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Collagen Fibrillogenesis in Tissues, in Solution and from Modeling: A Synthesis

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Collagen fibril formation has been studied in tissues by light and electron microscopy; in solution by light scattering and microscopy; and from modeling based on the amino acid sequence of type I collagen. Taken together these studies indicate that collagen fibril assembly involves a stepwise formation of intermediate aggregates in which each intermediate is formed from earlier aggregates. In this sequence, monomeric collagen contributes only to the formation of early aggregates; and fibrils grow in length by the addition of intermediate aggregates to the end of a subfibril and in width by lateral wrapping of subfibrils. Modeling based on amino acid sequence data of possible intermolecular charge-charge interactions indicate 2 different kinds, one which promotes linear aggregation and the other which promotes lateral aggregation. The effects of different collagens and coprecipitants such as glycoproteins and proteoglycans can begin to be explained by their influence on the character of intermediate subassemblies. Ultrastructural data from 2 tissues, embryonic cornea and tendon, indicate that the site of fibril growth and assembly is at the cell surface.

Collagen fibrils are the "warp and woof" of vertebrate tissues and their formation determines morphological form in development, growth, and repair. Our understanding of this important process derives from studies of intact tissues by morphological techniques; solution studies of purified collagens; and modeling based on primary structural data. In what follows we have attempted to briefly summarize recent reports in each of these areas and to synthesize the data into a common working hypothesis of collagen fibrillogenesis.

TISSUE STUDIES

Intracellular Packaging, Vectorial Discharge and Cell Surface Assembly

The intracellular compartmentalization and secretory pathway of collagen proceeds from synthesis in the endoplasmic reticulum to the Golgi apparatus where products are condensed in vacuoles which then are moved to the cell surface for discharge by exocytosis [1–3]. In some collagen producing cells the stiff-rope-like collagenous products come together in the vacuoles formed in the Golgi apparatus, like pencils in a box, into lateral aggregates which resemble SLS or segment long spacing crystallites [4–7]. Vacuoles are often found with 2 sets of such aggregates in which the ends of one set overlap the other [6,7]. Studies of cell culture media, in which SLS aggregates are released, as well as studies on intact tissues suggest these intracellular assembly forms are, in some manner, involved in fibril assembly [6,7]. These aggregates are the first indication of a collagen-collagen interaction of importance to fibrillogenesis and indicate that an early step in the fibril assembly sequence occurs inside the cell (Fig 1). These interactions which begin within the cell are limited by factors such as the size constraints of the vacuoles and by precursor forms, such as the procollagens, which require enzymatic processing.

In most cases, matrix cells synthesize more than one matrix component and the question of where and when these various components first mix is not known. In the embryonic chick cornea, for example, the epithelial cells produce several types of collagens and several types of proteoglycans [8–10]. Indirect evidence suggests that these different components are packaged together in the same secretory vacuoles [11]. The manner in which the cell regulates the stoichiometry of mixing during this compartmentalization will have important effects on fibrillogenesis in that the almost limitless diversity of tissue structure is established by a relatively small number of macromolecules and the behavior of intermediate aggregates comprised of mixed components will obviously be determined by the amounts and kinds of materials in such aggregates. By regulating the compartmentalization process, cells could also produce relatively similar biosynthetic products, but effect different morphologies by the manner in which these products were segregated or mixed within the cellular secretory pathways and then processed and assembled at the cell periphery.

Excellent examples of the differing morphologies produced with different mixtures of matrix components are the transparent cornea and the opaque sclera. In both tissues, type I collagen is the predominant matrix component, but the character of the proteoglycans (PGs) is different. Small diameter fibrils with constant interfibrillar spacing in the cornea are associated with a high keratan sulfate concentration with the remaining glycosaminoglycans (GAG) being chondroitin sulfate while the large range of fibril diameters and significantly less orderly fibrillar organization in the sclera is associated with an absence of keratan sulfate and the presence of chondroitin sulfate, dermatan sulfate and hyaluronic acid [12]. Intermediate morphologies and compositions are seen in the corneal-scleral junction. As one might expect, inhibitors of GAG synthesis applied to chick embryos have been shown to disrupt the development of the organized corneal matrix [13].

A second attribute of matrix cells which influences matrix morphogenesis is cellular polarity. Nearly all cells are structurally anisotropic as demonstrated by the intracellular distribution of organelles such as the Golgi apparatus and centrioles. Patterns of cellular polarity are readily recognized in epithelial tissues where most cells are present with their organelles in register and common orientation. The patterns of polarity in groups of mesenchyme cells are less readily recognized but can be detected by relatively simple histological means [14]. The position of an organelle within the cell determines the spatial consequences of that organelle's activities. For example, the position of secretory organelles can determine the vector of secretory discharge. The vector of discharge will determine the site(s) of initial entry of the secretory product into the extracellular space and can, if polymerization occurs in proximity to the site of discharge, partially determine the three dimensional geometry of the matrix.

The vectorial discharge of collagen from the embryonic chick corneal epithelium in particular illustrates this influence of cellular polarity. Prior to matrix secretion by the corneal epithelium, the Golgi apparatuses in all of the cells are present in

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Abbreviations:
GAG: glycosaminoglycans
PG: proteoglycans

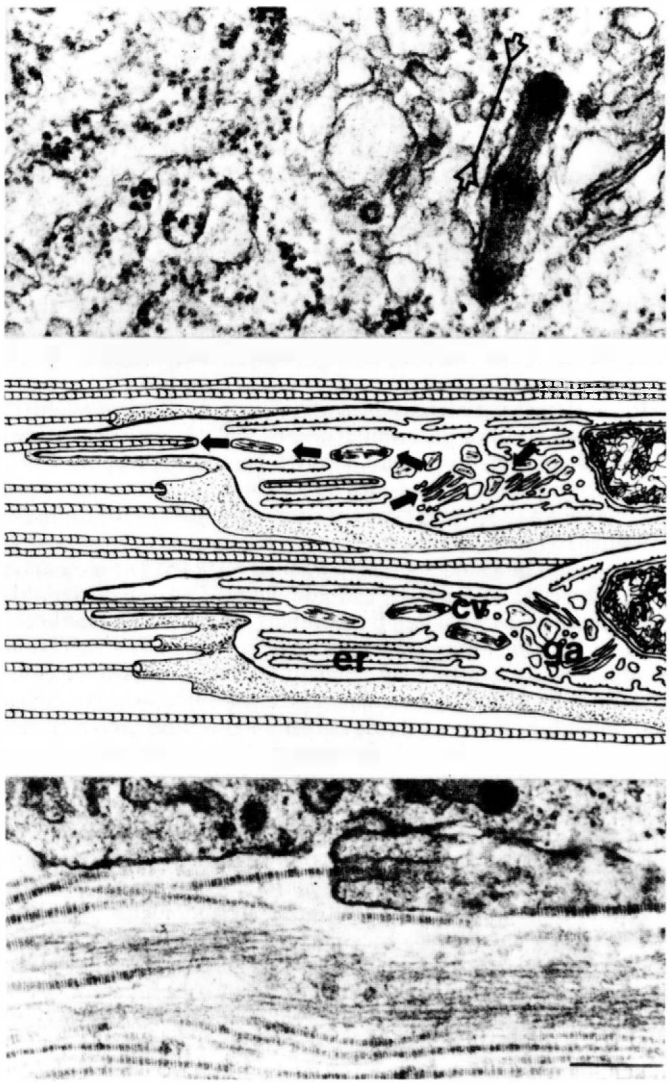

FIG 1. The top electron micrograph illustrates a collagen condensation vacuole in an embryonic chick tendon cell. These vacuoles contain a number of procollagen molecules, which are packed side by side. The *bar between the arrows* indicates the length of a fully processed collagen molecule (300 nm). At least 2 sets of molecules in some kind of end overlap configuration are apparent within the illustrated vacuole. The diagram illustrates the secretory route of collagen from fibroblasts in the chick embryo tendon. Following synthesis in the endoplasmic reticuluum (*er*), the procollagen is transported to the Golgi apparatus (*ga*) where the procollagen is packaged and where aggregation begins to occur. These aggregates are then moved to the cell surface in condensation vacuoles (*cv*) where they are discharged into the extracellular space. The site of discharge and initial fibril assembly is into deep recesses in the cell surface. This configuration allows the cell to control the growing end of the fibril and to add collagen aggregates in response to growth requirements. The bottom electron micrograph illustrates a cell recess from which a newly formed collagen fibril extends. (From Trelstad and Hayashi, 1979.) (Upper micrograph reduced from × 65,000; mark = 300 nm. lower micrograph reduced from × 56,000; mark = 300 nm.)

a supranuclear position [15]. Upon assumption of matrix secretory activities the Golgi apparatuses and centrioles in the epithelial cells move to an infranuclear position and remain there until the deposition of the collagens and proteoglycans is completed. By reordering their intracellular organelles, the cells establish a restricted zone across their basal surface from which they discharge their matrix products and thus give their secretory activity an anisotropic or polarized character. The polarized alignment of the organelles thus dictates, in part, the organization of the matrix formed.

Selective vectorial discharge of products from matrix cells is probably an integral step in regulating the manner in which much of the extracellular matrix is deposited. It is not likely that matrix constituents are discharged randomly into the extracellular space around the cell perimeter where they then engage in self assembly.

Following discharge from the cell the matrix components continue assembling to form the extended multimeric structures which constitute the formed matrix. In order for the collagens to engage in these interactions there are several enzymatic modifications which must occur first. For type I collagen, the removal of the amino terminal extension occurs first and probably inside the cell whereas the subsequent removal of the carboxy terminal extension occurs at the time of discharge from the cell [16]. In addition to this proteolytic processing, alterations in the conformation of the collagen, particularly the nonhelical ends, similar to those suggested from analysis of sequence data [17] might occur upon entry into the extracellular space because of the associated changes in pH, ionic strength and ionic species which will also take place during this process.

The site of matrix polymerization in the extracellular space in several well studied cases is close to or in association with the surface of the cell from which the materials are discharged. Ultrastructural studies of both the corneal epithelium and tendon fibroblast indicate that fibril assembly occurs in association with specialized features of the external surface of the cell membrane (Fig 1). In the cornea, this specialization is the basement membrane [8]; in the fibroblast this specialization consists of deep recesses in the cell surface [7]. In the cornea and tendon we know that matrix constituents do not diffuse any significant distance from the cell before they engage in later stages of matrix assembly, but rather that the assembly process occurs immediately at the surface of the cell [4,7]. Given this surface site of assembly, the vectorial discharge discussed earlier can be seen to influence matrix structure in that the site of matrix discharge is essentially the site of matrix assembly.

It is worth noting that the extracellular space in general is not a homogeneous compartment, but is comprised of multiple and variable domains. For example, the deep recesses in the fibroblasts in which fibril assembly apparently occurs are exterior to the cell membrane, but the content and properties of this space are most likely different from that space which surrounds fully mature collagen fibrils some microns distant from the cell. In the recesses in the fibroblast surface it is possible, for example, that enzymatic processing of the procollagen and the oxidative deaminations involved in cross-linking occur. These important steps might therefore occur outside the cell, but in a region in which continued regulation and influence by cellular activities is possible.

The ultrastructural features of fibril assembly in tendon and cornea support a model of multistep assembly of collagen into the fibril which involves intracellular compartmentalization of secretory components; vectorial discharge from the cells; and assembly at or near the cell surface. This dynamic process can only be inferred from the interpretation of static electron micrographs. In the following section some recent considerations of in vitro assembly will be discussed as they pertain to the multistep fibril assembly process.

SOLUTION STUDIES

Intermediate Forms and Heteropolymeric Interactions

Purified type I collagen in physiological solutions will spontaneously aggregate into fibrils when heated [18]. This *in vitro* assembly has been followed by monitoring changes in the light scattering of solutions and characteristically demonstrates three phases: lag, growth, and plateau. From ultrastructural and laser light scattering studies we now interpret these phases as follows [19,20]. Beginning with a solution of monomeric, fully processed type I collagen in which the nonhelical telopeptides are intact, the first aggregate to form in the gelation reaction is a dimer in

which the 2 molecules are staggered by 4D (4D dimer). This dimer grows in length by the addition of another monomer, also with a 4D stagger, to form a 4D trimer. Multiple 4D trimers then begin to associate laterally, in a D staggered manner, to form wider intermediate forms (Fig 2). Variable angle light scattering studies suggest that approximately five 4D trimers form a discrete intermediate and that such intermediates then add to each other, both linerally and perhaps laterally, to form a longer subfibrillar structure [21]. Further growth of the subfibrils has not been followed using light scattering methods, but ultrastructural studies of similar preparations have indicated that the lateral growth of the fibril occurs by the wrapping together of the subfibrils [19].

These studies have now provided a relatively simple model for assembly *in vitro* and explanations for the several phases of the turbidity curves of heat gelation. When collagen monomers interact to form 4D dimers and trimers, essentially no tubidity is generated by these long, narrow aggregates because light scattered from one end destructively interfers with light scattered from the other. Accordingly these intermediates have been "invisible" in ordinary turbidity studies and account for the lack of turbidity change during the lag phase of aggregation [22]. The growth phase of the *in vitro* process involves the lateral aggregation of the dimers and trimers into tactoidal structures with D period packing of at least 15 molecules. The addition of these intermediates to each other and to the ends of their adducts is responsible for the rapid increase in turbidity during the growth phase.

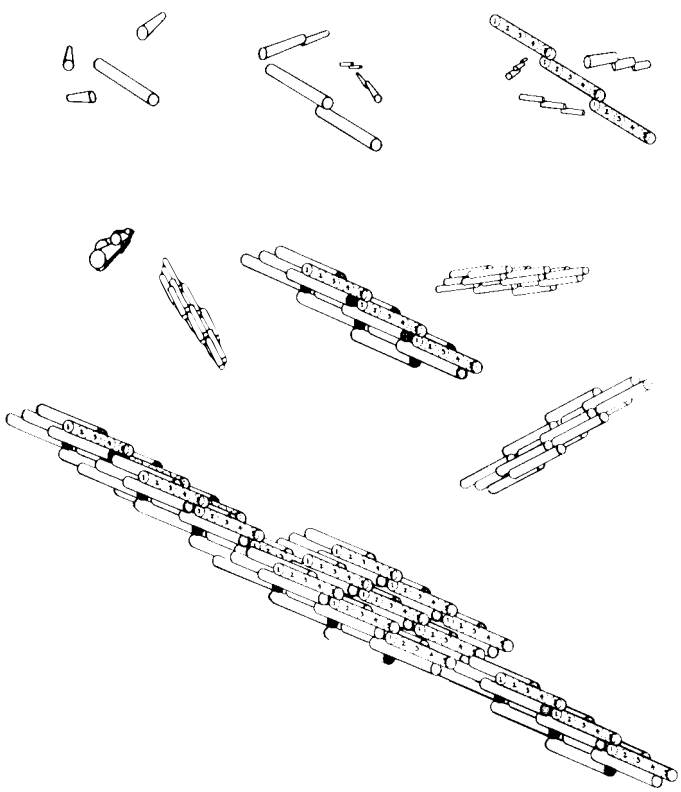

FIG 2. Proposed stages of Type I collagen fibrillogenesis derived from studies using light scattering and electron microscopy to identify intermediate forms. The individual monomers initially interact to form 4D staggered dimers and trimers (*top*). In the 4D trimer at the upper right and elsewhere, the numbers refer to D length segments along the 4.4D long molecular axis. Number *1* is at the amino terminus; *2* is at the interface of the first D segment with the second and so on. A *4*D staggered configuration is the same as an overlap of 0.*4*D. Following formation of the *4*D dimers and trimers, the next step is to laterally associate to form an aggregate consisting of approximately 5 trimers or 15 molecules. If the trimers overlap in a *1*D pattern, a relatively compact structure is formed (middle right) which can add to other like structures in a linear and/or lateral fashion to build a longer subfibril as illustrated by the larger central structure. (From Trelstad and Silver, 1981.)

The model of fibrillogenesis that derives from these *in vitro* studies does not involve the addition of monomeric collagen to a "nucleus," but rather requires that the monomer become incorporated into an intermediate subassembly before further fibril formation can occur. In our interpretation of this process, the monomer is not involved in the later stages of growth of the fibril. The implications of this model in respect to the observations based on tissue studies is that fibril assembly in vitro and in vivo proceeds by the formation of intermediate forms which add to each other in subsequent steps. Furthermore, since intermediates are the building blocks, their composition and acquired properties during the assembly sequence will dictate the nature of the subsequent steps. Accordingly the mixing of reactants such as collagens, glycoproteins, and proteoglycans, which apparently occurs within cells in secretory vacuoles, will create intermediates with unique properties and presumably unique intermediate and final morphologies.

Intermixing different collagen types or collagens with noncollagenous materials *in vitro* has significant effects on the rate of assembly and the morphology of the final product [23–25]. For example, corneal type I collagen forms fibrils significantly slower than scleral type I collagen [25]. Although the turbidimetric lag phase for the corneal collagen is approximately 6 to 7 times longer than that for the scleral collagen, the rate of fibril growth, as measured by the slope of the growth phase curve at half completion is similar for the collagens from both sources. We think that the difference in the lag phase is due to the increased glycosylation of corneal type I collagen in that other chemical parameters such as the state of the teleopeptides and the extent of covalent crosslinking are similar. The corneal type I collagen used in these studies was derived from the rabbit and contained more hydroxylysine and approximately four times as much bound hexose per molecule as the scleral collagen.

The influence of proteoglycans on *in vitro* fibrillogenesis has been studied using type I collagens from cornea and sclera and the PGs from corneal stroma and sclera [25]. Corneal and scleral type I collagen fibrillogenesis in the presence of corneal or scleral PGs are, in general, retarded in rate. However, the magnitude of the effect is different for corneal or scleral PGs. Scleral and corneal PGs lengthen the lag phase 2 times and 5 to 7 times respectively for both corneal and scleral collagen. Both PG preparations also retard fibril growth for corneal collagen, but only corneal PGs retard fibril growth for scleral collagen. These observations illustrate that specific molecular properties of the matrix components, as expected, are important in the regulation of collagen fibrillogenesis and tissue specific microarchitecture, but the exact mechanism(s) by which these molecular properties operate, for example, in respect to specific charge-charge or hydrophobic interactions remain to be defined.

MODELING

Charge-Charge and Hydrophobic Interactions

A number of studies have shown that the early stages of fibril formation *in vitro* are dependent on collagen concentration, pH, temperature, ionic strength and detergent concentration. The pH and ionic strength dependencies suggest that charge-charge interactions play a role in early stages of assembly. Recent modeling studies have proposed that linear aggregation, which occurs during the lag phase, is driven by interactions between a set of negative charges (− −) in the amino terminus and a set of positive charges (+ +) in the C terminus [17,26]. These studies indicate that this type of interaction is primarily responsible for the formation of 4D staggered dimers and trimers. Lateral growth is driven by interactions between sets of oppositely charged residues which form anti-parallel dipoles and are particularly stable at staggers of 1D. These interactions

stabilize the 5 membered 4D trimeric units as well as interactions between such units within the fibril. In both linear and lateral aggregation steps, with the side chains in an extended conformation, interactions between attractive charge pairs are both electrostatic and hydrophobic. Hydrophobic interactions occur by alignment of hydrocarbon elements of side chains forming a hydrophobic or "waterless" pocket between the molecules [26].

Thermodynamic measurements indicate that the process of fibril formation has a large entropy component which is due to the disruption of a hydration layer around the collagen monomers [27]. This layer is probably most intimately associated with the attractive charged pairs and must be removed before the antiparallel interactions can occur. Melting of this hydration shell is thus necessary for the lateral aggregation of the 4D dimers and trimers and the growth phase of fibril formation to occur. Other studies suggest that the large temperature dependence of early steps in fibril assembly is due to a thermally induced conformational change which may be associated with imino acid poor regions located 0.4D from each end of the molecule. This conformational change might also be influenced, as noted earlier, by the changes in the micro environment of the collagen as it is discharged into the extracellular space.

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