The developing organ of Corti contains retinoic acid and forms supernumerary hair cells in response to exogenous retinoic acid in culture

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SUMMARY

The mammalian organ of Corti has one of the most highly ordered patterns of cells in any vertebrate sensory epithelium. A single row of inner hair cells and three or four rows of outer hair cells extend along its length. The factors that regulate the formation of this strict pattern are unknown. In order to determine whether retinoic acid plays a role during the development of the organ of Corti, exogenous retinoic acid was added to embryonic mouse cochleae in vitro. Exogenous retinoic acid significantly increased the number of cells that developed as hair cells and resulted in large regions of supernumerary hair cells and supporting cells containing two rows of inner hair cells and up to 11 rows of outer hair cells. The effects of retinoic acid were dependent on concentration and on the timing of its addition.

Western blot analysis indicated that cellular retinoic acid binding protein (CRABP) was present in the sensory epithelium of the embryonic cochlea. The amount of CRABP apparently increased between embryonic day 14 and postnatal day 1, but CRABP was not detectable in sensory epithelia from adults. A retinoic acid reporter cell line was used to demonstrate that retinoic acid was also present in the developing organ of Corti between embryonic day 14 and postnatal day 1, and was also present in adult cochleae at least in the vicinity of the modiolus. These results suggest that retinoic acid is involved in the normal development of the organ of Corti and that the effect of retinoic acid may be to induce a population of prosensory cells to become competent to differentiate as hair cells and supporting cells.

Key words: retinoic acid, CRABP, hair cells, cochlea, hearing

INTRODUCTION

The geometric pattern of hair cells and supporting cells in the mammalian organ of Corti is one of the most highly ordered arrangements of cells in the vertebrate body. The factors that regulate the development of this unique tissue structure are largely unknown.

Retinoic acid is a member of the family of vitamin A derivatives classified as retinoids and has been shown to be biologically active during development. Although the actions of retinoic acid are not completely understood, retinoic acid has been shown to influence the determination of specific cell phenotypes (Roberts and Sporn, 1984; Simeone et al., 1990; DeLuca, 1991; Wanek et al., 1991; Johnson et al., 1992). In particular, it has been suggested that retinoic acid may be involved in the formation of patterns during the development of the limb and hindbrain structures and during regeneration in the limb (Tickle et al., 1982; Thaller and Eichele, 1987; Durston et al., 1989; Ruiz i Altava and Jessell, 1991; Brockes, 1990; Hogan et al., 1992). This investigation had four objectives: (1) to examine any

effects of the addition of exogenous retinoic acid to cultures of the developing organ of Corti, (2) to determine whether cellular retinoic acid binding protein (CRABP) is present in the organ of Corti during the period of determination and differentiation of the hair cells and supporting cells, (3) to determine whether retinoic acid is normally present in the organ of Corti during the same developmental time periods, and (4) to examine whether retinoic acid is present in other mammalian and avian hair cell epithelia.

MATERIALS AND METHODS

Culture of the organ of Corti

Cochlear cultures were established using a modification of the Sobkowicz technique (Sobkowicz et al., 1975). Pregnant ICR mice, as determined by the presence of a vaginal plug, were euthanized by cervical dislocation between the thirteenth and sixteenth days of gestation (E13-E16). Individual embryos were removed from the uterus into sterile Heps-buffered saline with Hank’s salts at pH 7.3 (HHBS) and staged according to the normal tables of Theiler (1972) based on the development of digits and the pinnae.
The head was cut along the mid-sagittal plane, the temporal bones were dissected from the skull and the cochleae were isolated. The stria vascularis was removed to expose the developing organ of Corti and each organ was transferred to a 45×50 mm coverglass that had been coated with Cell-Tak (Collaborative Research). The explants were oriented with the sensory epithelium up, covered with 100 µl of culture medium, sealed into Rosenthal chambers and maintained at 35°C. Subsequent media changes were made every 48 hours.

The culture medium contained 20% horse serum, 10% mouse embryo extract and 70% minimum essential medium (MEM) with Earle’s salts, 10 mM Heps, 5 mM L-glutamine, 26 mM sodium bicarbonate, 15 units/ml penicillin, and 0.09 µg/ml amphotericin B at pH 7.3. MEM, horse serum and amphotericin B were obtained from Gibco-BRL. The mouse embryo extract was prepared by homogenizing 15 to 20 mouse embryos at E14 in an equal volume of sterile HBBS. After standing at room temperature for 30 minutes, the homogenate was centrifuged at 10,000 g for 10 minutes. The resulting supernatant was used as mouse embryo extract.

Approximately 12 hours after the cochleae were set in culture, the medium was changed to either 125 µl of medium with 1×10⁻⁸ M all-trans-retinoic acid (RA, Sigma) or 125 µl of control medium containing an equal volume of dimethyl sulfoxide (DMSO), the retinoic acid vehicle. A 1×10⁻⁴ M stock solution of retinoic acid was made in DMSO and was stored in 5 µl aliquots in the dark at −20°C. Individual aliquots were thawed and diluted to the appropriate concentration in culture medium just prior to use.

25 cochleae received media containing RA and 30 cochleae received media containing vehicle. The viability and developmental progress of each culture was assessed daily using differential interference contrast (DIC) microscopy. After 5-7 days in culture, cochleae were fixed by immersion in 2.5% glutaraldehyde in 0.1 M phosphate buffer at pH 7.3. Cytocochleograms were generated from whole mounts of cochleae in glycerol and water (9:1) using a microscope equipped with a drawing tube.

In order to determine whether the addition of exogenous retinoic acid induced renewed proliferation within the sensory epithelium, 1 µCi/ml of [³H]methyl-thymidine (60 Ci/mmol., ICN Biologicals) was added to the culture medium for 8 pairs of control and RA-treated cochleae. After culturing, cochleae were fixed in 4% paraformaldehyde then processed as described. Prior to autoradiography according to a published procedure (Corwin, 1985) and counter-stained with thionin.

Dose-response experiments

Cochleae were removed from 19 mouse embryos at E13 and established in culture. Approximately 12 hours later, the culture medium was changed to a medium that contained retinoic acid at one of the following concentrations: 1×10⁻⁸ M, 5×10⁻⁸ M, 1×10⁻⁷ M, 5×10⁻⁷ M, or to a control medium that contained an equal volume of the retinoic acid vehicle (DMSO). Individual cultures were incubated until stereociliary bundles had developed on the hair cells along the entire length of the sensory epithelium (5 to 7 days). Cultures were then fixed and analyzed as described above. The numbers of inner and outer hair cells in all the supernumerary regions were determined for at least 4 cochleae at each concentration. The mean numbers of inner and outer hair cells in supernumerary regions and the standard error of the mean (s.e.m.) were determined for each concentration.

Temporal dependence

Embryos were removed from 26 timed pregnant mice on one of the following gestational days: E13, E14, E15, E17 and P0 (day of birth). Cochlear cultures were established as described and after 12 hours were changed to either medium containing 1×10⁻⁸ M all-trans-retinoic acid or to the control medium plus vehicle. Cultures were incubated until stereociliary bundles had developed on hair cells along the entire length of the sensory epithelium (3 to 7 days). Each cochlea was then fixed and processed as described.

There was variability in the rate of spontaneous occurrence of supernumerary hair cell regions in control cochleae over developmental time (E14: mean number of inner hair cells in supernumerary regions (mIHC)=3.27±1.65, mean number of outer hair cells (mOHC)=22±12.43; E15: mIHC=1.64±0.52, mOHC=8.38±2.65; E16: mIHC=15.32±5.79, mOHC=99.2±37.50; E18 and P0: mIHC=0.0±0.0, mOHC=0.0±0.0); however, overall the values were small in comparison to the experimental values. To compensate for this variability, the mean numbers from age matched controls were subtracted from the experimental values.

Western blots

Cochlear sensory epithelia, including the organ of Corti, the stria vascularis and the modiolus were dissected from 10 embryos on gestational days 14 and 17 (E14, E17), from 5 neonatal mice on postnatal day 1, and from 5 adults. At least 6 cochleae from each developmental time point were homogenized for each sample in a buffer consisting of 10 mM Tris-HCl, 5 mM MgCl₂, 1% Nonidet P-40, 1 mM 4-(aminomethyl)-benzene-sulfonyl fluoride (AEBSF), and 2 µg/ml aprotinin, at pH 7.4. The samples were combined with equal volumes of sample buffer containing 62.5 mM Tris, 2% sodium dodecylsulfate (SDS), 1% v/v glycerol, and 5% v/v 2-mercaptoethanol, at pH 6.8 and heated at 100°C for 10 minutes. Samples were then centrifuged for 10 minutes at 13,000 g and the resulting supernatant was removed. The total protein concentrations in the samples from cochleae at each developmental time point were determined using a bicinchoninic acid (BCA) assay with bovine serum albumin as the standard (Smith et al., 1985). In order to determine whether the amount of CRABP changed over developmental time, samples containing 25 µg of total protein were loaded into the lanes of a 15% polyacrylamide slab gel (SDS-PAGE) and proteins were separated by electrophoresis (Laemmli, 1970). Protein bands were electrophoretically transferred to nitrocellulose membranes (0.45 µm pore size) (Burnette, 1981). Prior to antibody staining, the samples were blocked in 0.5% nonfat milk in Tris-HCl buffer (10 mM Tris-HCl, 150 mM NaCl, 0.05% polyoxyethylene-sorbitan monolaureate (Tween 20)), at pH 8.0 and 4°C overnight. Membranes were then incubated with a purified monoclonal antibody specific for CRABP (a gift from Dr Jack Saari, University of Washington) at 1:500 dilution for 60 minutes at room temperature with agitation (Saari et al., 1982). This antibody was generated against CRABP I isolated from bovine retina (Crabb and Saari, 1986). However, cross reactivity of this antibody to CRABP II has not been determined and the possibility that this antibody also recognizes CRABP II cannot be eliminated. Primary antibody binding was detected using a monoclonal goat anti-mouse-IgG conjugated to alkaline phosphatase (Promega Inc.) at a 1:1000 dilution for 30 minutes at room temperature. The blots were then incubated with color-producing substrates: toluidine p-nitroetrazolium blue (NBT) and 5-bromo-4-chloro-3-indolylphosphate (BCIP) (Promega Inc.). The apparent molecular masses of labeled bands were determined by comparison with biotinylated molecular weight protein markers run in adjacent lanes (Bio-Rad).

The reporter cell line

The presence of retinoic acid in the developing organ of Corti between E14 and adulthood was determined using an F9 reporter cell line that had been modified to produce β-galactosidase in the presence of retinoic acid (Wagner et al., 1992). This reporter cell line was constructed so that the lacZ gene was driven specifically by...
the retinoic acid response element (RARE). Since the RARE is specific for ligand-bound nuclear retinoic acid receptors, the lacZ gene was susceptible to induction by activated retinoic acid receptors (Wagner et al., 1992). The reporter construct had been shown to be specific for retinoic acid; however the specificity for different isomers of retinoic acid had not been determined (Wagner et al., 1992).

Cochleae were dissected from E14 or E17 embryos or from P0 or adult mice and were placed individually onto confluent monolayers of the F9 cells. Since horse serum and mouse embryo extract contain retinoic acid, the F9 cells and the cochlear explants were maintained in a retinoic-acid-free modified L-15/CO2 medium (Specialty Media Inc). The medium contained glucose at 1600 mg/litre and the N-3 nutrient supplement at 3 ml/litre (Wagner et al., 1992). The F9/cocklear cultures were maintained for 18 to 24 hours at 37°C in a 5.0% CO2 atmosphere. Then, the cultures were fixed with 1% glutaraldehyde in 0.1 M phosphate-buffered saline (PBS) at pH 7.3 for 15 minutes at room temperature. Each culture was then washed twice in PBS and then exposed to 0.2% 5-bromo-4-chloro-3-indolyl-B-D-galactopyranoside (X-Gal) in PBS with 0.15 M NaCl, 0.1 mM MgCl2, 3.3 mM potassium ferrocyanide, 3.3 mM potassium ferricyanide at pH 7.3 and room temperature for 8 to 12 hours so that a blue reaction product would identify those cells that had produced β-galactosidase.

The reporter cell line was also used to test for the presence of retinoic acid in the sacculi of adult mice and in the cochleae and sacculi of hatching chicks. Three sacculi were removed from adult mice and eleven cochleae and seven sacculi were taken from 12-day-old chicks. After dissection, the epithelia were placed in chilled Medium-199 containing Hank’s salts and 25 mM Hapes. The tegmentum vasculosum and the lagena were dissected away from each cochlea and the otolithic membrane and otoconia were removed from each sacculus. The epithelia were then placed on confluent monolayers of the F9 cells with their sensory surfaces oriented downward, so as to contact the reporter cells. The epithelia were incubated in contact with the reporter cells for 24 hours at 37°C and were then fixed and processed as described above for visualization of β-galactosidase.

Several controls were run along with each run of cochlear explants (a total of five separate experiments) to confirm that the reporter cells were synthesizing detectable levels of β-galactosidase only in response to the presence of retinoic acid. All-trans-retinoic acid at $1 \times 10^{-8}$ M was added to a 35 mm plate of confluent F9 cells as a positive control. Two negative controls were used. First, skin from the dorsal surface of the embryonic head, which does not contain retinoic acid, was cultured in separate dishes of reporter cells at the same time as the cochlear explants. Second, E17 cochleae were cultured on modified F9 reporter cells that contained a portion of the reporter gene construct, including the β-galactosidase gene, but which did not have the retinoic acid response element upstream of that gene.

**RESULTS**

Control cochlear explants that were established between E13 and E16 developed a normal sensory epithelium with a single row of inner hair cells and three or four rows of outer hair cells after 5 to 7 days in culture. The pattern and the spacing of the hair cells and supporting cells appeared normal and regular (Figs 1A, 2A, C).

Small regions of supernumerary outer hair cells, which were defined for our purposes as regions that contained 6 or more rows of outer hair cells, were observed in 5 out of 30 control cultures that did not receive exogenous retinoic acid supplements. The overall density of inner and outer hair cells per length of sensory epithelium was similar to published densities of hair cells for adult ICR strain mice (Burda and Branis, 1988). In cochleae that had been cultured for 5 to 7 days, all of the hair cells had recognizable stereociliary bundles, most with the ‘’ or ‘’-type morphology that is characteristic for mature inner and outer hair cells respectively (Corwin and Warchol, 1991). Each hair cell was separated from the neighboring hair cells by the apical portions of supporting cells which also extended below the adjacent hair cells to overlie the basal lamina (Fig. 2A, C).

The developing supporting cells in the cultures closely resembled the Deiter’s cells that developed at those locations in control cochleae in vivo.

**Effects of retinoic acid**

Cultures exposed to medium containing $1 \times 10^{-8}$ M retinoic acid...
acid developed a significantly greater number of supernumerary hair cells than controls (Fig. 3). The supernumerary regions were characterized by six to ten rows of outer hair cells (Figs 1B, 2B,D). The entire sensory epithelium was affected in one culture, but in 24 others the extra rows developed in patches distributed along the epithelium. Inner hair cell duplications frequently occurred adjacent to the regions of supernumerary outer hair cells (Fig. 2B,D). Inner hair cell duplications were rarely observed in control cultures (5 cells in 21 cultures). All of the hair cells in the retinoic-acid-treated cultures, including those in the supernumerary regions, appeared normal. Each hair cell developed a stereociliary bundle and a cuticular plate (Figs 1, 2). As in the normal regions, even the hair cells that developed in the outermost supernumerary rows were separated from each other by intervening supporting cells (Figs 1, 2).

In order to determine whether the treatment with exogenous retinoic acid altered the spatial distributions of the hair cells in the cultures, all the hair cells in five pairs of treated and control cochleae from the opposite ears of the same five E13 mouse embryos were counted (Fig. 4). The cultures that were treated with retinoic acid showed a significant increase in the total number of inner and outer hair cells that had formed. A total of 22 supernumerary regions, which contained 2,189 hair cells, were observed in the five retinoic-acid-treated cochleae. Four supernumerary regions, which contained 249 hair cells, were observed in the five control cochleae. The spatial packing density of hair cells along the length of the epithelium increased in the retinoic-acid-treated cultures, and the ratio of outer hair cells to inner hair cells also increased in the treated cultures (Table 1; Fig. 3).

In order to determine whether the cells that differentiate as supernumerary hair cells were produced by cell divisions occurring after the addition of retinoic acid, tritiated thymidine was added to control cultures and to cultures that were treated with retinoic acid on E14 and E15. Most of the cells that differentiate as hair cells should have passed through terminal mitosis prior to the time of the addition of tritiated thymidine (Ruben, 1967), so the number of labeled hair cells in the control cultures was expected to be low (less than 5%). If the cultures that were treated with retinoic acid had showed an increase in the percentage of hair cells that
were labeled, as compared with controls, then that would have suggested that the addition of exogenous retinoic acid induced renewed proliferation. However, if no increase in the number of labeled hair cells was observed, then this would have suggested that the effect of exogenous retinoic acid was to alter the fate of cells that already existed within the epithelium at the time of retinoic acid addition. In eight pairs of retinoic-acid-treated cochleae and control cochleae (four established on E14 and four established on E15), some fibroblasts and other non-sensory cell types were labeled with an average of 30 to 50 silver grains above their nuclei, but no hair cells were observed with more than two silver grains over the nucleus. There was an absence of labeling even in supernumerary regions that contained as many as ten rows of outer hair cells. The results indicate that, under the culture conditions used, the terminal mitoses for all the cells that differentiated as hair cells had occurred prior to E14, supporting the hypothesis that the administration of exogenous retinoic acid alters the fate of existing cells within the sensory epithelium.

**Dose-response experiments**

The number of inner and outer hair cells in the supernumerary regions progressively increased as the concentration of retinoic acid was increased (Fig. 5). At $1 \times 10^{-9}$ M retinoic acid, there was an average, for each cochlea, of 58 outer hair cells and 9 inner hair cells in the supernumerary regions. The average number of supernumerary hair cells increased to 94 outer hair cells and 13 inner hair cells in cultures treated with $5 \times 10^{-9}$ M, and increased to 353 outer hair cells and 59 inner hair cells at $1 \times 10^{-8}$ M. The response was maintained or increased slightly in cultures treated with $5 \times 10^{-8}$ M (443 outer hair cells, 56 inner hair cells). However, at a retinoic acid concentration of $1 \times 10^{-7}$ M, the number of supernumerary hair cells decreased to 281 outer hair cells and 39 inner hair cells. At $5 \times 10^{-7}$ M retinoic acid, no supernumerary regions were observed, the cultures appeared to be unhealthy and they did not progress through the normal stages of structural maturation. It may be significant that in two of the five cochleae that received that dosage no hair cells were observed after 7 days in vitro.

**Temporal dependency**

The effect of retinoic acid on the development of supernumerary hair cells was directly related to the timing of its addition to the culture medium (Fig. 6). There was a dramatic decline in the number of hair cells in supernumerary regions when the beginning of the retinoic acid treatment was varied progressively between E14 and P1. Addition of $1 \times 10^{-8}$ M retinoic acid to E14 cochleae resulted in an average, for each cochlea, of approximately 60 inner hair cells and 350 outer hair cells in the supernumerary regions, while addition of the same concentration of retinoic acid on E15 resulted in only 15 inner hair cells and 150 outer hair cells in the supernumerary regions. Addition of retinoic acid on E16 resulted in the formation of only 7 inner hair cells and 65 outer hair cells in the supernumerary regions, and addition on E18 or P1 had no effect on the number of hair cells that developed in supernumerary regions, as compared to paired controls.

**Cellular retinoic acid binding protein**

Homogenates of whole cochlear sensory epithelia from different developmental time points were electrophoresed by SDS-PAGE, transblotted and probed for the presence of CRABP using a monoclonal antibody (Saari et al., 1982). The antibody specifically labeled a single band at a location that corresponded to an apparent molecular mass of 16x10$^3$ $M_t$ (Fig. 7). This molecular mass is in agreement with published findings for CRABP I and CRABP II, as well as with the predicted molecular masses based on the deduced amino acid sequences (Saari et al., 1982; Bailey and Siu, 1988). CRABP was detected at comparatively low levels on E14. However, the level of staining for CRABP per microgram of protein increased on E16 and, among the stages tested, it reached the highest level on P1. In this assay, CRABP was not detectable in homogenates of adult cochlear sensory epithelia.

In order to confirm that the apparent lack of binding of anti-CRABP in adult cochleae was a result of decreased expression of the protein and not a result of methodological...
problems, the level of binding of antibodies for CRABP and for α-actinin were compared in samples from P1 and adult cochleae on the same SDS-PAGE gel. The previously described procedure was followed, with one exception; in order to insure adequate binding, the quantity of total protein loaded in the sample containing adult cochleae was twice the total protein loaded for the sample from P1 cochleae (50:25 µg). After separation and transfer to nitrocellulose, the samples were incubated sequentially in anti-CRABP and a monoclonal antibody to α-actinin (Sigma Chemical Co.). Primary antibody binding was detected using the previously described method. The results indicated approximately equal staining intensity at 96×10^3 M_r, a position that corresponds with the known molecular mass for α-actinin (Endo).

![Fig. 4. Representative cytocochleograms of the entire sensory epithelium for control (A) and retinoic-acid-treated (B) cochlear cultures from the two ears of the same embryo. Open circles represent outer hair cells, closed circles represent inner hair cells. Supernumerary regions are marked by gray shading of the outer hair cells.](image)

Table 1. Effects of addition of 1×10^{-8} M retinoic acid to mouse cochlear cultures on E14 as compared with paired controls

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>R.A. treated</th>
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<tr>
<td>Mean length of sensory epithelium (µm)</td>
<td>1700±298 (5)</td>
<td>1932±203 (5)</td>
</tr>
<tr>
<td>Mean number of hair cells</td>
<td>772.6±106.3</td>
<td>1249.6±103.6*</td>
</tr>
<tr>
<td>Mean density of hair cells/µm of S.E.</td>
<td>0.47±0.03</td>
<td>0.66±0.04***</td>
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<tr>
<td>Mean number of inner hair cells</td>
<td>162.4±26.35</td>
<td>236.8±19.29*</td>
</tr>
<tr>
<td>Mean number of outer hair cells</td>
<td>610.2±80.19</td>
<td>1012.8±51.6*</td>
</tr>
<tr>
<td>Mean ratio of OHC/IHC</td>
<td>3.82±0.17</td>
<td>4.28±0.16**</td>
</tr>
<tr>
<td>Mean length of sensory epithelium with</td>
<td>59±33.26</td>
<td>345.6±35.63***</td>
</tr>
<tr>
<td>supernumerary hair cells (µm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean number of hair cells in supernumerary regions</td>
<td>49.8±27.86</td>
<td>437.8±64.26***</td>
</tr>
<tr>
<td>Mean OHC/IHC excluding supernumerary regions</td>
<td>3.63±0.08</td>
<td>3.64±0.10</td>
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*α=0.05, **α=0.01, ***α=0.005.

Based on the results of paired t-tests, the overall length of the sensory epithelium did not change significantly as a result of treatment with retinoic acid, but there was a significant increase in the number of hair cells and in the density of hair cells per micron of sensory epithelium. Also, as a result of the development of supernumerary regions, there was a significant increase the outer hair cell to inner hair cell ratio. If the ratio is calculated with the supernumerary regions excluded, the value is nearly equal to the ratio observed in adult mice (3.24 OHC/IHC) (Burda and Branis, 1988).
and Masaki, 1982). A second strong band was observed at $16 \times 10^3 \text{ M}_r$ in the lane containing cochleae from P1 mice, but no anti-CRABP staining was detectable in the lane containing the homogenate from the adult cochlea.

The retinoic acid reporter cell line
Explants of the organ of Corti from E14 and E17 embryos and from mice at P0 or adulthood were cultured for 18 to 24 hours on a confluent monolayer of the F9 reporter cells. The overall appearance of the explant cultures was good despite having been cultured in a serum-free medium. Approximately 75% of the explants became firmly attached to the F9 cells and, in many cases, fibroblasts grew out from the explants onto the monolayer. The remaining explants appeared to be alive, but they did not adhere to the F9 cells firmly, as a result they were easily dislodged from the F9 cells during fixation and subsequent processing. In these cases, it was still possible to preserve the explant and the F9 cells for later analysis, because each explant was cultured in a separate dish.

17 cochlear explants (8 at E14, 4 at E17, 3 at P0, and 2 from adults) were cultured on the F9 reporter cells. For each of the cultures established from the embryonic or P0 mice, blue cells were observed in the F9 layer in the regions immediately surrounding the explants after the X-gal reaction (Fig. 8). The intensity and number of the blue cells in the vicinity of the explants were distinct from any low-level staining due to a background of endogenous production of $\beta$-galactosidase. For all of the adult cochlear explants, the number and intensity of F9 cells that produced $\beta$-galactosidase was noticeably lower than for all of the embryonic or neonatal explants (Fig. 9A). The pattern of $\beta$-galactosidase staining in dishes containing adult cochlea suggests that

Fig. 5. Dose-response curve for retinoic acid induced supernumerary hair cells. Each data point represents the mean number of supernumerary hair cells for a minimum of 4 cochlear explants. Error bars are s.e.m. Both inner and outer hair cells show the same basic response characteristics. There is a gradual increase in the number of supernumerary hair cells between $1 \times 10^{-9} \text{ M}$ and $5 \times 10^{-9} \text{ M}$ retinoic acid, followed by a rapid increase at $1 \times 10^{-8} \text{ M}$. The number of supernumerary hair cells is constant or increases slightly at $5 \times 10^{-8} \text{ M}$ retinoic acid, but at retinoic acid concentrations of $1 \times 10^{-7} \text{ M}$ or greater, the number of supernumerary hair cells decreases.

Fig. 6. Temporal dependency of retinoic acid induced supernumerary hair cells. On the independent axis, Embryonic Age indicates the age of the organ of Corti on the day of addition of $1 \times 10^{-8} \text{ M}$ retinoic acid. Each data point represents the mean number of supernumerary hair cells from a minimum of 4 cochlear explants. Error bars are s.e.m. For both inner and outer hair cells, there is an approximately exponential decrease in the number of supernumerary hair cells with the age of the culture at the time of retinoic acid addition.

Fig. 7. Western blot analysis for the presence of CRABP in mouse cochleae at different developmental time points. Total homogenate (approximately 25 $\mu$g of protein per lane) of mouse cochlea from E14 (lane 1), E16 (lane 2), P1 (lane 3) and adult (lane 4) were separated on a 15% SDS-PAGE and electroblotted to nitrocellulose. The membrane was incubated with mouse anti-CRABP (1:500 dilution) followed by alkaline phosphatase secondary antibody. Molecular weight markers (Bio-Rad) are indicated on the figure. Note the single band in lanes 1, 2 and 3 at approximately $16 \times 10^3 \text{ M}_r$. This position corresponds to the known molecular mass for CRABP.
retinoic acid may not be present at detectable levels in the sensory epithelium at that stage of life.

Three sacculi from adult mice and 7 sacculi and 11 cochleae from P12 chicks were cultured on the reporter cell lines. In all cases, colored reaction product was present in the reporter cells that had contacted those epithelia, indicating that all of those epithelia contained retinoic acid (Fig. 9B-D). The β-galactosidase labeling appeared more dense in cells that were cultured with sacculi than in cells that were incubated with the adult cochleae of either species. This observation suggests that sacculi of adult mice and chicks may contain more retinoic acid than the mature cochlea.

The strength of the attachment between the explants and the F9 cells appeared to have an effect on the penetration of the X-gal solution into those F9 cells that were located directly beneath the explants. In those cultures in which the explants adhered to the F9 cells firmly, many blue cells were observed at the edge of the explants, but there were relatively few directly beneath the explants (Fig. 8). Similar effects on X-gal penetration have been reported in other fixed tissues (Sanes et al., 1986). In the cultures where an explant had been dislodged from the F9 cells during fixation, a single uniform blue patch was the only region in which there was an increase in the production of β-galactosidase.

Fig. 8. Detection of retinoic acid in embryonic and postnatal cochlear explants using an F9 reporter cell line. (A) Low magnification bright-field image of an E14 cochlea after incubation on a monolayer of the F9 reporter cells for 18 hours. (B) High magnification view of the same sample as in A. Note the blue cells around the outer edge of the explant. (C) The apical half of an E17 cochlea after incubation for 18 hours. (D) The basal half of the same E17 cochlea as in C. (E) The middle turn from a cochlea explanted on P1. (F) The apical third from a different cochlea than in E also explanted on P1. Scale bar in A, 200 µm. Scale bar in B (same for C-F), 100 µm.
over background after the X-gal reaction (Fig. 9C). For each of those cultures, the size of the blue spot approximately matched the size of the explant, as confirmed by positioning the explant over the spot (Fig. 9C).

In order to determine whether the distribution of retinoic acid in the organ of Corti became spatially heterogeneous at later developmental time points, cochleae that were dissected on E17 and P0 were cut into basal and apical (E17), or basal, middle and apical (P0) segments, and cultured separately. The results did not indicate a measurable spatial heterogeneity. Retinoic acid was present in all segments of the cochlea as late as P0 (Fig. 8). However, this particular reporter cell line was engineered to give a maximal response to the presence of even low levels of retinoic acid (Wagner et al., 1992). Therefore, it is possible that any retinoic acid heterogeneity or any gradients in retinoic acid concentration that might have existed would not have been detected by this cell line.

As positive controls, five 35 mm dishes of F9 cells from five separate experiments were exposed to $5 \times 10^{-8}$ M retinoic acid overnight. These dishes were then processed for detection of $\beta$-galactosidase. After the reaction with X-gal, the cells within these cultures were uniformly blue (data not shown). In each of eight negative controls from three separate experiments, embryonic head skin cultured on the retinoic acid reporter cells did not result in the reporter cells becoming positive for $\beta$-galactosidase (data not shown). Three E17 cochleae that were explanted onto monolayers of modified F9 cells, which contained the reporter construct minus the retinoic acid response element, did not result in cells that were positive for $\beta$-galactosidase (data not shown).

**DISCUSSION**

**Effects of exogenous retinoic acid on cochlear development**

In all placental mammals, the organ of Corti normally develops a single row of inner hair cells and three or four rows of outer hair cells, but the results of these experiments suggest that other cells located adjacent to the region of forming hair cells and supporting cells have a latent potential to develop as hair cells and supporting cells at early stages of cochlear development. This conclusion is most dramatically supported by the observation that the addition of exogenous retinoic acid resulted in significant increases in the numbers of cells that developed as hair cells and sup-

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**Fig. 9.** Detection of retinoic acid in adult mammalian and avian hair cell epithelia. (A) Bright-field photomicrograph of a section of an adult murine cochlea after incubation on F9 reporter cells for 18 hours. The X-gal staining appears diffuse because the level of focus has been adjusted to show the sensory epithelium. Note that there is very little staining near the sensory epithelium. (B) An adult murine saccus after incubation on the F9 reporter cells for 18 hours. Note the large region of X-gal staining. (C) A complete avian basilar papilla from a P12 chick after an 18 hour incubation. The tissue became detached during processing and has been repositioned over the stained region. (D) An avian saccus from a P12 chick after an 18 hour incubation. As in B, note the large region of staining. Scale bar in A (same in B), 10 µm. Scale bar in C, 1 mm. Scale in D, 200 µm.
porting cells. In addition, the results strongly suggest that the differentiation of cells as hair cells and supporting cells is influenced through environmental cues.

The normal fates of the cells that develop as supernumerary hair cells are unknown. One possibility is that these cells would normally differentiate as non-sensory cell types within the organ of Corti; another possibility is that these cells would be eliminated during a period of cell death. There appears to be no published evidence to support the hypothesis that a period of cell death occurs during the development of the organ of Corti, but time-lapse video recordings of developing mouse cochlea suggest that a large number of cells within the organ of Corti may be eliminated between E15 and E17 (Kelley et al., in preparation).

Along with the increase in the number of hair cells, the addition of exogenous retinoic acid resulted in an increase in the number of supporting cells. This supports the proposal that the development of these two cell types may be linked. It has been suggested that the cells within the developing sensory epithelium may in effect compete to become committed to differentiate as hair cells, and that once a cell has become committed to the hair cell phenotype, that cell inhibits its immediate neighbors from differentiating as hair cells (Corwin et al., 1991; Lewis, 1991). This lateral inhibition forces the cells surrounding the cell that has become committed to developing the hair cell phenotype to differentiate as supporting cells by default. If that hypothesis is correct, then a potential corollary is that retinoic acid is likely to influence cell fate prior to the determination of cells as hair cells and supporting cells. Retinoic acid may act to create a population of prosensory cells similar to the proneural cells that are created during the development of sensory bristles in Drosophila (Ghysen and Dambly-Chaudiere, 1988; 1989). Once these prosensory cells have been formed, the commitment decisions of hair cells and supporting cells would occur through cell-cell interactions. At an appropriate stage, the addition of exogenous retinoic acid could increase the number of cells within the epithelium that become prosensory, thereby leading to increases in the number of hair cells and supporting cells.

The results of the dose-response experiments agree with previous studies that have demonstrated a narrow functional range of retinoic acid concentrations with high levels of retinoic acid resulting in severe teratogenic effects (Petkovich et al., 1987; Giguere et al., 1987; Langman and Welch, 1967; Kochar, 1973; Cook and Sulik, 1988; Sulik and Dehart, 1988). In addition, the shape of the organ of Corti’s response curve is characteristic of a RAR-, rather than a RXR-, mediated event (Heyman et al., 1992).

There was a rapid decline in the formation of supernumerary hair cells in response to exogenous retinoic acid between E14 and E16. It is possible that sensitivity to retinoic acid may be expressed asynchronously at different points along the cochlea. Differentiation of the organ of Corti occurs as a wave that begins in the basal turn and proceeds apically (Kikuchi and Hilding, 1965; Anniko, 1983; Lim and Anniko, 1985; Kelley et al., 1991). If retinoic acid sensitivity were to occur in a similar developmental pattern, then we would predict that, in an E16 cochlea treated with retinoic acid, the majority of the supernumerary hair cell regions should occur in the apical end of the sensory epithelium. This hypothesis is supported by the observation that 76% of all supernumerary hair cells in the E16 cochleae were located in the apical half of the sensory epithelium (data not shown).

The specific mechanisms by which exogenous retinoic acid influences the commitment of a cell to differentiate as a hair cell are unknown. Bound RARs and RXRs may activate genes that regulate cell determination or differentiation. Retinoic acid influences the expression of several homeobox genes, including Hox 1.6, Hox 2.9 and the zinc finger gene Krox 20 (Petkovich et al., 1987; Giguere et al., 1987; Mangelsdorf et al., 1990; Cho and DeRobertis, 1990; Simeone et al., 1990; Lufkin et al., 1991; Chisaka et al., 1992; Papalopulu et al., 1991; Hunt et al., 1991). Defects in the development of the inner ear have been reported in mouse strains where expression of the Hox 1.6 or Hox 2.9 gene has been disrupted (Lufkin et al., 1991; Morriss-Kay et al., 1991; Chisaka et al., 1992).

Endogenous retinoic acid in the developing cochlea

Previous studies demonstrated the presence of mRNAs for CRABP I, CRABP II, RAR-α, and RAR-β, in the cochlea during embryonic development (Mendelsohn et al., 1991; Dolle et al., 1990; Ruberte et al., 1990; 1993). The results of the experiments using the retinoic acid reporter cell line clearly demonstrate that retinoic acid itself is present in the developing cochlea, but it is not possible to determine whether the retinoic acid in the cochlea was produced by cells there or had diffused or been actively transported into the cochlea from another source. The specific isomers of retinoic acid were not determined; however, the presence of RAR-α and RAR-β, as well as a response curve characteristic of a RAR-mediated response (Heyman et al., 1992), suggests that all-trans-retinoic acid may be the predominant isomer of retinoic acid in the developing cochlear sensory epithelium.

The antibody that was used to detect CRABP was raised against CRABP I, but may have also recognized CRABP II. In situ hybridization studies have demonstrated that mRNA for CRABP II, but not for CRABP I, is present in the mouse cochlear sensory epithelium between E13.5 and E14.5 (Dolle et al., 1990; Ruberte et al., 1993). These results suggest that the antibody binding observed on western blots, at least at E14, may have been to CRABP II. The function of CRABP II is unknown, however it has been suggested that expression of CRABP II along with CRBP I and RAR-β (all retinoic acid-inducible genes) may represent a retinoic acid controlled developmental pathway (Ruberte et al., 1993; Morriss-Kay, 1993; Gustafson et al., 1993).

Retinoic acid in other hair cell sensory epithelia

Retinoic acid was also detected in the chick’s basilar papilla, as well as in the vestibular epithelia from chicks and mice. The apparently high level of retinoic acid in the vestibular epithelia may be related to the observation that those epithelia are capable of producing new hair cells throughout the life of the organism (Jorgensen and Mathiesen, 1988; Roberson et al., 1991; Weisleder and Rubel, 1992; Forge et al., 1993; Warchol et al., 1993). Retinoic acid was not detected in the sensory epithelium of the adult mammalian
cochlea, a tissue that is not known to be capable of spontaneous hair cell regeneration (reviewed in Corwin and Warchol, 1991). Spontaneous hair cell regeneration has been observed in cultures of embryonic organs of Corti of mice (Kelley et al., 1991; Kelley, 1993) and it has recently been reported that addition of retinoic acid stimulates hair cell regeneration in early postnatal rat cochleae (Lefebvre et al., 1993).

Development of the organ of Corti

Based on the results of these experiments and other recent work in our laboratory (Kelley et al., 1991), we propose a time line for the developmental events in the organ of Corti of the mouse (Fig. 10). As terminal mitoses occur (E13-E14) (Ruben, 1967), all of the cells within the developing organ of Corti are likely to remain uncommitted. Immediately following terminal mitosis, the uncommitted daughter cells are likely to become sensitive to environmental cues that will influence their determination. In particular, some or all of the cells in the epithelium may produce RAR-α, RAR-β, CRBP I and CRABP II, and would be sensitive to retinoic acid (Dolle et al., 1990; Ruberte et al., 1990, 1993). During that time period, retinoic acid is present in the cochlea. The effect of binding of retinoic acid to its receptors would be to produce a subpopulation of cells within the developing sensory epithelium that are competent to commit to develop as hair cells or supporting cells. We have proposed to define this subpopulation of cells as ‘prosensory’ cells. Normally, the effect of retinoic acid would be limited to a narrow band of cells that runs along the abneural edge of the epithelium. The factors required for the formation of such a limited distribution of retinoic acid are unknown, but this type of distribution could occur through retinoic acid production by a specific population of cells that also run along the edge of the epithelium, or via diffusion from a structure, such as the spiral blood vessel, that runs underneath the sensory epithelium. Once a region of prosensory cells is defined, the subsequent commitment of individual cells within this population to become either hair cells or supporting cells would be determined through lateral cell-cell interactions (Corwin et al., 1991; Lewis, 1991).

In the organ of Corti, there are several distinct supporting cell phenotypes, suggesting that a sequence of cell-cell interactions may be required to produce its distinctive pattern. In fact, the apparently unique phenotype of the cell at each position along the width of the organ of Corti suggests that there may be a positional identity for each cell that is determined through sequential interactions with its neighbors, which may be analogous to interactions in other developing organisms (Tomlinson and Ready, 1987; Cagan and Zipursky, 1992). If these hypotheses are correct, then they suggest that the determination of specific cell phenotypes within the organ of Corti should occur as a wave that progresses across the width of the organ. Several authors have noted that during normal development of the organ of Corti, inner hair cells differentiate prior to outer hair cells, and first row outer hair cells differentiate prior to those in the second row, which differentiate prior to those in the third (Bredberg, 1968; Lim and Anniko, 1985). Overall, these data and hypotheses would suggest that commitment within the organ of Corti occurs along two gradients, a basal-to-apical wave that determines the population of prosensory cells, and a neural-to-abneural gradient that determines individual cell phenotypes.

The results of these experiments contribute to growing evidence of a role for retinoic acid during the development of the auditory system. Several classes of RAR receptors are present during development of the mouse ear (Dolle et al., 1990; Mendelsohn et al., 1991), and the addition of retinoic acid to stage 18 chick otocysts causes inhibition of cell proliferation and induces early differentiation of several structures including the sensory epithelium (Represa et al., 1990). Also, as discussed, a recent finding suggests that exogenous retinoic acid can stimulate hair cell regeneration in postnatal rat cochleae (Lefebvre et al., 1993).

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Fig. 10. Time line of cochlear development during embryogenesis in the mouse. The four proposed phases of cellular determination and differentiation of the sensory epithelium are illustrated. Note that for each of the final three phases there is a basal-to-apical gradient of onset that may result in overlap between the end of one phase in the apex of the cochlea and the beginning of the next in the base. See the text for further details.


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