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# THE DEVELOPMENTAL BIOLOGY OF VERTEBRATE COLLAGENS[1, 2]

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Collagen is present in the early embryo at stages of development prior to the appearance of differentiated connective tissue cells (15, 22, 33, 34). Our understanding of the function of this embryonic collagen is prejudiced, perhaps, by its more passive mechanical role in the adult. However, recent work has suggested more active roles for embryonic collagen in both morphogenesis and tissue interactions. For example collagen's capacity to self-assemble into higher ordered aggregates *in vitro* is well known (26) and it has been suggested that this process is also operative *in vivo* (63, 67) and of importance in tissue morphogenesis. In addition to this morphogenetic function, attention has focused on the possible role of extracellular macromolecules such as collagen and acid mucopolysaccharides in embryonic tissue interactions or inductions, a developmental event in which one tissue influences the subsequent differentiation of another (15, 25, 35). The following discussion considers both of these active functions of collagen, as a morphogenetic template and as a mediator of tissue interaction, in light of recent developments which indicate that vertebrate collagens are a heterogeneous population of molecules representing at least five different gene products.

## COLLAGEN HETEROGENEITY—PRIMARY STRUCTURAL DIFFERENCES

At least four different molecular species of collagen have been described in vertebrate tissues (32, 41, 43). The basic structure common to all of these collagens is a rodlike molecule approximately 3000 Å long and 15 Å in diameter composed of three polypeptide chains of molecular weight 100,000 called $\alpha$ chains, which are wrapped in a right handed super-helix (52). Other characteristic features of the $\alpha$ chains are the unusual amino acids, hydroxyproline and hydroxylysine, and the presence of glycine in every third residue (21, 52). The differences among the various collagens stems from differences in the primary structure and distribution of the constituent $\alpha$ chains. The characterization of these different collagens has been based on the following criteria: amino acid analysis of the native triple helical molecule as well as that of the isolated $\alpha$ chains (32, 40, 65); amino acid composition of peptides from these molecules derived by cyanogen bromide cleavage (41, 43); chromatographic behavior on carboxymethyl cellulose and diethylaminoethyl cellulose of both the denatured $\alpha$ chains and of the formaldehyde-cross-linked $\alpha$ chains (43, 65, 66); and the ultrastructural appearance of the segment long spacing (SLS) crystallites as well as the fibrils formed by the native molecules (65).

The most commonly studied molecule to date, that from the skin of various vertebrate species, consists of two identical $\alpha$ chains, which have been called $\alpha$1 type I or $\alpha$1 (I) chains (12, 14, 30). The third $\alpha$ chain in the skin molecule is different in its primary structure from $\alpha$1 (I) and is called $\alpha$2; the short hand for this molecular form is therefore $[\alpha 1\ (\mathrm{I})]_2\alpha 2$.[4]

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[4] The nomenclature stems from the elution position of these collagen $\alpha$ chains from columns of carboxymethyl cellulose, a commonly used cation exchange resin (50). The first molecule to be extensively studied from skin revealed an early eluting peak twice the size of a later eluting peak. The $\alpha$ chains in the first peak were thereby termed $\alpha$1 and those in the second, $\alpha$2. When subsequent studies of cartilage revealed an $\alpha$ chain which eluted from carboxymethyl cellulose like $\alpha$1 from skin, but which had a different primary structure, the term $\alpha$1 type II or $\alpha$1 (II) was introduced to differentiate it from the $\alpha$1 chain from skin which was renamed $\alpha$1 type I or $\alpha$1 (I) (43).

This molecular species is found also in bone, tendons and ligaments (42, 50). A second molecular species of collagen is found in cartilages from a variety of different tissues including the articular surface of the joint, the epiphyseal growth plate and the xiphoid process of the sternum (40, 41, 43, 58, 60, 65). This molecule consists of three identical $\alpha 1$ chains which because of their difference in primary structure from the $\alpha 1$ type I chain of skin and bone has been termed $\alpha 1$ type II. The shorthand for this molecular species is $[\alpha 1\ (II)]_3$. A third molecular species of collagen has been found in human fetal skin for which the precise molecular composition has yet to be fully defined. This collagen contains an $\alpha$ chain of the $\alpha 1$ type which has been termed $\alpha 1$ type III again because of its different amino acid composition from $\alpha 1$ (I) and $\alpha 1$ (II) (41). The fourth molecular species of collagen has been isolated from the basement membranes of various structures including the renal glomerulus and the lens capsule (32). This molecule also is composed of three identical chains which have not been formally numbered, but which by convention would be termed $\alpha 1$ type IV. The shorthand for this molecule would therefore be $[\alpha 1\ (IV)]_3$.

Recent studies of the collagens isolated from the lens and vitreous bodies of lathyritic chick eyes have suggested the possibility of yet another species of collagen composed only of $\alpha 1$ chains (31), but further investigation of this material is necessary. Finally codfish skin contains a collagen molecule in which each of the three $\alpha$ chains appears to be unique (49). The extent of collagen heterogeneity stemming from the unique primary structure of the component $\alpha$ chains has yet to be completely established.

The distribution of the five distinct $\alpha$ chains, $\alpha 1$ (I), $\alpha 1$ (II), $\alpha 1$ (III), $\alpha 1$ (IV) and $\alpha 2$, in the triple helical collagen molecule in mammals is apparently quite restricted. Only one molecule, the $[\alpha 1\ (I)]_2\alpha 2$ prominent in skin and bone, contains a mixture of two different chains. Both the cartilage molecule, $[\alpha 1\ (II)]_3$, and basement membrane molecule, $[\alpha 1\ (IV)]_3$, contain three identical chains. The precise chain composition of the molecule in fetal skin which contains $\alpha 1$ (III) has not been determined. Molecules with other theoretically possible mixtures of chains such as $[\alpha 1\ (I)\ \alpha 1\ (II)\ \alpha 1\ (III)]$, etc. have not yet been found but may exist.

### COLLAGEN HETEROGENEITY—POSTTRANSLATIONAL MODIFICATIONS

In addition to the collagen heterogeneity based on differences in primary structure there is also heterogeneity stemming from postsynthetic enzymatic modification. At least six different enzyme-mediated modifications of the collagen $\alpha$ chain occur after peptide bond formation: hydroxylation of proline (21, 54); hydroxylation of lysine (21, 44); galactosylation of hydroxylysine (21, 56); glucosylation of galactosyl-hydroxylysine (21, 56); oxidative deamination of lysine or hydroxylysine (21, 55); and proteolytic cleavage of extra N-terminal peptides (7, 21). This postsynthetic or microheterogeneity may represent the "fine tuning" of the collagen molecule for its ultimate biologic function. For example collagens of common primary structure such as those from skin and bone have variable degrees of hydroxylation of proline and lysine residues (6, 11, 42, 60, 66). Bone collagen $\alpha$ chains are more highly hydroxylated in younger bones than in those in the adult (42). In diet-induced rickets bone collagen is also exceptionally hydroxylated (60, 66). What property this increased lysyl hydroxylation might confer on bone collagen is presently under investigation.

The possible importance of such posttranslational modifications on the morphogenetic function of collagen is underscored by a recently described disease in two young sisters who have low levels of lysyl hydroxylase activity in their skin fibroblasts and skin collagen which is deficient in hydroxylysine (51). These children have significant skeletal anomalies, lax joints and unusually extensible skin. The precise mechanism by which these anatomical abnormalities are related to the hydroxylysine-deficient collagen is not yet known but this biologic accident emphasizes the potential importance of collagen microheterogeneity in normal morphogenesis.

The most recently described postsynthetic modification of collagen is the removal of extra $NH_2$-terminal peptides from the newly synthesized molecule which has been called procollagen (3, 17, 21, 36). Several functions have been suggested for the extra $NH_2$-terminal peptides on the procollagen including alignment of the three polypeptide chains during synthesis, transport through the tissues as a soluble form and control of fibrillogenesis. The $NH_2$-terminal extension is removed by a neutral protease, procollagen peptidase (7), and the importance of this proteolytic scission is demonstrated, again by a biologic error in an unusual disease found in cattle called dermatosparaxis (37). These cattle have poor tissue strength and form improperly packed fibrils. They die early in life usually from minor traumatic injuries which fail to heal and become septic. Interestingly these animals apparently do not have significant congenital anomalies. The relationship of the conversion of procollagen to collagen to developmental problems remains to be examined, but it potentially may involve a widely different number of collagen properties.

### EMBRYONIC COLLAGEN AND TISSUE INTERACTIONS

Embryonic tissue interactions operationally can be defined as the influence of one tissue on the ultimate differentiation of an adjacent tissue. This phenomenon has been examined in extensive detail in a variety of different epithelial-mesenchymal interactions in which one tissue induces another to differentiate. The specificity of this reaction resides either with the epithelium or the mesenchyme depending on the source of the tissues (1, 23–25, 53, 57, 68). The exact nature of this inductive interaction remains a major unsettled question in developmental biology. One hypothesis is that these interactions do not involve the direct transfer of information between the inducing and the reacting tissues but rather represent a permissive interaction in which one tissue merely stimulates another tissue, but does not instruct it. The nature of the molecules which might be involved in such permissive interactions are not known but it has been recognized for some time that connective tissue macromolecules including acid mucopolysaccharides and collagen are prominent in the intercellular space between interacting tissues (4, 5, 15, 25). The dependence of embryonic tissues on a collagen substratum for their development *in vitro* has also been described for both epithelial and mesenchymal cell types (18, 35), and taken together these observations have raised the hypothesis that connective tissue macromolecules may be mediators of tissue interactions (15, 25, 59, 64).

How these materials in the extracellular space might effect such changes as the biosynthesis in an adjacent cell is not known. However, an interesting and possibly relevant observation concerning the collagen platelet interaction may be of importance for understanding the role of collagen in embryonic inductions. It has long been known that platelets stick to collagen. A possible mechanism for this platelet collagen interaction according to recent work is that the platelet contains an enzymes(s) on its surface which can recognize either the hydroxylysine residue of collagen or its glycosylated derivatives (2, 8, 13). These data suggest that the collagen platelet interaction is dependent upon an enzyme substrate interaction. The interaction of the platelet with collagen causes dramatic changes in the subsequent biologic behavior of the platelet including release of adenosine diphosphate and viscous metamorphosis. Of potential relevance to developmental interactions is the fact that the biologic behavior of the platelet is altered by its encounter with an extracellular macromolecule.

Whether collagen and other stromal macromolecules might cause changes in the biologic behavior of embryonic cells by a mechanism analogous to the collagen-platelet interaction remains an open question. Recent circumstantial evidence, however, continues to support this hypothesis: hyaluronic acid influences chondroblast behavior *in vitro* (59); addition of exogenous chondromucoprotein to chondrocyte cultures stimulates production of more chondromucoprotein (47); and isolated spinal cords produce collagen during the period in development when they induce adjacent cells to become chondrocytes (15, 64). These and other examples (20, 46, 48) point to an active role of the extracellular matric in tissue interactions, but the specific biochemical steps in such interactions remain unknown.

The cells responsible for the collagen and acid mucopolysaccharide synthesis and excretion in tissue interactions can reside in either the epithelium or the mesenchyme. Collagen synthesis has been convincingly shown to occur in several different embryonic epithelia as well as in widely different types of mesenchyme (4, 15, 19, 25, 64). Possibly some of the specificity demonstrated in certain epithelial-mesenchymal interactions may reside not only in the nature of the extracellular matrix produced but also in the cell of origin of particular components.

### TISSUE DIFFERENTIATION AND COLLAGEN HETEROGENEITY

Tissue differentiation involves the elaboration of a specific and unique set of macromolecules by a group of cells. In many of the connective tissues the major protein synthesized is collagen, and thus differentiation means synthesis of a particular collagen species. For example during development of vertebrate long bones a cartilaginous analage or template is formed first which will define the shape of the bone. This cartilaginous structure is then gradually replaced by bone over the ensuing weeks and months of development and subsequent postnatal growth. The transition from a cartilaginous to an osseous skeleton requires a change from the cartilage species of collagen, $[\alpha 1\ (II)]_3$, to the bone species, $[\alpha 1\ (I)]_2\alpha 2$. Control mechanisms which are of fundamental importance to development must be operative in this change as it involves not only the new synthesis of one type collagen molecule but also the degradation of another. The timing, location and extent of such transitions from one molecular species of collagen to another may represent particularly significant developmental events.

Collagen turnover and degradation in embryogenesis presumably involves the neutral protease specific for collagen or collagenase. Coordinated collagenolytic activity would be particularly important during transitions when one species of collagen is being removed and another deposited such as occurs in long bone growth and remodeling (27, 28). Little is known about the control of collagenase, but recent work indicates that it involves multiple steps each of which might represent a site of regulation (29).

Another intriguing control mechanism for which at present there is no evidence is that changes may occur in the chain composition of a tissue collagen with development analogous to that which occurs in the shift from fetal to adult hemoglobin. The $\alpha 1$ (III) found in fetal skin has not been isolated in its triple helical native collagen molecule. Conceivably the $\alpha$ chain is present in a molecule, $[\alpha 1\ (III)]_2\alpha 2$, which shifts to the adult form, $[\alpha 1\ (I)]_2\alpha 2$, with maturation. Although this is raw speculation, the recent uncovering of collagen heterogeneity warrants raising such questions.

### MACROMOLECULAR SELF-ASSEMBLY AND MORPHOGENESIS

Self Assembly is a Thought
That's Inherently Complete
You Merely Have to State it Once
It'll Automatically Repeat . . . .
Repeat . . . .
Repeat . . . .

The capacity for the native collagen molecule to assemble into higher ordered aggregates *in vitro* has been well described (26). The exact final morphology of these aggregates as visualized in the electron microscope is dependent upon both the conditions as well as the molecular species of collagen employed. Under the appropriate conditions the molecule can be induced to precipitate in a near quarter staggered manner which is identical with that seen in the native cross-striated fibril in tissues, having a repeat banding period of 640 Å. If an acid solution of collagen is dialyzed against a low concentration of salt-free adenosine triphosphate the molecules will precipitate with their carboxy- and amino-terminal ends in register to form an aggregate which constitutes the molecules aligned in lateral register. This form has been called the SLS crystallite form. If $\alpha 1$ glycoprotein from the serum is used as a precipitant the molecules will precipitate in a configuration in which they alternate head to tail, head to tail, which has been termed fibrous long spacing. Other unusual forms have been described including a fibril with a banding pattern of 210 Å, and elaborate sheets of aligned molecules or even sets of molecules in an orthogonal array (26). All of these higher ordered patterns of the molecular aggregates appear to be inherent within the primary structure of the molecule and the conditions under which precipitation occurs dictates the manner in which the charged groups along the axis of the molecule interact to form the particular end product. In short collagen is an excellent example of a morphogenetic unit from which higher ordered structures can form by the self-assembly of the constituent molecules, perhaps in some cases in several discrete stages.

The nature of the precipitate is dictated not only by the conditions of precipitation but also by the nature of the molecular species of collagen employed. The SLS crystallites of the cartilage collagen molecule, $[\alpha 1\ (II)]_3$, have a uniquely different staining pattern from that of the SLS from skin collagen, $[\alpha 1\ (II)]_2\alpha 2$ (65). Recently we have found that the cartilage collagen will also precipitate in an unusual fibril which has an oblique staining pattern (10). The staggered or oblique pattern of the fibril appears to derive from an approximate 90-Å offset of adjacent sets of subfibrils. The width of each set of subfibrils is approximately 130 Å. These observations suggest at least two different packing arrangements; first the molecules pack laterally with adjacent molecules to form a finite subfibrillar bundle with a diameter of 130 Å, and these bundles then pack with adjacent microfibrillar bundles with a 90-Å offset to form the staggered fibril. Although precise analysis of this new stacking pattern is not yet complete, it underscores the principle that higher orders of molecular organization can derive spontaneously from the molecule itself or from aggregates of molecules. Presumably all of these examples of packing order are inherent in the primary structure of the collagen molecule.

If cartilage collagen molecules can organize into bundles which then pack in respect to each other with a staggered repeat, is it not likely that yet higher orders of organization can derive from such aggregates? For example, do fibrils in the tissue interact with one another such that they influence each other's packing pattern? At such higher levels of organization such as the fibril it seems likely that molecular species other than collagen may also be involved since normally tissue fibrils are separated by a significant distance over which more direct interactions would be unlikely. One possibility for such a noncollagenous macromolecule is the acid mucopolysaccharides, a common constituent of connective tissues, which have been shown to interact with collagen and influence its precipitability *in vitro* (39, 61). Is it possible then that self-assembly at macromolecular levels may play an important role in normal morphogenetic phenomena?

### CORNEAL MORPHOGENESIS

From recent studies of the morphogenesis of the chick cornea we have suggested that macromolecular self-assembly is partly responsible for the three-dimensional architecture of the connective tissue stroma of this tissue (63). The collagen in the adult chick cornea is organized into 50–60 layers, each approximately 4–5 $\mu$ thick. The collagen fibrils in each layer lie parallel to the corneal surface and all course in the same direction. The fibrils in adjacent layers lie approximately at right angles to each other. In addition to this orthogonal arrangement of adjacent layers, the matrix undergoes a gradual clockwise shift from the outer to the inner layers of over 200°. The direction of this shift is clockwise in both eyes and is thus asymmetric about the midbody axis of the animal. This is an unusual anatomical configuration since all other known structures in the eye are bilaterally symmetrical. The morphogenesis of this matrix in the embryo has been studied ultrastructurally and biochemically and has been shown to derive from a collagenous matrix deposited very early in development by the corneal epithelium (19, 62, 63). As each wave of collagen in excreted by the epithelial cells the collagen polymerizes into 640-Å banded collagen fibrils within 2000 Å of the cell surface. This matrix precipitates in a cell-free zone so that mesenchymal cells contribute neither to the collagenous content nor to its anatomical configuration.

The lateral spacing between fibrils within an orthogonal set interestingly is equal to the macroperiod of the collagen fibril, that is 640 Å. This similarity of distance between fibrils and the macroperiod of the collagen may suggest that the exact spacing of one set of collagen fibrils is somehow influenced by the collagenous matrix itself, that is that it represents macromolecular self-assembly. Additional indirect evidence for matrix self-assembly is that the angular shift of the orthogonal collagenous matrix from the outer to inner layers is clockwise in both eyes. The inherent asymmetries of the collagen and protein polysaccharides in the matrix (9, 38, 52) may actually be causing this asymmetry of the orthogonal pattern in the corneal stroma. Thus the same handedness of the two corneas might be established because of the same handedness of the constituent molecules. The exact manner by which fibrils forming in one direction influence the spacing pattern of the fibrils forming in adjacent layers in unknown. In an effort to investigate this process we are currently examining fibrils forming *in vitro* on flat surfaces. Gross has published several examples of such precipitates one of which shows an orthogonal pattern (26).

The formation of the orthogonal matrix occurs at the interface of the epithelium with the developing corneal stroma. The flat basal surface of the epithelial cells and the organization of the basement membrane are also likely to be important in the morphogenesis of the orthogonal matrix. In fact, remarkable changes in the corneal epithelial cell shape during deposition of the first portions of the matrix strongly suggest a major cellular influence on the early matrix orientation and pattern (63). Experiments with Azetidine, an analogue of proline which temporarily interfers with matrix production when given to the whole embryo, have shown that normal morphogenesis can resume at the epithelial surface despite large gaps in the matrix (16). Thus if the matrix is getting spatial cues from itself this process must be occurring within the region defined by the basal cell surface and the basement membrane.

The collagen molecules synthesized by the corneal epithelial cells are probably packaged within the cell in vacuoles which range from 6,000 to 10,000 Å in length and 1000–1500 Å in diameter (62). These packages of collagen are then transported to the basal cell surface and excreted from the cell by exocytosis. The collagen molecules which enter the extracellular space are immediately enmeshed in the epithelial basement membrane and must traverse it and begin assemblying into fibrils within 2000 Å of the basal cell surface. The influence of the basement membrane on the pattern of the matrix is not yet understood. In developing fish skin it has been suggested that the subepithelial orthogonal pattern of collagen may be directed by the epithelial basement membrane (45). Staining of the chick corneal basement membrane with ruthenium red and lanthanum indicates that a pattern is present within this structure. The manner in which the pattern in the basement membrane influences the pattern of deposition of the subepithelial collagen fibrils is under investigation.

The molecular type(s) of collagen produced by the corneal epithelium is probably also important in the genesis of the orthogonal pattern. Histologically there are two distinct types of corneal epithelial collagen: that in the amorphous basement membrane and that in the 640-Å cross-striated fibrils. Biochemically the epithelium also appears to be producing two species which are currently being characterized. The collagen produced by the corneal fibroblasts is the $[\alpha 1\ (I)]_2\alpha 2$.

## CONCLUSION

Since tissue form and function develop together in a coordinated manner in the embryo, it seems worthwhile to consider whether some of the factors which influence morphogenesis might also simultaneously influence cell differentiation. Such factors could serve to orchestrate the multiple contemporaneous events which occur in the embryo and would thus be an important focus of biologic control. Collagen clearly plays an important role in morphogenesis and, as discussed above, probably also in embryonic tissue interactions. In like manner acid mucopolysaccharides have also been implicated in these embryonic phenomena. Our current understanding of the way in which these extracellular materials operate in these developmental events is fragmentary. Future investigation in particular to the role of collagen must now take into account the variety of different molecular species which exist representing both different gene products and different postsynthetic modifications. This diversity of collagen species is well suited for the multiplicity of roles it may play in embryogenesis.

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