Expression of Insulin-like Growth Factor I in Developing Lens Is Compartmentalized*

(Received for publication, June 25, 1991)

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We, and others, have recently reported that insulin-like growth factor I (IGF-I) mRNA is expressed in multiple tissues during embryogenesis and in whole embryos during early organogenesis. Therefore, it is likely that, in addition to any effect on embryo growth, IGF-I plays a paracrine/autocrine role in development. The embryonic chicken lens, an avascular organ composed by a single type of cell that undergoes differentiation in vivo and in vitro, is an ideal model to characterize the paracrine/autocrine action of IGF-I. The lens cells express IGF-I receptors, and respond to exogenous IGF-I with induction of fiber cells differentiation and stimulation of ß-crystallin gene transcription. Whether embryonic lens cells express IGF-I was uncertain. In the present study, we used a sensitive semi-quantitative method (reverse transcription of RNA followed by amplification with the polymerase chain reaction) to analyze IGF-I gene expression. An amplified product of the expected length (209 base pairs) was found in days 8, 12, 15, and 19 lenses. At all embryo ages studied, the product was more readily detected in the lens than in the liver, while in eye tissues (excluding lens), IGF-I expression was relatively high. After microdissection of the epithelial cells from the fully differentiated fiber cells, IGF-I expression was detected exclusively in the epithelial cells, IGF-I immunoreactivity was found using high performance liquid chromatography followed by radioimmunoassay in the days 8–19 lens extracts, and in primary cultures of isolated epithelial cells. Our previous and present findings show that the lens has all the elements for IGF-I autocrine/paracrine action in development.

The lens, an encapsulated organ: that develops early in embryogenesis, lacks blood vessels and nerves. It is a tissue of epithelial origin, formed by a front layer of cubical epithelial cells that divide, migrate, elongate, and finally become fully differentiated fiber cells that constitute the main body of the mature lens (reviewed in Ref. 1). Lentripectin, a protein that stimulates elongation, was partially purified from chick embryo vitreous humor, and was found by Beebe et al. (2, 3) to be related to IGF-I.1 A similar differentiation effect had previously been observed with fetal serum, vitreous humor (1, 4), and with high concentrations of insulin (5–7). Thus, growth factors of the insulin family and, perhaps, fibroblast growth factor (8, 9) and platelet-derived growth factor (10), may be essential for normal development of the lens. Fiber cell differentiation during organogenesis of the chick embryo lens is typically associated with an increase in ß-crystallin expression (7, 11). We have previously shown that exogenous IGF-I stimulates transcription of the endogenous ß-crystallin gene, as well as of a transfectd ß-crystallin/chloramphenicol acetyltransferase hybrid gene (11, 12). We, and others, have demonstrated that both epithelial and fiber cells display IGF-I receptors that are highly regulated in development (13–15).

While IGF-I has been largely considered a postnatal growth factor in mammals, there is evidence suggesting that IGF-I also has autocrine/paracrine actions during development. IGF-I mRNA and IGF-I have been detected in multiple fetal tissues in mammals (16–21). In the chick embryo, IGF-I mRNA and IGF-I are expressed in the whole embryo during gastrulation and neurulation, before circulation is established (22, 23). Immunoactive IGF-I is detected in serum by day 6, peaks at days 14–16, and then decreases markedly by day 20 (hatching is at day 21) (23). In a model of chick embryo cultured ex ovo, with marked retarded growth, we have shown that the IGF-I midembryogenesis serum peak is abolished (23). While in this situation and others (18) the serum concentration of IGF-I appears to relate to the overall growth of the organism, to understand the local action (paracrine or autocrine) of IGF-I we need information on the levels of IGF-I mRNA and IGF-I in individual tissues.

The goal of our present study was to document that embryonic lens contained IGF-I mRNA and IGF-I, and to study the changes in ontogeny. We found IGF-I expression in the lens at all ages studied, from late organogenesis until near birth. Furthermore, the changes in IGF-I mRNA and IGF-I during development in the lens do not correlate with serum IGF-I. Within the lens, only the epithelial cells, and not the fully differentiated fiber cells express the IGF-I gene. This selectivity is a clear example of cell-compartmentalized expression of IGF-I within a developing tissue that has the ability to differentiate in response to IGF-I.

**Experimental Procedures**

*Chickens Embryos and Dissections—Fertilized eggs from White Leghorn chickens (Truslow Farms, Inc., Chestertown, MD) were incubated in a Petersime egg incubator at 38 °C and 40–60% relative humidity.

† The abbreviations used are: IGF-I, insulin-like growth factor-I; DMEM, Dulbecco’s modified Eagle’s medium; HPLC, high performance liquid chromatography; bp, base pair(s); RT-PCR, reverse transcription of RNA followed by amplification with the polymerase chain reaction; RIA, radioimmunoassay.
humidity. On appropriate days of development, the embryos were morphologically staged (24) and the lens was microdissected from the rest of the eye.

The embryos were decapitated and a lateral incision at the corneal limbus of the eye was made. The vitreous body with the attached lens was gently extruded to the outside of the eye and was grasped with fine forceps (25). The lens was extracted from the eye with curved capillary forceps rolled over filter paper to remove adherent iris, washed in unsupplemented DMEM, and then frozen at ~70°C until the protein or RNA was extracted.

**Primary Lens Cells Culture**—Patches of lens epithelial cells from day 13 chick embryos (E13), were prepared as described (36). Epithelia from nine lenses were used per 35-mm dish (total of 90 epithelia). After 24 h of incubation in 2 ml of DMEM, supplemented with 10% fetal calf serum, at 37°C in 5% CO2, the cells were washed three times with 5 ml of DMEM. The cells were then harvested by adding 0.5 ml of 0.01% trypsin/0.02% EDTA solution for 2 min, scraped from the dish and centrifuged for 1 min at 500 ×g. The cell pellet was washed twice in DMEM and then homogenized in 0.25 M Tris, pH 7.5. The homogenate was desalted on a column of Sephadex G-25 and the eluate was lyophilized and resuspended in HPLC buffer (see later).

**Isolation of RNA**—Whole lenses (either 10 of E8, E12, and E15 or 5 E19) were homogenized in RNazol (2 ml/100 mg of tissue) for 1 min with a glass-teflon homogenizer, and total RNA was extracted according to the method of the RNazol manufacturer (Cinna/Biotec, Friendswood, TX, Ref. 27). For the study of epithelial and fiber cells, 15 lenses of E15 were microdissected and washed in DMEM. The epithelial cells were added to the capsular portion separated from the fiber cells. Each group of cells was homogenized and processed as described above for the whole lenses. The eye of a day 19 embryo was microdissected and the vitreous body and the lens were discarded; the residual tissue was processed as described for the lens. In all preparations the RNA concentration was determined by absorption at 260 nm and the purity by the 260 nm:280 nm ratio. The integrity of ribosomal RNA was confirmed by gel electrophoresis and ethidium bromide staining (results not shown).

**Design of Oligonucleotides**—Oligonucleotides complementary to the sequence of chicken IGF-I determined previously (22, 28) were synthesized in a Coder 300 automated DNA synthesizer (E.I. Du Pont). They were 24 bp long and spanned most of the coding region of IGF-I, including a portion of the B-, C-, A- and part of the D-domain (Fig. 1A). The sequence of the upstream primer (U, sense) was 5′CCAGAAAACACTGTGTGGTGCTGAG3′, corresponding to amino acids 2 through 9 of the mature peptide. The sequence of downstream primer (D, antisense) was 5′GTTACAGAGTATCTATGTTGAG3′, corresponding to amino acids 63 through 70. For the expression studies the probe was the oligonucleotide 5′AGGACGAGACCCGTTACACCAAGG3′, corresponding to amino acids 34 through 41. Reverse Transcription Followed by the Polymerase Chain Reaction (RT-PCR) of Total RNA from the Lens—Total RNA from the lens at each stage of the development (see later) was amplified using XAR-2 Kodak film, with one intensifying screen at ~70°C. Autoradiograms were generated after exposure for 4 weeks at ~70°C. The arrow indicates the 209 bp fragment. The autoradiograms were generated after exposure for 4 weeks at ~70°C.

Reverse-phase HPLC—Chromatography was performed on a Gilson system, using a Vydac C-18 hydrophobic interaction TP104 column as described (30). The two-solvent linear gradient consisted of solvent A (0.1% trifluoroacetic acid in H2O) and solvent B (80% acetonitrile/20% H2O/0.1% trifluoroacetic acid). Aliquots of the extracts (as indicated in the corresponding figures) were injected in 25% solvent B/75% solvent A, and solvent B was maintained at 25% for 20 min. A linear gradient was then established from 25 to 65% over 40 min. Fractions were collected every min (1 ml) for the 60-min period. They were evaporated in a vacuum centrifuge and then resuspended in HPLC buffer (750 μl of solvent A and 250 μl of solvent B, described below).

**DNA Blot Hybridization**—An aliquot of the PCR-amplified product was fractionated on a 2% agarose gel containing ethidium bromide in Tris-borate-EDTA (TBE 1×) buffer. After electrophoresis the products were visualized by UV transillumination and the position of the expected bands was compared to a x174 ladder (Promega). The gels were denatured for 1 h in 0.5 N NaOH in 1.5 M NaCl, and washed for 1 h in 1 M Tris containing 1.5 M NaCl. The DNA was transferred to nylon filters (Nytran, Schleicher and Schuell; Keene, NH) by capillary action, using 5× SSC. The filters were prehybridized and then hybridized (29) to a 32P-labeled oligonucleotide (Fig. 1A), at 37°C for 16−20 h. The filters were then washed in 2× SSPE with 0.05% sodium dodecyl sulfate at 37°C. Autoradiograms were generated using XAR-2 Kodak film, with one intensifying screen at ~70°C.

**Screening and Expression of Candidate Proteins**—Prior to the chromatographic step to remove the IGF-I binding proteins (23), the lenses were homogenized in 1–2 ml of 0.25 M HCl using a hand-held glass/teflon homogenizer with 10 strokes. The homogenate was centrifuged in an Eppendorf microcentrifuge for 5 min at 14,000 rpm at 4°C, and the supernatant was transferred to a tube and diluted 1:1 with 0.5 M NaCl. After incubation for 1 h at 20°C the mixture was loaded onto a C-18 SepPak cartridge; the eluate was recycled once, the column was washed with 4% acetic acid, and the retained material was eluted with 10% methanol. The eluate was evaporated to dryness in a vacuum centrifuge. All the samples were dissolved in 1 ml of HPLC buffer (750 μl of solvent A and 250 μl of solvent B, described below).

**Reverse-phase HPLC—Chromatography**—Chromatography was performed on a Gilson system, using a Vydac C-18 hydrophobic interaction TP104 column as described (30). The two-solvent linear gradient consisted of solvent A (0.1% trifluoroacetic acid in H2O) and solvent B (80% acetonitrile/20% H2O/0.1% trifluoroacetic acid). Aliquots of the extracts (as indicated in the corresponding figures) were injected in 25% solvent B/75% solvent A, and solvent B was maintained at 25% for 20 min. A linear gradient was then established from 25 to 65% over 40 min. Fractions were collected every min (1 ml) for the 60-min period. They were evaporated in a vacuum centrifuge and then resuspended in HPLC buffer (750 μl of solvent A and 250 μl of solvent B, described below).

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RESULTS

IGF-I mRNA and IGF-I Are Expressed in Developing Whole Lens—We searched for IGF-I mRNA in the lens during ontogeny from day 8 through day 19 (hatching is at day 21). Suspecting that the level of IGF-I gene expression might be very low, based on our experiments in whole embryos (22), we used a very sensitive RT-PCR strategy to amplify IGF-I mRNA followed by gene-specific hybridization (Fig. 1A). We detected IGF-I mRNA in the whole lens from embryos at all ages studied (Fig. 1B). The signal at day 19 was higher in several independent experiments when compared with lenses obtained from younger age embryos. The product amplified was of the expected size, and RNase pretreatment of the samples prevented its appearance, indicating that the band represented mRNA in the sample and not contaminating DNA.

To determine the expression of IGF-I, whole lenses from the same ages were studied. All the samples tested contained immunoactive IGF-I after HPLC (Fig. 2). The immunoactivity eluted in a single peak in a position similar to that of chicken serum IGF-I (which in this type of gradient also coincides with the position of human IGF-I) (23). The content of IGF-I per lens increased slightly during the second week of embryogenesis and there was a further increase at day 19 (Table I), consistent with the increase in IGF-I mRNA at that age.

Differential IGF-I Gene Expression in the Embryo Lens Cells, Eye, and Liver—Since the lens is composed of a single layer of undifferentiated epithelial cells located next to the anterior part of the capsule and a large mass of fiber cells, the two cell groups can be studied separately. To analyze whether both epithelial and fiber lens cells expressed IGF-I mRNA, the RNA extracted from the cell layers after careful microdissection of the cell layers was amplified by RT-PCR. The epithelial lens cells, but not the fiber cells, were found to express the IGF-I gene (Fig. 3). In addition, the eye tissues minus the lens also expressed IGF-I mRNA in relatively high levels (Fig. 3). Although our RT-PCR protocol does not allow strict quantitation of mRNA, the amount of RNA in the reaction had a direct relationship with the signal obtained after amplification. Serial dilutions of the eye total RNA resulted in a progressive decrease in the specific signal (Fig. 3). The lens of the same age embryos (day 19) had a signal that fell between the 1:25 and 1:150 dilutions of the whole eye minus lens, i.e., the level of IGF-I mRNA in the lens is probably 2 orders of magnitude lower than in the structures of the eye minus the lens. In contrast with the IGF-I mRNA in the lens and the eye during embryogenesis, its presence in developing liver was barely detectable, using similar amounts of total RNA in the RT-PCR reaction (Fig. 3C). This suggests that the liver is not an important source of IGF-I prenatally.

To confirm that the epithelial cells of the lens contained IGF-I, we established primary cultures with patches of epithelial cells. After 24 h in culture an extract was chromatographed on C-18. The HPLC reactions contained immunoactive IGF-I (Fig. 4). The concentration of IGF-I per lens-equivalent was slightly higher than in the extract of the whole

**Table 1**

| Embryo age | 8 days | 12 days | 15 days | 19 days | E<sub>12</sub> epithelial cultures |
|------------|--------|---------|---------|---------|---------------------------------|
| Experiment 1 | 0.36 (n = 109) | 0.81 (n = 73) | 0.58 (n = 73) | 2.6 (n = 40) | 1.06 |
| Experiment 2 | 0.30 (n = 65) | 0.54 (n = 65) | 0.57 (n = 82) | 14.4 × 10<sup>4</sup> | 8.1 × 10<sup>4</sup> |
| Average | 6.6 × 10<sup>-6</sup> | 5.1 × 10<sup>-6</sup> | 3.8 × 10<sup>-6</sup> | |

*n, number of lenses used for the extract in each of the two experiments.

*To obtain these relative values the mean IGF-I concentration of experiments 1 and 2 was divided by the number of epithelial cells at each age calculated by Persons and Modak (41).
lens, eye, and liver. The initial RNA ethidium bromide staining of rRNA (results not shown). Samples were either untreated or treated with RNase A (+ lanes). The exposure of the autoradiogram was for 3 days at −70°C; a short exposure (4 h) of the eye lanes is also shown. The presence of an amplified product in RNA from epithelial cells and the absence in RNA from the fiber cells were confirmed in several independent RT-PCRs, and with another series of RNA preparations. Integrity of the RNA from fiber cells had been confirmed by gel electrophoresis and ethidium bromide staining of rRNA (results not shown). B, RNA concentration dependency of the hybridization signal. Prior to the RT-PCR, 10 μg of total RNA from day 19 eye (minus lens and vitreous humor) were diluted as indicated. As negative control we used distilled water (W). 10 μg of total RNA of the day 19 lens were also analyzed. The amplification reaction and the hybridization were performed as described in the legend for Fig. 3. The amplified band corresponds to the 209-bp product and is indicated by an arrowhead. The autoradiogram was exposed for 18 h at −70°C. C, IGF-I gene expression in developing liver. RT-PCR was performed using 10 μg of total RNA from liver of embryos at days 12, 14, 16, 19, and 50-day-old chicken. The samples were either untreated or treated with RNase A (+ lanes) prior to RT-PCR. The amplified products were processed and the hybridization performed as described in the legend for Fig. 3. The exposure time of the autoradiogram was 3 days. The results presented in all panels (A–C) were obtained with products from the same PCR experiment.

Fig. 3. Differential IGF-I gene expression in the embryo lens, eye, and liver. A, RT-PCR was performed using 10 μg of total RNA from either day 19 whole lens, epithelial cells, fiber cells, or the eye minus lens, as described in the legend for Fig. 1. The initial RNA samples were either untreated (− lanes) or treated with RNase A (+ lanes). The exposure of the autoradiogram was for 3 days at −70°C; a short exposure (4 h) of the eye lanes is also shown. The presence of an amplified product in RNA from epithelial cells and the absence in RNA from the fiber cells were confirmed in several independent RT-PCRs, and with another series of RNA preparations. Integrity of the RNA from fiber cells had been confirmed by gel electrophoresis and ethidium bromide staining of rRNA (results not shown). B, RNA concentration dependency of the hybridization signal. Prior to the RT-PCR, 10 μg of total RNA from day 19 eye (minus lens and vitreous humor) were diluted as indicated. As negative control we used distilled water (W). 10 μg of total RNA of the day 19 lens were also analyzed. The amplification reaction and the hybridization were performed as described in the legend for Fig. 3. The amplified band corresponds to the 209-bp product and is indicated by an arrowhead. The autoradiogram was exposed for 18 h at −70°C. C, IGF-I gene expression in developing liver. RT-PCR was performed using 10 μg of total RNA from liver of embryos at days 12, 14, 16, 19, and 50-day-old chicken. The samples were either untreated or treated with RNase A (+ lanes) prior to RT-PCR. The amplified products were processed and the hybridization performed as described in the legend for Fig. 3. The exposure time of the autoradiogram was 3 days. The results presented in all panels (A–C) were obtained with products from the same PCR experiment.

D and C showing a linear acetonitrile gradient was established as described in Fig. 2 legend. Fractions 30 through 50 were analyzed in duplicate in the IGF-I RIA. Similar results were obtained with another separate culture. The sensitivity of the RIA is indicated by a horizontal line. The position of an IGF-I standard is indicated by an arrow.

In the present study we show evidence that the embryonic lens, a tissue responsive to IGF-I, expresses the IGF-I gene. IGF-I mRNA is found in the lens in a subset of its cells, the epithelial cells, that are precursors of the differentiated fiber cells. The embryonic lens contains also IGF-I. The developmental profile of the IGF-I detected in lens extracts roughly parallels the levels of IGF-I mRNA in whole lens. The highest values of both peptide and mRNA are found at day 19, 2 days before hatching (Table I and Fig. 1B). It is worth noting that this is not the developmental pattern exhibited by serum IGF-I, that increases from day 6 to a peak concentration at day 15 and then decreases markedly the days before hatching (25). This divergence between circulating IGF-I and lens IGF-I supports the local origin and autocrine/paracrine function of IGF-I in the lens. Since IGF-I mRNA appeared to be restricted only to the epithelial, mitotic cells and absent from the fiber cells (Fig. 3), the peptide found in whole lens extracts most likely was produced in the epithelial layer. We could find IGF-I immunoreactive protein in primary cultures of lens epithelial cells from day 12 embryos in comparable amounts to the IGF-I from fresh-frozen lens, supporting the concept of cell-specific expression of IGF-I (Fig. 4). Apparently, IGF-I is expressed by the least differentiated cells, suggesting that it may stimulate their differentiation process in vivo. (We have not excluded that partially differentiated cells in vitro or equatorial cells in vivo also expresses IGF-I.) Parenthetically, the expression of IGF-I in one subset of cells, while the neighbor cells also respond to the peptide, occurs postnatally in the ovary; only the granulose cells contain IGF-I mRNA, but not theca cells, while both respond to IGF-I (33).

Lentropin, a factor related immunologically to IGF-I and clearly distinct from insulin, had been partially purified from day 15 chick embryo vitreous humor (2, 3) and proposed as a physiological factor responsible for the differentiation of epithelial lens cells into fiber cells. The reported size of lentropin, M, ~60 kDa (3) is much larger than the chicken IGF-I gene primary translation product (prepro-IGF-I, M, ~17.5 kDa) (28). It remains to be clarified whether lentropin represents IGF-I bound to binding proteins, or an IGF-I binding protein alone, with IGF-I-like effects, or another molecule. We have confirmed that the vitreous humor from chicken embryo as early as day 6 contains both IGF-I and IGF-I binding proteins.2

Several other aspects related to IGF-I differ between fully differentiated lens fiber cells and epithelial cells. While both cells contain IGF-I receptors, epithelial cells did, but fiber cells did not, internalize gold-labeled IGF-I (34). The level of IGF-I binding to membrane preparations was slightly lower

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DISCUSSION

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in fiber cells than in epithelial cells at all ages studied. The concentration of receptors decreased in both cells with embryo age from day 6 to day 19 (13). Therefore, the effect of IGF-I in vivo is modulated by changes not only in the local peptide levels, but also changes in the level of cell receptors and, probably, the level of IGF-I binding proteins.

The whole eye (minus the lens) contains significant amounts of IGF-I mRNA (Fig. 3). We do not know the specific location of this mRNA or the peptide. (Preliminary attempts to localize IGF-I mRNA by in situ hybridization or IGF-I by immunocytochemistry in the eye of day 7 embryos have not been successful, perhaps due to their low concentration.) In the human fetus, IGF-I mRNA has been found in the sclera (19) and in the rat fetus in the iris (35). We were surprised that the IGF-I mRNA in the eye was markedly higher than in the embryonic liver (Fig. 3). However, in a previous study we had obtained similar results in liver using different oligonucleotides as primers for the RT-PCR (22); others have confirmed the very low expression of the IGF-I gene in developing liver during chicken embryogenesis by an independent technique, solution hybridization, and RNase protection assay (36). It is intriguing that in the only species in which a complete developmental profile for circulating IGF-I is available, the chicken, the contribution by hepatic IGF-I appears negligible and the regulation by growth hormone seems very unlikely during embryogenesis (36). Since multiple embryonic tissues from both fetal rat (19, 21) and mouse (37, 38) as well as chick embryos (22) express IGF-I mRNA, there are multiple potential sources for IGF-I in the circulation. Whether the eye is a contributor to IGF-I serum levels in the embryo remains to be studied.

The panoply of effects of IGF-I in developing lenses include stimulation of protein and RNA synthesis, morphological elongation and β-crystallin gene transcription, i.e. IGF-I is both a growth and a differentiation factor (2, 7, 11, 12, 39). It should be interesting to analyze in detail the dose dependency of these effects at different developmental points. Perhaps small differences in IGF-1 concentration in the lens local milieu (aqueous humor versus lens, versus vitreous humor) can regulate both growth and differentiation of the lens cells in an orderly manner, as it has been proposed for fibroblast growth factor (8, 9). We speculate that a concentration gradient may exist between the IGF-I locally produced by the epithelial cells and the vitreous humor IGF-I, that is in contact with fiber cells. Differences in IGF-I concentration may induce the lens cells either to divide or to differentiate. The embryonic lens, thus, provides a good example of cell-compartmentalized IGF-I gene expression and a confined model for IGF-I autocrine/paracrine action in early embryogenesis.

Acknowledgments—We thank Jesse Roth for his support for this project and Maxine A. Lesniak, Peggy S. Zelenka, and Carolyn A. Bondy for critical reading of the manuscript.

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