Regeneration and Mammalian Auditory Hair Cells

Until recently, it was assumed that deafness in mammals resulting from the loss of auditory receptors (by administration of ototoxic drugs or by physical injury) was permanent, although such is not the case for cold-blooded vertebrates and birds (1). P. Lefebvre et al. report that retinoic acid (RA) stimulation of rearing hair cells on the cochleas of neonatal rats maintained in vitro after ototoxic poisoning (2). Finding a molecule or molecules that could initiate regeneration of hair cells would have a great impact on the treatment of deafness.

To test whether RA can stimulate hair cell regeneration, we performed tests with RA and neomycin separately and then compared the effects of RA and neomycin together (Fig. 1). Corti explants from 3-day-old rats were maintained for 10 to 13 days in vitro with fetal bovine serum (FBS) and N1 (3). Normal cochlear organization with one row of inner hair cells (IHCs) and three rows of outer hair cells (OHCs) was observed by phalloidin staining (4). The apical surface of hair cells with their cuticular plates and stereocilia bundles were normal. However, some qualitative differences were observed compared to in vivo control cochleae. For example, stereocilia of IHCs were more elongated and stereocilia of OHCs sometimes still had an immature shape, irrespective of in vitro conditions. The mean number density of hair cells in vitro compared to 3 days after birth (DAB) in vivo control cochleae is slightly lower, because an increase in the intercellular spaces between hair cells. No dying hair cells were observed. Postnatal cochlea explants may have the potential to produce supernumerary hair cells in vitro, (5), but previous observations (6) have shown that explants from postnatal cochleae, even from the apical part, do not reliably produce supernumerary hair cells. Moreover, our control explants from three DAB, maintained 10 to 13 days in vitro, have less hair cells whatever the origin of the explant in the cochlea (Fig. 2).

We investigated whether RA is able to stimulate production of supernumerary hair cells in postnatal explants (7). Explants exposed to RA alone for 7 days (8) did not show an increase in the number of hair cells and no extra rows of hair cells were observed (Fig. 1).

Is antibiotic treatment, as used by Lefebvre et al. (2), able to destroy virtually all hair cells? After treatment (9), the apical surface of the former sensory epithelium is replaced bypolygonal cells. Few hair cells were visible in the basal turn (Figs. 1 and 2A). However, explants from the last half of the apical turn showed remaining hair cells. Over half
of the hair cells survived the drug treatment compared to control explants (Fig. 2B). The cochlear area containing surviving cells varied in length from 160 to 1800 μm (the average was 750 μm).

Explants from 34 cochleas were treated with neomycin and subsequently treated with 10−8 M RA for 7 days. The explants were examined by phalloidin staining and by scanning electron microscopy (SEM) (10). The surface of the former sensory epithelium in all cultures showed the same cells with polygonal borders as observed with neomycin treatment alone (Fig. 3, A and B). We did not observe at any time, with any explantation methods or culture solutions, any signs of hair cell regeneration caused by RA. There were no differences between explants treated with only neomycin and those treated with neomycin plus RA. Phalloidin-stained dots were often present in the center of cells that replaced lost hair cells in both conditions. Quantitative analyses (Fig. 1) indicated that few hair cells persisted in the basal turn after either treatment, and a high percentage of hair cells in the last half of the apical turn survived.

Differentiation and regeneration are likely to depend on a number of factors such as the culture methods, the presence of chemical mediators, and the microenvironment to which the explant is exposed. Some of these factors could explain why we were not able to replicate the observations of Lefebvre et al. (2). One answer may come from technical differences. However, our conditions did preserve the organotypy of the explant, as well as hair cell integrity and the afferent innervation (11).

One difference may be culture supports; we used dialyzed rat tail collagen or glass and plastic coated with poly-D-lysine, while many other coating substances exist such as other molecules involved in the extracellular matrix (12). We know of no information about the effect of such substances on the preservation of cochlear organotypy and hair cell integrity, although there are many observations concerning the good preservation, over many days, in vitro of hair cells in collagen matrices (13).

A further critical point is the culture medium. One medium corresponds as closely as possible to the one used by Lefebvre et al. (2), with a cocktail of hormones mainly used for neurons in vitro. Despite this, we did not find indication of hair cell regeneration due to RA, and there was negligible difference in these cultures compared to the use of other media. We were unable to find hair cell regeneration with different explanation techniques and different media; possible minor differences between our culture techniques and those of Lefebvre et al. (2) seem unlikely to account for the difference in the results.

The previous evidence presented of hair cell regeneration in figure 3, C and D, of the report by Lefebvre et al. (2) is not convincing. An apical cell surface with microvilli and a kinocilium cannot be taken as proof of hair cell regeneration because cells of Kolliker’s organ (progenitor cells of hair cells) and young hair cells as well as supporting cells present microvilli and a kinocilium. The kinocilium was seen from the embryonic stage to at least 1 week after birth in rat (14). Moreover, cells with polygonal shape after only neomycin treatment also presented a kinocilium (Fig. 4).

Finally, the survival of apical hair cells must be considered as a possible reason for the difference between our results and those reported earlier. We and others (15) have
shown that hair cells from the apex of the cochlea are less sensitive to neomycin and other ototoxic drugs (Figs. 1 and 2B). It is possible that remaining apical hair cells after neomycin treatment could have been taken as regenerated cells in Lefebvre’s study (2).

With regard to auditory receptors, regeneration may be possible. However, how hair cells differentiate from progenitor cells needs to be understood first, as well as the signals responsible for it. The finding of spontaneous supererythroid hair cells after explantation of fetal mammal cochleae (6) looks promising. The nature of the trigger signal of this enhanced proliferation remains to be investigated in order to design new approaches to regeneration strategies in adult mammals which finally may become applicable in humans.

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REFERENCES AND NOTES


3. Sprague-Dawley rat cochleae were explanted 3 days after birth (DAB). Cultures were prepared according to several procedures because the protocol described by Lefebvre et al. (2) was not complete. First, we used the recently described organotypic method (D. Rastel, A. Abdou, D. Dahl, R. Romand, J. Neurosci. Methods 47, 123 (1993), which gives good results on the preservation of the organotypy of the explant including its innervation. The distinguishing feature of this method is that the explants are put on a drop of photo-crosslinked collagen floating over a feeding medium. In addition, we used other procedures; explants were placed in a 24-mm COSTAR transwell-coll transparent collagen-coated membrane insert with 0.45-μm pores, or on glass lamellar and plastic lamellae (Teflon-coated) with poly-D-lysine. In all cases, the spiral lamina was dissected in minimum essential medium. Stria vascularis and Reissner’s membrane were removed. The spiral lamina was cut in two pieces (base included the hook and the first turn; apex the second and apical turns) and each part was cultivated in a separate well. Explants were cultivated with two different batches of heat-inactivated fetal bovine serum (Sigma F 4135). For some experiments, we used Dulbecco’s modified Eagle’s medium (DMEM) (Sigma D1152) 50%/Ham’s balanced salt solution (Sigma H6135) 40% with high glucose and L-glutamine. In experiments that served as a basis for Fig. 1, we used the same feeding solution as described by Rogister et al. (4). L-glutamine and 1.6% L-glutamine. Cultures were changed every days.

4. For tetramethylrhodamine isothiocyanate (TRITC)-labeled phalloidin (Sigma) staining, explants were fixed 45 min in 4% paraformaldehyde in phosphate-buffered saline (PSS) and after washing stained with phalloidin (3 μg/ml in PSS) for 45 min at room temperature. Observations were made with a Nikon microscope with a blue excitation filter.


8. Control explants with normal cochleae were cultured simultaneously using the same procedures and medium as described (3). After 4 days in culture, 10-8 M RA (all-trans, ref. R. 2065, Sigma) was added for 6 to 9 days. Two different batches of RA were used, and contact with light was avoided as much as possible.

9. Explants DAB from 25 cochleae, after 48 hours in vitro were exposed to neomycin 10-3 M for an additional 48 hours and seven more days in culture with different neomycin-free feeding solution. This should have been an effective dose for the destruction of 99% of auditory hair explants in organotypic cultures per Lefebvre et al. (2).

10. Cultures were fixed for 1 to 4 hours in 2% (w/v) paraformaldehyde-2% (v/v) glutaraldehyde buffered in a 0.1 M Sorenson phosphate buffer, (pH 7.4). After four washes and postfixation with 2% (w/v) osmium tetroxide in 0.1 M phosphate buffer, the explants were dehydrated with ascending concentrations of ethyl alcohol and acetone before being critical point-dried with liquid CO2, and plated with gold. The specimens were observed with a Cambridge stereoscop 360 SEM.


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Response: The fact that Chardin and Romand did not replicate the results of our report (1) may be explained by essential differences in technique. The organotypic explants in our study were maintained as free floating cultures of whole Corti’s organs. In a series of 45 cultures (where the basal, mid, and apical segments were assayed for hair cell counts) these explants consistently showed regeneration-repair of the auditory sensory epithelium (1). The cultures of Chardin and Romand were divided into three portions, thereby introducing additional trauma to these explants. In our initial attempts to stimulate regeneration-repair of otoxin-damaged auditory hair cells, we used organotypic cochlea cultures fixed to either a polyvimidine-coated plastic substratum or a collagen type I matrix to allow for the sequential observation of any regeneration-repair that occurred in response to treatment with retinoic acid (RA) and fetal calf serum (FCS).

None of the 30 cultures grown while fixed to a substratum showed signs of regeneration or repair despite the use of the same medium and supplements that were described in our report (1). The explants in the study of Chardin and Romand were all adhered to a substratum. Adherence to a substratum, and most particularly interaction with matrix molecules such as collagen type I, can have profound effects on cellular behavior and even result in changes in cellular phenotype, for example, epithelial-mesenchymal transformation (2). There are also differences in the culture medium that could effect the outcome of the regeneration-repair process. We have found that handling of the insulin supplement and the selection of batches of FCS to be used as a growth supplement affected our results (3).

Insulin has been shown to be an important factor for potentiating the stimulation of cell proliferation by two members of the epithelial growth factor (EGF) family. EGF and transforming growth factor-α (TGF-α), in organotypic cultures of utricles (4); therefore, proper handling of insulin would be a critical factor. To overcome the problem of variable quality of FCS batches, we have attempted to replace serum with a growth factor.

Western blot studies of noise- and ototoxicity-induced juvenile chick cochlea suggest that a protein with TGF-α-like immunoactivity may play a role in the regeneration of chick auditory hair cells (5). Initial histological studies localized EGF receptor to the inner and outer hair cells of the 3-day-old (PP) rat Corti’s organ and free floating 3 PP rat organ of Corti cultures exposed to neomycin 10-3 M for 48 hours showed immunolocalization of EGF receptor to the area of ototoxic damage (6). Two separate studies have also shown that TGF-α may participate in the regeneration-repair of vestibular hair cells in mammals (4, 7). These observations prompted us to test whether any members of the EGF family of trophic factors could be substituted for FCS.

A series of 3 PP rat Corti’s organ cultures were exposed for 48 hours to neomycin 10-3 M followed by an additional 8 days in Dulbecco’s modified Eagle’s medium + N + glucose (8) plus RA (10-8 M) and either EGF, TGF-α, platelet derived growth factor (PDGF), or basic fibroblast growth factor (bFGF) at varying concentrations, in 10 to 500 ng/ml amounts (9). Control cultures

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