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, hair cells differentiate from progenitor cells needs to be understood first, as well as the signals responsible for it. The finding of spontaneous supernumerary 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. Abdouh, D. Dahl, R. Romand, J. Neurosci. Methods, 47, 123 (1993), which gave 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-reacted collagen floating over a feeding medium. In addition, we used other procedures; explants were placed in a 24-mm COSTAR transwell-coil transparent collagen-coated membrane insert with 0.45-μm pores, or on glass lamellae and plastic lamellae (ThermoX) 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 D 1152) 50% Hank's balanced salt solution (Sigma H 6135) 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 Ro-geliet et al. (2) and Lefebvre et al. (2) (DMEM-DF 1152: 1:1 mixture Sigma D 86900) plus a cocktail of hormones (N1) usually used for neuron cultures with final 0.6 g of D-glucose and 4 mM L-glutamine. Cultures were changed every 3 days.

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


8. Control explants with rat cochleae were cultured simultaneously using the same procedures and mediums as described (3). After 4 days in culture, 10-4 M RA (all-trans, ref. R. 2025, 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. Explants 3 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 cells in explants in organotypic cultures per Lefebvre et al. (2).

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

aptification of ototoxic-damaged auditory hair cells, we used organotypic cochlea cultures fixed to either a polynor-thine-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 urocytes (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 ototoxic lesioned juvenile chick cochleae 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 + N2 + glucose (8) and 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
not exposed to neomycin showed maintenance of hair cell integrity (Fig. 1A), whereas cultures treated with neomycin 10⁻³ M for 48 hours followed by either 8 days in defined medium (Fig. 1B) or 8 days in defined medium supplemented with RA 10⁻⁸ M showed almost complete destruction of all hair cells in the sampled midportion of Corti’s organ. In agreement with the observations of Chardin and Romand, we observed that there were auditory hair cells that survived the 48 hours of exposure to neomycin (10⁻³ M) located only in the most apical portion of Corti’s organ. Therefore, these apical areas were excluded from our counts of stereocilia bearing cells. Addition of EGF, PDGF, or bFGF (10 to 500 ng/ml) to the RA 10⁻⁸ M supplemented medium did not result in any detectable regeneration-repair of hair cells. Addition of TGF-α at concentrations of 10 to 50 ng/ml (9) resulted in the presence of cells bearing disorganized bundles of stereocilia in the mid-portion (Fig. 1C) of the explants (Table 1). Retinoic acid could be entirely eliminated in these cultures without impairment of the TGF-α induced hair cell regeneration-repair process, however higher doses of TGF-α were needed to achieve a similar degree of regeneration-repair (Table 1). This suggests that retinoic acid potentiates, but does not initiate, the regeneration-repair process observed in these cultures. Several recent studies on epithelial regeneration have observed the interaction of TGF-α-EGF receptor in regeneration-repair of gastric mucosa, liver, and kidney (10), thus setting a biological precedent for our observations.

To determine if the TGF-α initiated regeneration-repair process is the result of cell division as shown for avian hair cell regeneration (11), TGF-α treated organotypic cultures were labeled with bromodeoxyuridine (BrdU) (12). Uptake of BrdU was low in both control and neomycin exposed cultures (Table 2). A 19-fold increase in BrdU labeling was seen in the combined epithelial and mesenchymal layers of ototoxic exposed organ of Corti explants that were treated with TGF-α during the period of BrdU exposure (Table 2), however, no labeled hair cells have been observed in serial sections of explants. This supports our earlier observations that treatment with cytosine arabinoside could inhibit the regeneration-repair process (1). A mitotic event may be involved in the regeneration-repair process, but the regenerated-repaired auditory hair cells themselves do not appear to be a direct product of a mitotic event. There may be a fundamental difference between the process occurring in our cultures (1) and the process of avian hair cell regeneration (11).

It appears that the regeneration-repair observed in our cultures is mediated by TFGFa and the EGF receptor and that retinoic acid modulates, but does not initiate this process. This data potentially provides further clarification as to why Chardin and Romand did not reproduce our results (1). Potentially, the serum used in their cultures may not have had adequate levels of TFGFa. With the use of the free-floating culture system under the conditions described, we have consistently observed the regeneration-repair of stereocilia bearing cells in hundreds of explants using either FCS 10% + RA 10⁻⁸ M or TGF-α + RA 10⁻⁸ M as supplements. Whether or not this process represents regeneration from a stem cell population or a regenerative-repair process as reported by Sobkowicz (13) remains unclear, as does whether or not this process can occur in the adult animal. However, in agreement with Chardin and Romand, we believe that an increased understanding of the molecular basis of hair cell differentiation signals and the process of hair cell regeneration-repair are essential for progress in this field.

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**Table 1.** Effect of TGF-α concentration with and without retinoic acid 10⁻⁶ M on the number of hair cells in the mid-portion segments of ototoxic-damaged Corti’s organ explants.

<table>
<thead>
<tr>
<th>TGFα (ng/ml)</th>
<th>Hair cells per millimeter*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>−RA, 0 M</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>29 ± 6†</td>
</tr>
<tr>
<td>25</td>
<td>145 ± 29</td>
</tr>
<tr>
<td>50</td>
<td>330 ± 52</td>
</tr>
<tr>
<td>Unlesioned</td>
<td>428 ± 47</td>
</tr>
</tbody>
</table>

*Hair cell counts were determined by averaging total number of stereocilia bundles in three 250-µm segments of the mid-portion of Corti’s organ explants. Number of specimens per group is 15 explants. † ± Sample standard deviation.

**Table 2.** Cells labeled with BrdU in whole mount cultures of 3 PP rat Corti’s organ Corti explants.

<table>
<thead>
<tr>
<th>Culture condition</th>
<th>BrdU-labeled nuclei/×40 field*</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>2.7 ± 5.2†</td>
<td>—</td>
</tr>
<tr>
<td>Neo &gt; DMEM</td>
<td>3.8 ± 7.4</td>
<td>P &gt; 0.05</td>
</tr>
<tr>
<td>Neo &gt; DMEM+TGF-α</td>
<td>53.1 ± 28.4</td>
<td>P &lt; 0.001</td>
</tr>
</tbody>
</table>

*Each group represents the average of counts from 12 specimens. Four ×40 fields were counted from each whole mount specimen representing the basal, mid, and apical portions. † ± Sample standard deviation.
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3. Each of the N1 components was prepared separately as either 500 × (insulin, progesterone) or 1000 × (putrescine, transferrin, Na₂SeO₄) solutions. These N1 components with the exception of insulin were combined into 1.25-mL aliquots and stored at −20°C for a maximum storage period of 1 mon. Insulin stock solution was acidified with 1 N HCl until the suspension went into solution and was stored at 4°C. (Insulin solution cannot be frozen and must be prepared fresh every week. We sample six batches of FCS at a time, and of these only one on average is suitable as a culture supplement.) FCS batches were selected with the use of several neuronal survival and cell proliferation assays (G. Moonen, University of Liège, Belgium). All trans retinoin acid (Sigma, St. Louis, MO) stock solution was prepared at a concentration 0.01 M in ethanol, stored in a light-proof container at −20°C, and thawed immediately before use. RA was freshly diluted each time the medium was exchanged and cultures were protected from light.

9. Cort’s organ explants from 3-day-old rats were set up as previously described (1). The explants were exposed to neomycin (10⁻³ M) in defined medium (DMEM + N1 + glucose) for 48 hours and subsequently incubated for another 8 days in defined medium; defined medium supplemented with RA 10⁻⁸ M and growth factor; or defined medium supplemented with only TGF-α. Growth factors: PDGF, EGF, and bFGF (R&D Systems, Minneapolis, MN) were added in concentrations of 10, 25, 50, 100, and 500 ng/ml defined medium. After 10 days in vitro cultures were fixed, stained with FITC-phalloidin and analyzed with the aid of Bio-Rad MRC 600 confocal microscope.
12. Cort’s organ explants from 3-day-old rats were prepared as previously described (1). Explants were cultured for 48 hours in either DMEM + N1 + glucose or in this defined medium plus neomycin 10⁻³ M. During the second 48 hours of culture, explants were either exposed to defined medium plus BrdU [2 μg/ml] or BrdU plus a supplement of TGF-α (25 ng/ml).
After 4 days of culture, all explants were methanol fixed, permeabilized with a solution of Tween 80 and Sigma, St. Louis, MO) stained with a monoclonal antibody against BrdU, mounted in glycerin and analyzed for immunolabeled nuclei (counts per ×40 field) in the area of Cort’s organ with the use of a Zeiss Axiopt microscope.

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Response
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