The morphogenesis of the chick primary corneal stroma

I. New observations on collagen organization in vivo help explain stromal deposition and growth

JONATHAN B. L. BARD and MOHINDER K. BANSAL
MRC Clinical & Population Cytogenetics Unit, Western General Hospital, Edinburgh EH4 2XU, UK

Summary

The primary stroma of the avian cornea contains collagen fibrils in orthogonal array. While investigating the processes underlying its morphogenesis, we have found that stromal organization is not as expected in three important respects. First, the fibrils are not uniform: those near the epithelium (newly laid down) have a maximum diameter of about 20 nm (mean: 17.7 nm), while those near the endothelium (laid down for approx. 40 h) have diameters up to 40 nm (mean: 22.8 nm). Fibrils thus grow rapidly to 20 nm and then continue to enlarge slowly, presumably by diffusion of collagen molecules from the epithelium. Second, the collagen, although orthogonally organized, does not contain layers of parallel fibrils. Instead, SEM observation shows that only a few fibrils lie in a parallel array before this short-range order is broken by orthogonal fibrils in the same plane. Furthermore, fibrils in corneas that had been freeze dried but not critical-point dried for SEM were widely spaced and the intervening gaps were filled by an extensive matrix that was probably composed of the proteoglycans known to be in the stroma. Third, we have shown experimentally that the stromal undulations seen in sections are not present in vivo but are shrinkage artifacts: the less corneas were shrunk for SEM preparation, the less pronounced were the stromal undulations. We also noted that, even after the distortions required for the stroma to undulate, the constituent fibrils remained orthogonally organized.

These results give insight into the mechanisms underlying stromal morphogenesis and growth. The observations on the growth of collagen fibrils and on collagen organization show that stromal deposition is a more stochastic process than previously thought and, hence, provides support for the view that a complex self-assembly mechanism underlies both fibrillogenesis and the generation of orthogonal organization. The experiments on, and the analysis of, stromal folding show that fibrils slide over one another as undulations form, with the extensive matrix of hydrated proteoglycans being the likely lubricant. This fluidity of the stromal components probably explains how growth can occur without the structure being distorted.

Key words: collagen, cornea, fibrillogenesis, glycosaminoglycans, growth, morphogenesis, self-assembly, chick.

Introduction

Although collagen and its associated proteoglycans provide the extracellular matrix of the embryo and are essential for normal development, little is known of the mechanisms responsible for their morphogenesis. The primary stroma of the chick cornea is probably the best system for studying how connective tissue is laid down: first, the structure is well-defined, comprising orthogonally organized collagen fibrils of relatively uniform diameter (approx. 20 nm) whose axes are parallel to the corneal surface; second, the geometry is simple: as the orthogonal collagen is laid down by anterior epithelium of the cornea rather than by mesenchymal cells, the matrix is completely separate from the cells that synthesize it; and, third, the cornea is, for an embryonic tissue, large, simple and accessible. It is not therefore surprising that the cornea has attracted a considerable amount of interest and many of the morphological and biochemical aspects of its development are now well known (Hart, 1978; Hay, 1980).
The main feature of stromal morphogenesis not yet understood is the mechanism responsible for the orthogonal arrangement of the collagen fibrils. In this context, the detailed structure of the stroma is important because it defines the problem that the mechanism has to solve. On the basis of TEM micrographs, it is generally held that the collagen of the primary stroma is laid down in orthogonally ordered layers and that, within each layer, the collagen is organized in 'bundles which are generally 1–3 fibrils thick and 5–25 fibrils wide. The layers of the primary stroma consist of these belt-shaped bundles of fibrils which are laterally associated at similar levels within the stroma' (Trelstad & Coulombre, 1971). If this description is correct, then stromal morphogenesis is difficult to comprehend because it requires that the corneal epithelium coordinates its synthesizing ability so that layers of similarly oriented fibrils will extend across the whole cornea. Such a mechanism is probably incompatible with the suggestion of Trelstad & Coulombre (1971) that self-assembly plays an important role in generating orthogonality. More recently, and perhaps to circumvent this problem, Trelstad & Silver (1981) have suggested that, because corneal fibrillogenesis takes place in the immediate vicinity of the basement membrane of the epithelia, the membrane itself may help generate orthogonality. This latter suggestion is not, however, parsimonious because, in mammalian corneas, the orthogonally organized fibrils are only laid down by the fibroblasts (Haustein, 1983) and an epithelial basal lamina is thus not necessary for the generation of orthogonality. There is a further problem associated with the well-defined and precise organization that the primary stroma is supposed to have: it is hard to see how it can grow without causing its layered structure to distort.

In order to resolve these contradictions, we have re-examined the organization of the collagen in the primary stroma of the stage-26 (5-day) cornea, a stage chosen because it is just before the stroma is colonized by fibroblasts (Bard & Hay, 1975) and, hence, when the primary stroma is at its largest. We report here observations and experiments at three levels of collagen organization: fibril diameters, fibril orthogonality and the morphology of the whole stroma. The results are not in accordance with expectation and hence provide new constraints on the mechanisms responsible for stromal morphogenesis.

**Materials and methods**

**Embryos**
Fertile eggs of randomly mated, S-line White Leghorn chicks were obtained from the Poultry Research Centre, Roslin, and incubated in a 38°C humidified incubator.

Embryos were staged as required by age or according to the criteria of Hamburger & Hamilton (1957).

**Dissection techniques**
Corneas with a small amount of adjacent tissue were dissected out from embryos under a Wild M40 stereomicroscope. To remove the epithelia, corneas were incubated for 20 min at 20°C in dispase (0.25 mg ml⁻¹, Calbiochem, UK) made up in Dulbecco’s A saline and then washed three times for 2–3 min in saline. After this, the epithelia could be removed from the stroma with sharpened forceps. If the epithelia were to be removed from intact eyes, a ring was scratched around the periphery of the cornea with a sharpened tungsten needle and the eyes were incubated in dispase. The corneal epithelium was then removed with needles and fine forceps.

**Microscopy**
For transmission electron microscopy (TEM), corneas were routinely fixed in 2.5% cacodylate-buffered glutaraldehyde and postfixed in osmium tetroxide. Specimens were dehydrated and routinely flat-embedded in Araldite (Bard & Ross, 1982). Blocks were cut and split perpendicular to the choroid fissure. Thick and thin sections were cut on an LKB Ultratome V and viewed on a Phillips 300 TEM.

For measurements of fibril diameters, specimens (a minimum of three for any one stage) were photographed at a plate magnification of ×50 000 and contact prints made. Lines were then drawn on these positives and a Brinel lens, which has a built-in scale, was used to measure fibril diameters at the point where they intersected the lines.

Specimens for scanning electron microscopy (SEM) were critical-point dried (CPD) or freeze dried (FD), either unmouted or, if a support was required, on 5 mm discs of filter paper. Stromas to be CPD were routinely fixed, dehydrated in graded acetones and CPD from liquid carbon dioxide in a critical-point drier (Balzer, UK). They were then mounted on aluminium stubs with 'twistick' Sello-tape, coated with gold–palladium in a sputterer (Polaron, UK) and examined in a Cambridge S180 SEM. Specimens to be FD were routinely fixed and frozen in liquid nitrogen slush. After freeze drying overnight under high vacuum (10⁻⁴ Pa) in an Edward’s (UK) vacuum coater (for details, see Bard, McBride & Ross, 1983), they were treated routinely.

**Results**

**Stromal morphology in vivo**
The morphological events that take place as the primary stroma of the chick cornea forms are well known (see Fig. 1 for morphological details and Hay, 1980, for review) and relatively simple: following lens formation, the anterior epithelium starts to lay down the collagen of the primary stroma (approx. stage 22) and the first signs of orthogonal organization are apparent soon afterwards (Fig. 2). Neural-crest cells from the periphery of the cornea then migrate between the stroma and the subjacent lens to form the
endothelium (stage 24). The epithelium continues to lay down collagen on the existing stroma so that the further the collagen is from the epithelium, the older it is. On the sixth day (stage 27), the primary stroma swells and is colonized by more neural crest cells which differentiate into fibroblasts and lay down the secondary stroma on the scaffold of the primary stroma.

**Fibril diameters in the primary stroma**

The mechanisms governing fibrillogenesis in the primary stroma are not understood, but collagen synthesized by the epithelium is assumed to aggregate in the subepithelial space to form fibrils with approx. 20 nm diameter. A histogram of fibril diameters in the stage-24 eye (Fig. 5) supports this view as fibril diameters are sharply peaked (s.d. 3-0 nm) about a mean of 20-5 nm. In these eyes, the stroma is thin (approx. 5 μm) and the population from which the diameters are measured extends from the anterior to the posterior of the stroma. At first sight, therefore, the fibril diameter range is in accord with expectation.

Examination of the stage-26 stroma, however, shows that the situation is more complex than expected. Because its thickness is approx. 15 μm, it is possible to distinguish a newly formed population of fibrils near the epithelium (Fig. 3) and an older population (approx. 48 h) near the endothelium (Fig. 4). The former is orthogonally organized while the latter is less organized and more spaced. In addition, measurement shows that these populations differ in fibril diameter. Newly formed fibrils within 2-3 μm of the epithelium (Fig. 6) have a mean diameter of 17-7 nm (s.d. 2-6 nm); furthermore, a much smaller proportion of these fibrils has a diameter greater than 20 nm (8%) as compared with that in the stage-24 stroma as a whole (42%). The population of older fibrils, those within 5 μm of the endothelium (Fig. 7), contrasts sharply with the anterior one: the mean is higher (22-8 nm) and the spread (s.d. 5-5 nm) is much broader (57% having diameters greater than 20 nm) with the largest fibrils being wider than 40 nm. These results show that, while most collagen rapidly forms fibrils of 20 nm subjacent to the epithelium, some appears to move through the stroma to enlarge existing fibrils. The difference in the diameter means of the epithelial and endothelial fibril populations suggests that this growth is about 0-1 nm h\(^{-1}\) (approx. 5 nm in approx. 48 h).

**Collagen organization within the stroma**

On the basis of TEM micrographs, it is generally held that the collagen of the primary stroma is laid down in extensive layers which, it must be said, are not easy to see as only short-range order is usually apparent (e.g. Fig. 2). Because it is hard in the TEM to analyse fibril organization in planes parallel to the epithelium, we have used the SEM to examine large areas of newly laid-down collagen. To do this, we have removed from stage-26 corneas the epithelium and its associated basement membrane and then either CPD or FD the corneal stromas.

SEM micrographs of the anterior surface of the CPD primary stroma of 5-day (stage-26) corneas do not support the view that collagen fibrils are laid down in layers (Figs 8, 9). Most of the fibrils are indeed aligned approximately perpendicularly and orthogonal to the axis of the choroid fissure, but there is no evidence to suggest that the fibrils in a single plane are either grouped in bundles loosely associated with one another or are all oriented in the same direction. Were such layers present, one would expect neighbouring fibrils both in and directly below...
the anterior surface to show the same orientation over a considerable distance. In fact, the anterior surface of the stroma contains fibrils in both orientations and fibrils immediately below the anterior ones may also lie in both directions. Examination of many such specimens showed that, typically, a belt of about 10 fibrils (Fig. 9) could be considered as aligned before the organization was interrupted by orthogonal fibrils in the same plane. The structure seen in the SEM is thus less well organized than that deduced from TEM micrographs.

Such SEM micrographs of CPD corneas give an incomplete picture of the stroma as they show only the collagen and not the proteoglycan matrix which is known to be present in the primary stroma (for review, see Hay, 1980). This is because dehydration, a necessary step in the CPD process, causes highly hydrated molecules to condense (Bard et al. 1983). Freeze drying, in contrast, retains some proteoglycan morphology and also causes considerably less shrinkage than does critical-point drying (see later). FD specimens of primary stroma show a structure that is very different from that seen in CPD corneas: they contain an amorphous matrix in which the fibrils are embedded (Fig. 10). At higher magnification (Fig. 11), it can be seen that the collagen is still arranged orthogonally, but the fibrils are more loosely packed than they were in the CPD specimens with there being interfibrillar gaps of up to several hundred nanometres.

One particular FD specimen provided direct evidence that the stroma does not contain layers in
which all the fibrils are aligned in the same orientation. In this specimen, the needle removed the epithelium but penetrated into the stroma and this disturbance caused the stroma to form thin flakes, probably because the needle caused the stroma to shear along transverse planes where forces between superior and subjacent fibrils would be relatively weak (Fig. 12). Close examination showed that the

![Fig. 3](image1.png)  
**Fig. 3.** A TEM micrograph of freshly laid-down collagen in a stage-26 stroma immediately subjacent to the epithelium. The collagen is orthogonally organized and the fibrils have diameters of about 20 nm. ×65,000; bar, 0.2 μm.

![Fig. 4](image2.png)  
**Fig. 4.** A TEM micrograph of the same cornea as that in Fig. 3, but of fibrils near the endothelium. These fibrils are not well organized, but are rather wider than those near the epithelium. ×65,000; bar, 0.2 μm.

![Fig. 5](image3.png)  
**Fig. 5.** A histogram of fibril diameters from three stage-24 corneal stromas. The distribution is approximately normal with a mean (arrow) of 20.5 nm (n = 100).

![Fig. 6](image4.png)  
**Fig. 6.** A histogram of fibril diameters from five stage-26 corneas. All the fibrils (n = 468) were within 3 μm of the epithelium. In this population, the mean diameter was 17.7 nm and very few fibrils were wider than 20 nm.

![Fig. 7](image5.png)  
**Fig. 7.** A histogram of fibril diameters from five stage-26 corneas. In this case, all the fibrils (n = 422) were within 5 μm of the endothelium. In this population, the mean diameter was 22.8 nm and 57% of the fibrils were wider than 20 nm.
fibrils both within such a flake and immediately subjacent to it were orthogonally arranged (Fig. 13). Again, there was only local order and no evidence to suggest that all fibrils in a single plane were aligned. This result shows that, if there are layers in the stroma, they do not contain fibrils with the same orientation.

**Stromal undulations**

There are folds in sections of young corneal stromas even though the epithelium and endothelium do not fold (Figs 1, 2) and it is not yet known whether these folds are a feature of normal morphogenesis or an artifact of fixation (Trelstad & Coulombre, 1971; Bard, Hay & Meller, 1975). If the former is so, then a mechanism for their morphogenesis is needed; if the latter, then the fibrils can move with respect to one another and the cornea is more fluid than usually thought. It is therefore important to resolve this question and we test here the hypothesis (Bard et al. 1975) that these undulations are shrinkage folds caused by removing the cornea from the hydrostatic

**Fig. 8.** An SEM micrograph of the anterior collagen fibrils of a critical-point-dried stage-26 stroma. The fibrils are in the main orthogonally organized and it is possible to see bundles of similarly-ordered fibrils, but it is hard to see any evidence of layering. ×9,300; bar, 1 μm.

**Fig. 9.** A higher-power SEM micrograph of the CPD anterior stroma; the spaces between the fibrils seem empty. Again, the fibrils appear randomly oriented along one or the other of the preferred axes. The mean centre–centre spacing of the fibrils is about 120 nm. ×33,000; bar, 0.3 μm.

**Fig. 10.** An SEM micrograph of the anterior stroma after freeze drying. The fibrils are still orthogonally organized, but the fibrils are embedded in an amorphous matrix. ×3,700; bar, 3 μm.

**Fig. 11.** A higher-power SEM micrograph of the FD anterior stroma: many of the superficial fibrils appear proud of the matrix but several disappear into it (arrows). In such FD stromas, the fibrils are well separated (compare with Fig. 8 which is printed at the same magnification) and there are interfibrillar spaces of 300 or more nanometres. ×9,300; bar, 1 μm.
pressure exerted by the vitreous (if the retina is cut, the eye rapidly collapses). To do this, we have prepared corneas for SEM examination in ways that cause differing amounts of shrinkage.

For 'normal' shrinkage, corneas had their epithelia removed and were fixed, dehydrated and critical-point dried. Such specimens shrunk markedly (approx. 35%) and had deep and extensive folds (Figs 14, 15; see legend for details). For intermediate shrinkage, corneas were similarly treated, but were freeze dried rather than CPD. These corneas only shrank by about 15% and had relatively small folds on their surface (Figs 16, 17). For minimal shrinkage, the dissected corneas were mounted on pieces of filter paper to provide an adherent support and touch dried on single-ply Kleenex before fixation and freeze drying. These corneas barely shrank at all (<5%) and, while there was some tearing of the stroma (Figs 18, 19), there was almost no folding. An identical result was obtained with intact eyes whose epithelia had been removed. These observations show that the less was the shrinkage in the stroma the less pronounced were the folds. The almost complete lack of distortion in corneas that were both FD and supported implies that there were no folds in the original eye.

Discussion

Our observations on the primary stroma demonstrate that older collagen fibrils are wider than newer ones, that collagen is more randomly organized than expected and that the stromal undulations are a shrinkage artifact. These results help explain the mechanisms underlying fibrillogenesis, fibril organization and growth in the cornea, and we consider each aspect in turn.

Fibrillogenesis in the primary stroma

The presence of 40 nm fibrils in the distal part of the primary corneal stroma is quite unexpected as such wide fibrils have only been reported in damaged corneas (Jakus, 1964) and in fibrils of soluble corneal collagen aggregating in vitro (Bard & Freeman, cited in Bard, 1969). The mechanism by which these distal fibrils enlarge is not at first sight clear: it could either be through the redistribution of existing fibrillar collagen or through the diffusion of new collagen from the epithelium. The facts that, even in stage-24 corneas, there are fibrils wider than the newly formed fibrils in the stage-26 cornea and that the fibril-diameter distribution in the secondary stroma is sharp argue that fibrils, once formed, are stable and that a proportion does not break down and adds to other existing fibrils. It therefore seems that, although most collagen synthesized by the epithelium immediately forms new fibrils in the immediate vicinity of the epithelium, a minority does not. Instead, some molecules diffuse distally and either slowly add to existing fibrils or form new ones (it can be seen (Fig. 7) that some of the fibrils near the stage-26 endothelium are narrow).

It is at first sight hard to see how collagen molecules could diffuse through the densely packed fibrils of the primary stroma. However, the SEM observations on freeze-dried stroma (e.g. Fig. 11) demonstrate that the fibrils are much further apart than usually...
expected. In FD samples that shrink by about 15%, there are interfibrillar spaces of up to 300 nm or more which are filled by a matrix which is probably composed of highly hydrated proteoglycans (see next section). If the long, thin molecules of collagen can diffuse through the bound water associated with such molecules, they will be able to move through the stroma and so enlarge distal fibrils.

**Organization in the primary stroma**

Our observations on stromal organization show that the collagen fibrils are not layered and that the
stomal matrix is unexpectedly extensive. While the nature of this matrix is not apparent from the micrographs, it is probably proteoglycan. First, its disappearance after alcohol dehydration and critical-point drying suggests that it has the high water content associated with proteoglycans. Second, a similar matrix, seen on cultured mesenchymal cells that were FD but not CPD, is the hydrated glycosaminoglycan, hyaluronic acid (Bard et al. 1983). The stage-26 stroma lacks hyaluronic acid (Toole & Trelstad, 1971), but contains chondroitin and heparan sulphates and possibly other proteoglycans (Hart, 1978; Hay, 1980) that have been identified in TEM micrographs of corneas fixed in the presence of cetylpyridinium chloride (CPC) and ruthenium red (Trelstad, Hayashi & Toole, 1974). However, as CPC causes proteoglycans to condense, this technique underestimates the extent of the matrix.

The SEM results also show that the collagen is not laid down in successive orthogonal layers that extend across the cornea. Instead, organization is far more random with the primary stroma containing only belts of fibrils aligned along the two axes and embedded in a matrix. In any one plane, relatively few fibrils align parallel to one another before the array is disturbed by orthogonal fibrils. The evidence of flaking (Figs 12, 13), perhaps the best evidence to support the view that fibrils in the primary stroma are in layers of parallel fibrils, turns out to contradict it. It is of note that, in terms of the Maurice’s model of corneal transparency whereby the orthogonal arrangement of fibrils acts as a diffraction grating (Maurice, 1957), it makes no difference whether the fibrils are arranged in layers or not. It is only important that the fibrils align in two preferred orthogonal directions. In terms of morphogenesis and growth, however, this difference is crucial.

Consider the implications of the traditional view of stromal morphogenesis which holds that all collagen is laid down as fibrils with uniform diameters in orthogonal layers of similar orientation. First, collagen that passed through the basement membrane would assemble rapidly into approx. 20 nm fibrils. Second, a mechanism is required to ensure that the epithelium lays down the fibrils in arrays that remain aligned over long distances, but whose orientations switched regularly. Third, an alternative mechanism is needed to explain how fibroblasts in the mouse cornea lay down orthogonal collagen, for its epithelium does not lay down a primary stroma to act as a scaffold (Haustein, 1983). Finally, growth would be hard to explain. In short, morphogenesis would be very difficult.

The observations reported here suggest, in contrast, that the process of stromal morphogenesis is not as complex as expected. The fact that stromal collagen does not aggregate immediately means that it self-assembles much like it does elsewhere, with diameter regulation being achieved by the particular mix of collagen and other compounds present (Hay, 1980; Linsenmayer, Fitch, Gross & Mayne, 1985) and by the operation of kinetic factors (Wood & Keech, 1960). The absence of layers extending across the cornea means that the epithelial cells need not be coordinated. The same mechanism could thus organize both the primary collagen laid down by the avian epithelia and the secondary collagen laid down by avian or mouse fibroblasts.

Trelstad & Coulombre (1971) have argued that this self-assembly is not only the basis for fibrillogenesis, but also for generating orthogonal organization. Our observations on the lack of precision in stromal organization support this thesis because self-assembly is intrinsically a stochastic process. A recent observation showing that fibrils can organize themselves without cellular intervention supports the self-assembly hypothesis: if the medium from cultured fibroblasts is centrifuged, spun-down collagen will form a pellet in which groups of fibrils align along the same axes (Gross & Bruns, 1984). Although the evidence for self-assembly remains circumstantial in the absence of a detailed mechanism, the hypothesis remains, by default, the most likely way to generate orthogonality.

**Growth of the cornea**

There is an intriguing aspect to the traditional view of stromal morphogenesis outlined earlier: it cannot
allow growth to occur. Were collagen in the primary stroma laid down in stable layers, the cornea could not expand as it grew: its lateral extension would either cause the cornea to be cone shaped (Fig. 20) or would disrupt the structure by pulling apart the distal layers where there is no new and major source of collagen. The absence of layers of aligned fibrils simplifies the problem of growth, but a solution requires an understanding of what happens to fibril organization as the stroma grows.

It is in this context that the artifactual nature of the stromal undulations is important: the observations show not only that stromal collagen is orthogonally organized in the normal eye but that it remains so even after distortion (Fig. 2). As stromal bending causes the fibrils to move with respect to one another (see legend to Fig. 21), it is clear that stromal fibrils can slide over one another without pulling the structure apart. The fluidity required for corneal bending can also play a role in its enlargement because it will allow the cornea to grow without distorting its organization. Epithelial expansion can thus be compensated for by the collagen fibrils of the stroma, perhaps in groups, sliding over one another, breaking local order without distorting the general organization.

Such movement requires a lubricant and the obvious candidate is the proteoglycan matrix and its associated water (Hascall & Hascall, 1981). The extent to which the stromal proteoglycan is hydrated remains unknown, but the fact that dehydration and critical-point drying causes a 35% decrease in the corneal diameter suggests that more than 70% of the original volume was water. It is therefore possible that here, as in cartilage (Walker, Unsworth, Dawson, Kowski & Weight, 1970), high GAG hydration will permit slippage.

Limitations

While the mechanisms outlined in this paper provide a framework in which to consider stromal morphogenesis and growth, they cannot explain two aspects of collagen organization: first, the mechanism that constrains fibrils to lie parallel and perpendicular to the choroid fissure of the eye and, second, the reasons why these orientations should be rotated anticlockwise outwards from the seventh day onwards (Trelstad & Coulombre, 1971). Nothing is known about the origins of the preferred axes, but Coulombre & Coulombre (1975) have demonstrated that GAG production may play a role in axis rotation. Their observations and much of the analysis here impute important morphogenetic properties to the proteoglycan component of the stroma. We are studying the effects of altering their synthesis on collagen fibrillogenesis and stromal morphogenesis in vitro and hope to report our results soon.

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References


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