A Synchrotron X-Ray Diffraction Study of Developing Chick Corneas

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ABSTRACT To study some ultrastructural aspects of developing chick corneas we performed a synchrotron x-ray diffraction analysis of 22 specimens obtained daily from developmental day 10 through day 19. Before day 12 of development in chicks we were unable to detect a meridional x-ray diffraction pattern from cornea. Neither were we able to record a first-order equatorial x-ray reflection at this time. Normally, these reflections are present in corneal x-ray patterns, arising from, respectively, the periodic axial electron density of fibrillar collagen and the lattice-like arrangement of the fibrils. By day 12 of development we could detect the third- and fifth-order meridional reflections (indicating increased amounts of collagen) and a first-order equatorial reflection (implying that more collagen was regularly arranged). The third- and fifth-order meridional reflections became more intense as the tissue matured, suggestive of a continued deposition of fibrillar collagen, and the scattering angle of the interfibrillar maximum increased, suggesting that regularly arranged collagen was becoming more closely packed with maturation. In embryonic chick corneas, the establishment of an orderly, fairly compacted matrix of collagen fibrils may be one of the main events underlying the acquisition of corneal transparency.

INTRODUCTION

After 5 days of development the primary chick corneal stroma, consisting of fibrillar collagen types I and II and the fibril-associated type IX (Fitch et al., 1994), is invaded by presumptive fibroblasts. Soon thereafter, along with the transient appearance of collagen type IV (Fitch et al., 1991) and hyaluronate (Toole et al., 1971), the cornea swells noticeably and attains its maximal thickness (approximately 220 μm) around developmental day 9 (Hay and Revel, 1969). Presumably, some of this thickening can be ascribed to the secretion of heterotypic type I/V collagen fibrils (Linsenmayer et al., 1984; Birk et al., 1986) into what is now the secondary stroma, but a significant portion is undoubtedly due to the edema that starts to occur at this time. After reaching its maximal thickness, the embryonic chick cornea starts to dehydrate and become progressively thinner; by developmental day 12 it is less than 200 μm thick and is approximately 150 μm thick by day 14 of development (Hay and Revel, 1969).

The acquisition of corneal transparency in chick embryos has often been attributed to this dehydration of the stromal matrix and the attendant compaction and reorganization of collagen, even though the cornea begins to thin (and presumably reorganize its collagen) a couple of days before the initial increase in transparency is noticed (Coulombre and Coulombre, 1958). In an attempt to appreciate more fully the dynamics of collagen deposition and reorganization during embryogenesis, we initiated a synchrotron x-ray diffraction study of chick corneas in the latter stages of development. This approach has an inherent advantage over electron microscopic studies in that tissue can be examined without the need for fixation, dehydration, or embedding, procedures known to induce changes in the corneal ultrastructure (Fullwood and Meek, 1993).

MATERIALS AND METHODS

Chick corneas (n = 22) were obtained at various stages of development from embryonic day 10 through day 19 and immediately frozen in liquid nitrogen. They were wrapped in plastic wrap and stored frozen until they could be analyzed using a low-angle fiber diffraction set-up at the National Synchrotron Light Source (NSLS), Brookhaven National Laboratory (Capel, 1993). Freezing is an appropriate method for storing corneas before examining the extracellular matrix by synchrotron x-ray diffraction because we know that structural changes caused by freezing are reversible upon thawing (Fullwood and Meek, 1994). To obtain diffraction patterns, each specimen was removed from the freezer, unwrapped, and immediately placed (at room temperature) between two mylar windows in a sealed specimen holder suitable for beamline X12B at the NSLS (Quantock et al., 1996). The use of this apparatus, along with the relatively short time required to mount the cornea and place it in the path of the x-ray beam (a minute or two) and record the diffraction pattern (4 min), minimizes the natural dehydration of the cornea, which, if it were to proceed unchecked would cause the collagen fibrils to move closer together (Meek et al., 1991; Fratzl and Daxer, 1993). Indeed, previous work with the same set-up at the NSLS (Quantock et al., 1997) has shown that corneas lose only a few percent of their wet weight during this type of x-ray exposure. To obtain a diffraction pattern, an x-ray beam of wavelength 1.59 Å was passed through the cornea parallel to its optical axis, and the diffraction pattern recorded on a two-dimensional, position-sensitive x-ray detector located approximately 2.5 m behind the specimen. A similarly obtained background pattern from the mylar windows of the empty specimen holder was then subtracted from the corneal x-ray diffraction pattern after scaling for differences in incident flux.

Received for publication 28 August 1997 and in final form 20 October 1997.

Presented, in part, at The Association for Research in Vision and Ophthalmology annual meeting, Ft. Lauderdale, FL, May 11–16, 1997.

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0006-3495/98/02/995/04 $2.00

RESULTS

We found that the third and fifth orders of the meridional x-ray diffraction pattern from developing chick corneas, reflections normally present on x-ray patterns from more mature corneas, first appear at day 12 of development (although there is a hint that these two reflections may be present in the pattern from one of our two day-11 corneas, but only very faintly). Meridional reflections arise because of the axial electron density of fibrillar collagen. They do not depend upon its lateral arrangement and, as such, lead us to conclude that the amount of new collagen being deposited into the secondary chick stroma reaches a threshold by developmental day 12 whereby a detectable meridional x-ray diffraction pattern is formed. After day 12 of development, the intensity of the third- and fifth-order meridional reflections increases steadily up to developmental day 19 (Fig. 1), indicating the continued deposition of collagen (Daxer et al., 1994). Also, the position of the third-order meridional reflection, when calibrated against the meridional x-ray reflections arising from the axial 67-nm repeat of collagen in moist rat-tail tendon, indicates that the axial D-period of fibrillar corneal collagen in chicks at developmental days 12 and 13 measures 64.4 nm ($n = 4$; SD = 0.4 nm) whereas the same value at day 19 of development is 63.4 nm ($n = 3$; SD = 0.3 nm).

As with the meridional findings, before day 12 of development in chicks we were unable to detect a first-order equatorial x-ray reflection from cornea (two day-10 corneas and two day-11 corneas were examined). At developmental day 12 this reflection became evident but was too weak to measure reliably in one of our two day-12 corneas. Thereafter, the first-order equatorial reflection was observed on all x-ray diffraction patterns obtained from developing chick corneas older than 12 days, indicating the presence of increased amounts of regularly arranged fibrillar collagen. Moreover, we noticed that the first-order equatorial reflection, especially in older corneas, was invariably lobed (Fig. 1) with increased scattering in two orthogonal directions; this phenomenon, also seen in mature human corneas (Meek et al., 1987; Daxer and Fratzl, 1997), is indicative of the tendency of collagen fibrils within different lamellae to lie at right angles to each other. At present, it is unclear whether or not this orthogonal orientation effect, an effect that seems to be present in the earlier stages of development, becomes more pronounced as the chick cornea develops.

The position of the first-order equatorial x-ray reflection in developing chick corneas older than 12 days, again calibrated against the meridional x-ray reflections arising from the axial 67-nm repeat of collagen in moist rat-tail tendon, enabled us to obtain an estimate for the mean center-to-center interfibrillar Bragg spacing of the regularly arranged collagen in these tissues. It is clear from the data (Fig. 2) that this value drops ($R^2 = 0.421$) as the developing chick cornea matures, measuring 71.6 nm ($n = 3$; SD = 1.7 nm) on developmental days 12 and 13 compared with 57.4 nm ($n = 3$; SD = 5.4 nm) on day 19 of development. We

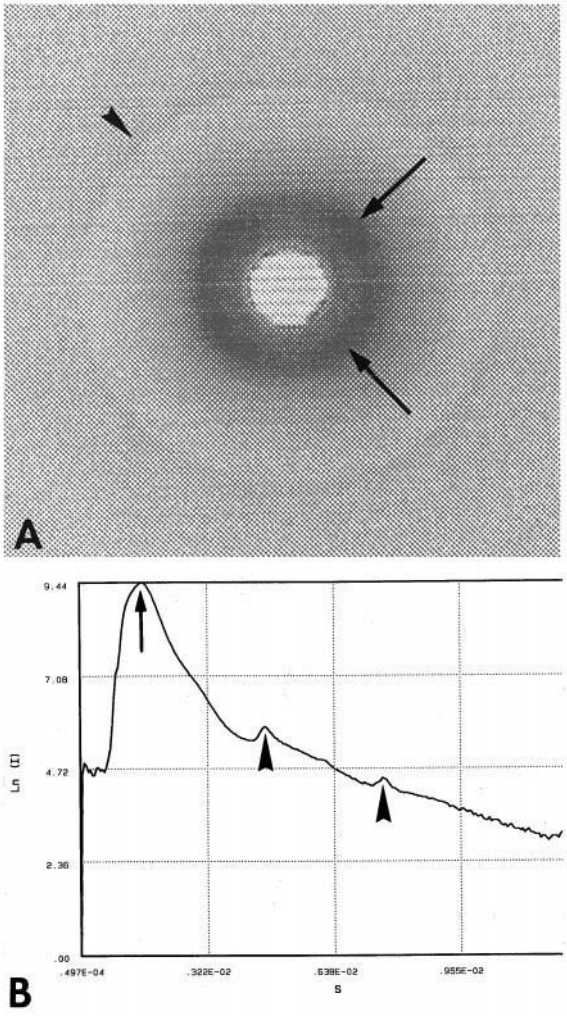

FIGURE 1 (*A*) An x-ray diffraction pattern from one of the developmental day 19 chick corneas showing the third order of the meridional pattern (*arrowhead*) and the two axes (*arrows*) along which the four lobes in the first-order equatorial reflection lie. (*B*) A plot of Ln intensity (I) versus scattering angle S ($Å^{-1}$) in which peaks corresponding to the first-order equatorial reflection (*arrow*) and third- and fifth-order meridional reflections (*arrowheads*) are indicated.

should remember here some of the limitations of estimating a value for the interfibrillar Bragg spacing using the position of the first-order equatorial (i.e., interfibrillar) reflection as we have done. The accuracy with which we could measure the position of a particular interfibrillar maximum converted to real space was ±2 nm. Also, if we bear in mind the incoherent scattering from matrix elements other than collagen, scattering that exhibits power-law behavior and thus shifts the interfibrillar maximum toward smaller scattering angles (Sayers et al., 1982; Fratzl and Daxer 1993), it is evident that our data may represent an overestimate of the actual values. Nevertheless, as profiles of the background scatter from all of our specimens are similar, it seems reasonable to assume that any overestimate will be systematic and that regularly spaced collagen does indeed become more closely packed in older developing chick corneas.

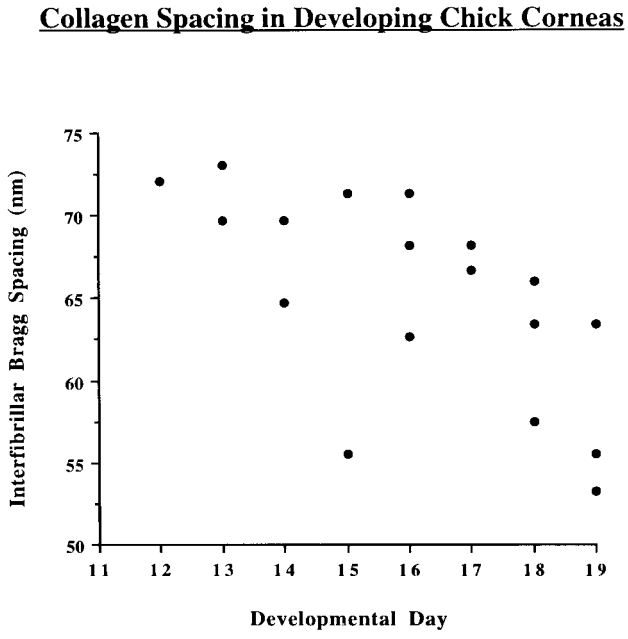

FIGURE 2 The interfibrillar Bragg spacing of fibrillar collagen in chick corneas at various stages of development.

DISCUSSION

An inspection of published electron micrographs from 14-day-old embryonic chick corneas (see Fig. 5.7 in Hay and Revel, 1969) reveals that the collagenous arrangement of the corneal stroma is in some disarray at this stage of development. Collagen fibrils seem to exist in groups with numerous, fairly large spaces in between that are devoid of collagen. It is thought that, if their dimensions approach half the wavelength of visible light, collagen-free spaces in the corneal stroma will be to the detriment of corneal transparency (Benedek, 1971). Given this, it seems reasonable to assume that, as the chick cornea develops, the resolution of spaces between groups of collagen fibrils should aid the acquisition of corneal transparency. Despite the irregular lamellar organization of the stromal collagen as a whole at 14 days of development (Hay and Revel, 1969), within the individual groups of fibrils there does seem to be some semblance of order, an impression shared by Cornuet and associates (1994). The fact that we are able to obtain a first-order equatorial x-ray reflection from developmental day-14 corneas indicates that the packing of fibrils within their groups is indeed fairly regular. That no interfibrillar reflection was evident before day 12 of development tells us 1) that edema is more widespread at this time and collagen fibrils tend not to exist in groups, 2) that collagen does exist in groups but within these groups the interfibrillar spacing is not particularly uniform, or 3) that collagen fibrils exist in groups and are regularly spaced, but not enough fibrils are present to give rise to a detectable first-order interfibrillar reflection. When considering which of these interpretations is more likely it is useful to follow the reasoning of Daxer et al. (1994) who showed that a qualitative inspection of meridional x-ray reflections can help shed some light on the relative amounts of collagen in corneal tissue. In doing so, it becomes clear that, because the third- and fifth-order meridional reflections appear at around the same time as the first-order equatorial reflection, new collagen is being laid down at developmental day 12 in chicks while, at the same time, the fibrillar lattice is becoming more organized. Furthermore, as the third and fifth meridional orders become more intense while the scattering angle of the interfibrillar reflection changes, it seems as though deposition and reorganization (i.e., compaction) of collagen proceed hand in hand as the embryonic chick cornea matures, although it is not evident from our data whether this new collagen is deposited in a regular array or is deposited irregularly and subsequently becomes more properly organized.

After the developing chick cornea has reached its maximal thickness (around day 10 of development), the subsequent dehydration (Coulombre and Coulombre, 1958) and compaction of fibrillar collagen is initially more prevalent in the posterior stroma (Hay and Revel, 1969). As the x-ray beam provides an average value throughout the whole thickness of the cornea we can offer no evidence as to the specific location (anterior or posterior) of the initial stromal compaction, although our data indicate that this compaction of the secondary stroma must proceed for approximately 2 or 3 days before sufficient order is achieved to give rise to an interfibrillar x-ray reflection. An additional 2 days then pass before corneal transparency starts to increase (between developmental day 14 and day 15 (Coulombre and Coulombre, 1958)). This increase in transparency continues until day 19 of development, a day or so before hatching, when the transparency of the developing chick cornea reaches adult levels (Coulombre and Coulombre, 1958). In the mature cornea, the arrangement of stromal collagen is known to have a huge bearing on the transmission of light (Maurice, 1957; Benedek, 1971). Given this, it seems reasonable to assume that the closer packing of collagen that exists by day 19 of embryogenesis in chicks may well be a major factor underlying the acquisition of corneal transparency. Although data for the spacing of collagen in adult chicken corneas are not available, our value for the interfibrillar Bragg spacing in chicks at developmental day 19 (57.4 ± 5.4 nm) is, within confidence limits, the same as that for six of seven species of adult bird examined (Meek and Leonard, 1993). Even though it is clear from this study that regularly arranged collagen in the embryonic chick cornea becomes more closely packed as the tissue matures (Fig. 2), it is not obvious whether this occurs in a linear fashion from day 12 to day 19 of development or takes place more rapidly over certain intervals. More data will be required to answer this question.

We conclude that as the secondary chick stroma dehydrates and compacts it lays down new fibrillar collagen, while at the same time a proportion of collagen becomes sufficiently organized to give rise to an interfibrillar x-ray reflection after 12 days of development, even though collagen-free spaces may still exist between adjacent groups of

fibrils. In the week that follows, the deposition of new collagen fibrils continues, and those that are arranged regularly become packed more closely as time proceeds, achieving adult levels of packing by developmental day 19 concomitant with the onset of transparency. We contend that this establishment of a closely packed collagenous matrix is an essential step for the acquisition of corneal transparency, although the important question remains as to the nature of factors that initiate and modulate this change. At present, these are not understood fully; however, chief among the likely contenders are links to endothelial function and thyroid activity (Coulombre and Coulombre, 1964), the pattern of corneal innervation (Clark and Bee, 1996), and changes in the proteoglycan metabolism (Anseth, 1961; Hahn and Birk, 1992; Cornuet et al., 1994; Cai et al., 1996).

We thank Drs. Keith Meek, Nigel Fullwood, and Albert Daxer for helpful advice.

This work was supported in part by Research to Prevent Blindness, Inc., New York, NY (D. J. Schanzlin). The research was carried out in part at the National Synchrotron Light Source, Brookhaven National Laboratory, which is supported by the U.S. Department of Energy, Division of Materials Sciences and Division of Chemical Sciences (user grant 95-X-1028 to A. J. Quantock).

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