrillar aggregates in sponges are stabilized by covalent cross-links, but these cross-links are of unknown chemistry [86]. In other animals tyrosine-, quinone-, aldimine-, ketoimine- and cysteine-derived cross-links exist [87]. An obvious extrapolation of these observations is that the pathways for the secretion and extracellular assembly of collagen molecules into fibrils and the processing enzymes required for converting the insoluble aggregates into mechanically and chemically stable structures have, from the beginning, been essential components of collagenous matrices.

From their first appearance in sponges, collagen fibrils have been associated with other macromolecules, and have been organized into fibre-reinforced composite tissues consisting of stiff and strong collagen fibrils in an isotropic matrix dominated by proteoglycans and water. As is true of all fibrous composites, the mechanical properties of collagenous matrices are determined by the physical–chemical properties, concentrations, spatial

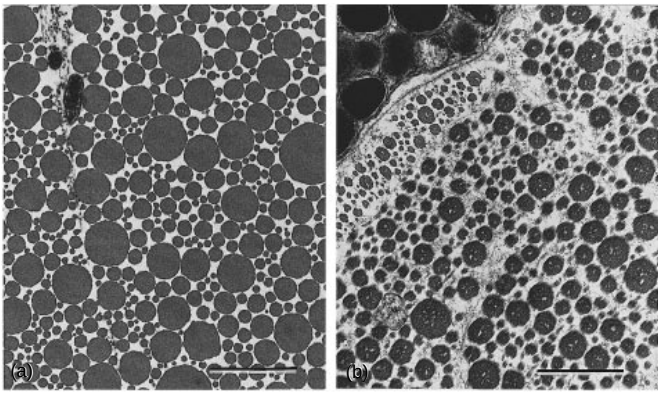

Figure 8 Transmission electron micrographs of echinoderm collagenous tissues

(**a**) Sea urchin ligament. Fibrils have approximately circular cross-sections and uniform electron density. Diameters are highly variable. (**b**) Sea cucumber dermis. Fibrils are somewhat irregular in outline and possess internal electron-lucent regions. Scale marker = 0.5 μm. Reproduced from [92], with permission.

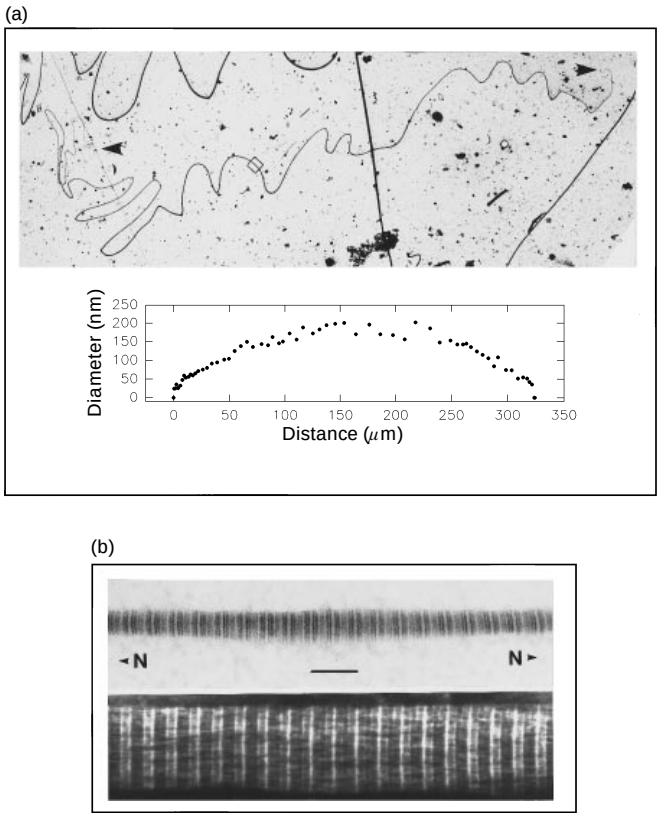

Figure 9 Isolated collagen fibrils from sea urchin ligament and sea cucumber dermis

(**a**) Electron micrograph of an entire positively stained sea urchin fibril. The ends of the 170-μm-long fibril are marked by arrowheads. The central region of the fibril is boxed. The scatter plot shows the relationship between diameter and length along a similarly prepared sea cucumber fibril. (**b**) The central regions of a positively stained sea cucumber fibril (top) and a negatively stained sea urchin fibril (bottom) are shown. At the midpoint of these fibrils (indicated by the bar), as in all others examined, the molecular polarity undergoes a reversal (compare with fibril in Figure 4). Reproduced from [51], with permission.

arrangements and interactions of the separate constituents. The phylum *Echinodermata* has evolved collagenous tissues with mutable mechanical properties that make them interesting from the perspective of their unique physiology, as well as providing a unique opportunity to study collagen fibril structure and interfibrillar interactions. The connective tissues of these animals are mechanically regulated by the secretions of resident neurosecretory cells, and change rapidly from stiff and strong to weak and compliant states [88,89]. These mechanical changes are thought to be due to changes in the strength of associations between adjacent fibrils.

The organization of collagen fibrils in the spine ligaments of sea urchins resembles that in vertebrate ligaments; the relaxed spine ligaments even have a crimp pattern. The fibrils are allowed to slide when the ligament is plastic, and are strongly interconnected when it is elastic (stiff) [90,91]. Electron micrographs of *Eucidaris tribuloides* ligaments show round fibrils with a uniform interior (Figure 8). Their diameter distribution is unimodal; the range is 20–750 nm, with a modal value of 100 nm [92]. The diameter distribution is not unlike the distributions seen in vertebrate tissues composed of type I collagen fibrils, such as tendon and ligament [93] and, in fact, the isolated collagen resembles type I collagen [94]. The fibrils in the dermis of the sea cucumber *Cucumaria frondosa* are basically circular but with irregular perimeters and with numerous electron-lucent interiors [92], which is true of some other echinoderm fibrils as well [93,95,96]. They also have a wide range of diameters in tissue sections, up to a maximum of about 400 nm [92]. The isolated collagen is an α1 trimer, with no compositional or solubility similarities to vertebrate type I collagen, and with no immunological similarity either to vertebrate type I collagen or to *E. tribuloides* collagen [97].

The collagenous tissues of several different echinoderms have been disaggregated into suspensions of separate intact fibrils [51,92,97–99]. The fibrils have tapered ends (Figure 9). Those isolated from the spine ligaments of *E. tribuloides* and the dermis of *C. frondosa* have lengths of 30 μm–1.2 mm and 39–436 μm respectively [92,99]. Surprisingly, the aspect ratio (length/diameter) is constant in the fibrils from each species, regardless of length: about 2000 in *C. frondosa* fibrils and about 2500 in *E. tribuloides* fibrils, where the diameter was measured at the midpoint of the fibrils. The constant aspect ratio means that growing fibrils must increase their diameter in proportion to their increase in length, with the result that fibrils of all lengths have the same shape. This observation is all the more interesting when it is considered in the light of the fact that the fibrils are not cylindrical, but are rather symmetrically spindle-shaped, which may be related to their role as the reinforcing fibres in a discontinuous composite material [51,92,99].

Free fibrils are isolated from *C. frondosa* dermis in solutions of both high and low ionic strength [100]. Similarly, biomechanical tests on *C. frondosa* dermis have shown that its stiffness is many times lower at both low and high ionic strengths than it is at intermediate values (J. A. Trotter, J. P. Salgado and T. J. Koob, unpublished work). The isolated fibrils form aggregates *in vitro* when the ionic strength is in the physiological range. These experiments indicate that the fibrils interact with one another *in vivo* and *in vitro* largely by electrostatic interactions.

The fibrils of echinoderms have sulphated glycosaminoglycan moieties associated with their surfaces. The presence and locations of these moieties have been identified using Cupromeronic or Cuprolinic Blue, or Ruthenium Red [97,99,101–103]. In sea urchin and sea cucumber fibrils the precipitates are associated with the middle of the gap zone, where charged amino

Table 1 Summary of the growth characteristics of early collagen fibrils

Abbreviation: AMD, axial mass distribution. See the text for further details.

Fibril assembly characteristics	Developing vertebrate tendon (*in vivo* 1)	Acid-soluble type I collagen (*in vitro* 1)	Type I pCcollagen plus C-proteinase (*in vitro* 2)	Echinoderm ligament and dermis (*in vivo* 2)
Linear AMDs of fibril tips	+	+	+	+
Tip shape set at early stage	?	+	+	−
Limiting diameter at early stage	+	+	−	−
Unipolar fibrils	+	+	−	−
Bipolar fibrils (N–N)	+	−	+	+

acids are concentrated in bands corresponding to the **d**/**e** bands of vertebrate fibrils [97,99]. In crinoid fibrils there are apparently two glycosaminoglycan sites per *D*-period, but the banding pattern has not yet been well defined for these fibrils [104].

The proteoglycans on the surfaces of echinoderm fibrils could be involved in the control of fibril shape and size, as is postulated to occur in vertebrate type I collagen fibrils. They could also be involved in the electrostatic interactions that determine the stiffness of echinoderm tissues, although definitive evidence for this role is lacking. It has recently been shown that the salt-dependent aggregation of *C. frondosa* fibrils depends on the presence of a protein that is bound to the isolated fibrils [100]. Fibrils that lack this protein do not aggregate in univalent salt solutions. The protein has been purified and partially characterized. Full characterization, including the definition of its binding sites on fibrils and the mechanism by which it induces fibril aggregation, should prove useful. This protein is the first to have been isolated that plays a crucial role in creating the fibril–fibril interactions that convert an array of individual fibrils into a mechanically functional tissue.

When compared with vertebrate fibrils, echinoderm collagen fibrils have greatly reduced intensities in their **a3** and **b2** bands, and greatly increased intensity in the **c3** band [92]. It is not known whether these changes in charge density are related to fibril functions, for example the binding of the aggregating protein mentioned above. The similarities between vertebrate and echinoderm collagen molecules, including their segment long spacing and fibril-positive and -negative stain patterns, allowed the banding pattern of echinoderm fibrils to be related to the orientation of molecules in the fibril [92]. This information was used to analyse the molecular orientation in the symmetrically spindle-shaped echinoderm fibrils [51]. Ultrastructural analyses of many fibrils from both sea urchins and sea cucumbers showed that they are all composed of molecules that have their N-terminal ends oriented towards the nearest fibril end. It follows that the centre of each fibril contains a region in which the polarity is reversed. In fibrils from both species this region contains between one and three *D*-periods in which the banding pattern is symmetrical, and is identical to the DPS-III [50] banding pattern that occurs when vertebrate collagens are induced to form fibrils under certain conditions, such as elevated phosphate concentrations (as discussed above). This pattern results from the alignment of equal numbers of anti-parallel molecules in an arrangement that brings the C-telopeptides of anti-parallel molecules into register, where they can both participate in trivalent cross-links with the same helical hydroxylysine residue. In contrast, the N-telopeptides are not in register, and thus could not participate in cross-links between anti-parallel molecules. On either side of the symmetrical *D*-periods are transitional zones of about three *D*-periods wide, in which the DPS-III pattern changes into the normal one. These fibrils from adult echinoderms thus resemble those isolated from embryonic chick tendons [13,54].

Although it is clear that equal numbers of molecules of each polarity are present in the DPS-III regions, it is not clear whether the total number of molecules in that region is different from the number in adjacent regions. This question is being approached using mass determinations, but no definitive answers yet exist. The answers are required in order to produce a realistic model of the molecular packing in that region.

As was noted above, all the fibrils from sea urchins and sea cucumbers are similarly spindle-shaped, regardless of their lengths. Preliminary results have indicated that the fibrils have linear axial mass distributions, and are therefore similar to the ends of fibrils isolated from foetal chicken tendons and the fibrils formed *in vitro* from vertebrate type I pCcollagen using C-proteinase to control assembly. This means that the fibrils have an essentially paraboloidal shape, with a plane of mirror symmetry through their centres. The observation that all echinoderm fibrils, regardless of size, are symmetrical about a plane through the central anti-parallel *D*-period suggests that the assembly of echinoderm bipolars is different from that of vertebrate bipolars. A specific model to account for this growth process has yet to be developed.

CONCLUSIONS

The evolution of multicellular organisms depended on the formation of an extracellular matrix. Collagen fibrils, which can be several millimetres in length, have played a central role in extracellular matrices for at least 500 million years. Current evidence suggests that collagen fibril formation is intrinsically a self-assembly process but that considerable cellular control is exerted on the process *in vivo*. In this review we have surveyed four different systems of fibril formation, two *in vivo* and two *in vitro* (Table 1). The summary shows that fibrils, whether they occur *in vivo* or *in vitro*, all have growing tips with linear axial mass distributions. In the two systems *in vitro* the tip shape is established at an early time in the assembly pathway and remains unchanged during growth of the fibrils. Our understanding of the corresponding behaviour *in vivo* is incomplete and must await further quantitative studies.

A new observation is that early fibrils in developing tissues can be either unipolar or bipolar. This finding has major implications for our understanding of fibril fusion in young tissues and in healing wounds. Models based on existing experimental data suggest that fusion of a bipolar fibril to a bipolar fibril is unlikely to occur and that the presence of such fibrils may impose a limit on fibril fusion. The exclusivity of short bipolar fibrils in a cell-free system in which pCcollagen is cleaved with procollagen C-

proteinase suggests that the concentration of pCcollagen during early fibril assembly may contribute to the formation of bipolar fibrils. Conversely, long and exclusively unipolar fibrils form when pCcollagen is absent during early fibril formation of acid-soluble collagen *in vitro*.

Their occurrence not only in vertebrates but also in invertebrates suggests that bipolar fibrils could be widespread throughout the animal kingdom. The observation that invertebrate bipolar fibrils are centro-symmetrical (with the molecular switch region at the midpoint of the fibril), whereas those in chick tendon are shape-asymmetrical, implies that the mechanism of assembly of bipolar fibrils could be different in vertebrates and invertebrates. Nevertheless, the occurrence of bipolar fibrils in organisms as diverse as echinoderms and chickens points to a common, but as yet unknown, physiological function of bipolar fibrils.

FUTURE PROSPECTS

A major direction of collagen fibril research will be to understand, at the molecular level, the principles governing the self-assembly of unipolar and bipolar fibrils, to understand how cells regulate this process, to learn how the deposition of early collagen fibrils is orchestrated in embryonic tissues, to understand the processes by which early fibrils are converted into larger fibrils in newly forming tissues, and to understand the role of other collagens and macromolecules in these processes. A number of short-term goals have been set, and these include the understanding of the occurrence of bipolar fibrils in tissues and in healing wounds, and the identification of cell-mediated factors in determining collagen fibril size and shape. The longer-term objective must be to place collagen fibril assembly in a broader context involving gene regulation, cytokine induction and cell-matrix signalling events that underpin tissue patterning and the generation of the body form. A recent observation has been that the gene for procollagen C-proteinase [105] is the same as that for bone morphogenic protein-1, which is related to the patterning gene *tolloid*. Thus the identification of a proteinase which is involved in collagen fibril formation, in determining the shape of collagen fibrils *in vitro*, and which also plays a major role in pattern formation in the early embryo, begins to provide new insights into the relationship between extracellular matrix deposition and vertebrate development.

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