Induction of Cell Proliferation in Avian Inner Ear Sensory Epithelia by Insulin-Like Growth Factor-I and Insulin

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ABSTRACT

Postembryonic production of inner-ear hair cells occurs both normally and after insult in lower vertebrates and avians. To determine how this proliferation is controlled, several growth factors were tested for effects on progenitor-cell division in cultured avian vestibular sensory epithelium. Mitogenic effects of bombesin, epidermal growth factor, insulin-like growth factor-I (IGF-I), insulin, and transforming growth factor-α were assayed in organotypic cultures of utricles from the mature, undamaged (normal) chicken inner ear. Tritiated thymidine and autoradiographic techniques and 5-bromo-2′-deoxyuridine (BrdU) immunocytochemistry were used to identify cells synthesizing DNA. IGF-I stimulated DNA synthesis in the vestibular sensory receptor epithelium in a dose-dependent manner. DNA synthesis was also stimulated by insulin. These results suggest that stimulation of the IGF-I receptors by IGF-I or insulin binding stimulates cell proliferation in the mature avian vestibular sensory epithelium. J. Comp. Neurol. 380:262–274, 1997. © 1997 Wiley-Liss, Inc.

Indexing terms: vestibular; hair cell regeneration; growth factors; insulin-like growth factor-I

Hair cells are the sensory receptors of the auditory, vestibular, and lateral-line end organs and are involved in the detection of sound, acceleration, and substrate vibration. Sound is transduced by hair cells in the cochlea, and hair cells in the vestibular sensory receptor epithelia detect head movements. New hair cells are produced throughout life in the ears and lateral-line organs of fish, amphibians, and birds either during continued growth (Tester and Kendall, 1969; Corwin, 1981, 1983, 1985; Jørgensen, 1981; Popper and Hoxter, 1984, 1990) or through turnover (Jørgensen and Mathiesen, 1988; Roberson et al., 1992; Weisleder and Rubel, 1993). Inner-ear hair cells are damaged by disease, infection, exposure to loud sound or ototoxic drugs, and processes associated with aging. It is now well established that fish, amphibians, and birds have the capacity to regenerate hair cells after damage (Stone, 1933, 1937; Cotanche, 1987; Cruz et al., 1987; Corwin and Cotanche, 1988; Ryals and Rubel, 1988; Baird et al., 1993a,b; Lombarte et al., 1993; Weisleder and Rubel, 1993). The regenerated hair cells become innervated and mediate functional recovery (for review, see Cotanche et al., 1994). Mature mammals may have a low level of reparative capacity in their vestibular sensory epithelia (Forge et al., 1993; Warchol et al., 1993; Rubel et al., 1995; Tanyeri et al., 1995; Kuntz and Oesterle, 1996), but regenerative hair-cell replacement does not normally occur in the auditory epithelium (Sobkowicz et al., 1992; Roberson and Rubel, 1994). Supporting cells within the sensory receptor epithelium (SE) are the probable progenitors of the postembryonic hair cells (Corwin and Cotanche, 1988; Girod et al., 1989; Balak et al., 1990; Raphael, 1992; Jones and Corwin, 1993; Weisleder and Rubel, 1993; Stone and Cotanche, 1994; Tsue et al., 1994).

The factors responsible for controlling the proliferation and differentiation of hair-cell progenitors in either developing, renewing, or regenerating inner ears remain to be determined. Most likely, mechanisms that regulate cell proliferation in hair-cell progenitors are similar to the overwhelmingly conserved mechanisms that control proliferation in other eukaryotic cells, and mitogenic growth factors undoubtedly play a fundamental role. Several growth factors are known to be mitogenic for the developing otocyst as a whole, but little is known regarding factors

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mitogenic specifically for hair-cell progenitors. Epidermal growth factor (EGF), platelet-derived growth factor (PDGF), insulin-like growth factor-I (IGF-I), and bombesin are mitogenic for the chick otocyst, and several of these factors are potentiated by insulin (Represa et al., 1988; León et al., 1995). Basic fibroblast growth factor (bFGF) has a mitogenic effect and plays a role in inducing the invagination of the chick otic vesicle in vitro (Represa et al., 1991). Antisense oligonucleotides and antibodies targeted against *int*-2, which is an FGF-related proto-oncogene, inhibit proliferation and formation of the otic vesicle (Represa et al., 1991). It has been suggested (Lefebvre et al., 1993) that retinoic acid may act synergistically with serum to stimulate proliferation in the developing auditory SE (the organ of Corti) of drug-damaged neonatal rats. However, Chardin and Romand (1995) were unable to find any retinoic acid-induced stimulation of proliferation in the developing organ of Corti.

With regard to the mature inner ear, recent reports indicate that transforming growth factor-α (TGF-α) stimulates a low level of proliferation in normal (Yamashita and Oesterle, 1994, 1995) and drug-damaged (Lambert, 1994) murine vestibular SE grown in culture. Its mitogenic effect is potentiated by insulin (Yamashita and Oesterle, 1995), and TGF-α supplemented with insulin stimulates cell proliferation in the in situ vestibular sensory epithelium (Kuntz and Oesterle, 1996). EGF, when it is supplemented with insulin, also stimulates a low level of proliferation in normal mature murine vestibular SE grown in culture (Yamashita and Oesterle, 1995).

At present, virtually nothing is known regarding the factors controlling the proliferation and differentiation of hair-cell progenitors in the mature avian inner ear. Mature avian inner ears are useful structures to address problems of hair-cell genesis and differentiation for several reasons. First, the regenerative capabilities of the mature avian inner ear are extensive, and they are well characterized (Corwin and Cotanche, 1988; Ryals and Rubel, 1988; Weisleder and Rubel, 1993). Second, there is continued hair-cell genesis in the mature avian vestibular epithelium (Jørgensen and Mathiesen, 1988; Roberson et al., 1992); therefore, the cellular environment must have the constituents necessary to sustain proliferation and differentiation of hair-cell progenitors. Finally, studies of the effects of putative mitogenic growth factors on the avian vestibular epithelium will assist in better understanding how sensory-receptor cell numbers are regulated in continually renewing and regenerating tissues and will aid in better understanding the roles of growth factors in the development and regulation of sensory receptors.

IGF-I (formerly referred to as somatomedin C) is one member of a family of structurally related peptides that also includes insulin-like growth factor-II (IGF-II) and insulin. IGF-I is a growth factor involved in regulating growth and development of the whole organism as well as differentiated functions in specific tissues, whereas insulin is primarily a metabolic hormone involved in regulating carbohydrate, fat, and protein metabolism. The structure of IGF-I is very similar to that of insulin, and IGF-I shares about 50% sequence identity with proinsulin (Rotwein, 1991). Functions of IGF-I and insulin are mediated by specific tyrosine kinase receptors expressed on the cell surface. The insulin and IGF-I receptors, although they are separate gene products, are very similar (Czech, 1989). Actions of IGF-I are mediated by the type I IGF receptor, which binds IGF-I with high affinity and binds IGF-II with slightly lower affinity. The type I IGF receptor also binds insulin with an affinity that is 500–1,000 times lower, which explains, in part, the well-known role of insulin as a growth factor at high concentrations (for reviews, see Lowe, 1991; Werner et al., 1992). The insulin receptor binds insulin with high affinity and also binds IGF-II (with a tenfold lower affinity) and IGF-I (with a 50- to 100-fold lower affinity).

IGF-I is synthesized by a number of tissues in the body, including the central nervous system (CNS) and muscle, but the majority of circulating IGF-I is secreted from the liver (Daughaday and Rotwein, 1989; Rechler and Nissley, 1990). Cell types that are known to express IGF-I include hepatocytes, neurons, astrocytes, fibroblasts, vascular smooth and skeletal muscle satellite cells, Schwann cells, macrophages, and neutrophils (for review, see Lewis et al., 1993). Although circulating IGF-I proteins can act in an endocrine mode, most tissues synthesize IGF proteins, which then can act locally in an autocrine/paracrine manner (Lewis et al., 1993). Proliferation of a variety of cell types, at least in culture, requires the presence of IGF-I (e.g., fibroblasts, neuroblasts, osteoblasts, hemopoietic cells, astroglia, satellite cells, neuronal cells, retinal pigment epithelial cells, and others; for reviews, see Goldring and Goldring, 1991; Macaulay, 1992; Baserga and Ruben, 1993).

To investigate the signals that regulate the mitotic activity of hair-cell progenitors in the mature avian vestibular epithelium, we assayed IGF-I, insulin, bombesin, EGF, and TGF-α for effects on hair-cell progenitor proliferation. Here, we report that IGF-I and insulin are mitogenic for vestibular SE progenitor cells in vitro. Preliminary accounts of portions of these data have been presented in abstract and summary forms (Oesterle et al., 1994; Oesterle and Rubel, 1996).

MATERIALS AND METHODS

Animals

Eight- to-eighteen-day-old White leghorn chickens (*Gallus domesticus*, H and N, Redmond, WA) served as experimental subjects. Animals were killed by rapid decapitation, and their utricles were dissected free from the head by sterile technique. Entire utricles were cultured. All experiments were approved by the University of Washington Animal Care Committee under PHS grants DC00395 and DC02388.

Growth factors and hormones

Bombesin and insulin were obtained from Sigma (St. Louis, MO). EGF was purchased from Sigma and Collaborative Biomedical Products (Bedford, MA), IGF-I was obtained from GIBCO (Grand Island, NY) and Boehringer Mannheim (Indianapolis, IN), and TGF-α was purchased from Collaborative Biomedical Products.

Organ culture

Previously described culture techniques (Oesterle et al., 1993), which have been demonstrated to maintain sensory receptor epithelia from the mature avian inner ear for a 7-day period, were employed. Isolated utricles were placed free floating into wells of 24-well tissue culture plates filled

with 1.5 ml of basal medium Eagle (BME) supplemented with Earle's balanced salt solution, 0.5% D-glucose, and 0.1% fetal bovine serum (FBS). All standard medium components were from GIBCO.

Growth factor effects on proliferation

To examine the efficacy of a growth factor on hair-cell progenitor proliferation, growth factor (0.1–200 ng/ml) was added to the experimental cultures at the start of the culture period. Growth factor was not added to the control cultures, which were otherwise cultured identically to the experimental cultures. A cell proliferation marker, [methyl-^{3}H]thymidine (1 mCi/ml; 5 Ci/mmol as supplied, Amersham (Arlington Heights, IL); 1 Ci = 37 GBq), was added to all cultures at 1 μCi/ml at the start of the culture period to label cells that passed through S phase (the phase of the cell cycle during which the replication of DNA occurs) during the experiment, thereby identifying mitotic cells and their progeny. Utricles were incubated for 2 days at 37°C in a 5% CO_2/95% air environment. Culture medium was not exchanged during the culture period, and each organ was cultured individually.

For screening, four to six utricular explants per experimental paradigm were studied in each individual experiment, and each factor was tested at two or three different concentrations. When a factor was observed to be mitogenic for sensory epithelial cells, three or four separate replications of the experiment were then conducted.

IGF-I kinetics

To examine the time course of the effect of IGF-I on mitosis, IGF-I was added to experimental cultures at 100 ng/ml at the start of the culture period. It was not added to control cultures, which were otherwise treated identically to the experimental cultures. Cultures were fixed with 4% paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.4, for 30 minutes after 4, 24, or 48 hours of in vitro growth. The cell proliferation marker 5-bromo-2′-deoxyuridine (BrdU; Sigma) was added to all cultures at 10 nM 4 hours prior to fixation (a pulse-fix paradigm) to identify cells synthesizing DNA. Four to six organs per experimental paradigm were studied.

Cell survival

To assess the survival rate of mitotic SE cells in our organ explants, utricles were placed into the previously described BME culture medium supplemented with 0.1% FBS. The explants were then treated in one of the following ways: 1) pulse labeled with [^{3}H]thymidine for 5 hours then immediately fixed; 2) pulse labeled with [^{3}H]thymidine for 5 hours then thoroughly rinsed four times with [^{3}H]thymidine-free medium and grown for an additional 2 days in [^{3}H]thymidine-free medium; or 3) pulse labeled with [^{3}H]thymidine for 5 hours then thoroughly rinsed four times with [^{3}H]thymidine-free medium and grown for an additional 2 days in [^{3}H]thymidine-free medium supplemented with 100 ng/ml IGF-I.

Histological methods and autoradiography

Cultures were immersion fixed in 3.5% glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4, overnight and postfixed in 1% OsO_4. They were dehydrated in a graded ethanol series and embedded in Spurr's epoxy resin. Transverse serial sections (3 μm) were cut through each utricular explant, and all sections were mounted and processed for autoradiography by using standard techniques (Oesterle et al., 1992). Sections were lightly counterstained with 0.01% toluidine blue before coverslipping.

BrdU immunohistochemistry

Wholemount preparations were analyzed for BrdU immunohistochemistry according to techniques developed by Stone and Cotanche (1994). Following fixation, cultured organs were rinsed in 0.1 M phosphate-buffered saline (PBS), pH 7.4, and the otoconia were removed. Next, organs were placed in 1 N HCL in 0.1% Triton X-100/PBS buffer at 37°C for 30 minutes to denature DNA. After HCL treatment, utricles were washed with PBS, pH 8.5, prior to washes with PBS, pH 7.4. Utricles were then washed with 70% ethanol and incubated in 0.5% hydrogen peroxide (H_2O_2) in methanol for 15 minutes to inactivate endogenous peroxidase. After a wash with 70% ethanol, utricles were placed in a blocking solution of 10% normal horse serum/0.1% Triton X-100 in PBS/1% BSA for 10 minutes to block nonspecific binding of the antibody. Following these steps, utricles were immersed in mouse anti-BrdU monoclonal antibody (Becton Dickinson, San Jose, CA) for 1 hour, horse anti-mouse biotinylated secondary antibody (Vector Laboratories, Burlinghame, CA) for 30 minutes, and avidin-biotin-horseradish peroxidase reagent (Vector Laboratories) for 45 minutes. Thorough rinses with PBS followed each antibody treatment, and each antibody treatment was diluted in a 0.1% Triton-X in PBS/1% BSA solution. Utricles were transferred to 0.05 M Tris, pH 7.6, for 5 minutes and placed in 0.1% diaminobenzadine (DAB) in 0.05 M Tris for 5 minutes. Next, utricles were placed in an identical DAB solution with 0.01% H_2O_2 until the appropriate staining density was attained (usually 5 minutes). Following the immunocytochemistry, the utricles were washed in 0.05 M Tris, mounted in 90% glycerol in 10% PBS, coverslipped, and analyzed as wholemounts by using a Leitz Aristoplan equipped with differential interference contrast (Nomarski) optics.

Data analysis

Estimation of SE volume. The Cavalieri method of estimating volume (Cavalieri, 1966) was used to determine the volume of the vestibular SE in the cultured utricles. Systematic random sampling was used such that, with a random start, every 30th section (90 μm apart) through the SE was analyzed. This resulted in 11–15 sections being examined per organ. A Dage MTI model 68 video camera was attached to a Zeiss Universal photomicroscope, and images of the sections (under a ×25 objective) were displayed with a MacIntosh IIcx computer. A Datatranslation QuickCapture board (Datatranslation, Marlboro, MA) was used to capture the image from the microscope. NIH Image 1.45 (image analysis software; Research Services Branch, NIMH, Bethesda, MD) allowed analysis of digitized images. A test grid of 30 μm × 30 μm squares was randomly superimposed over the captured image on the computer screen, and the number of points (P_i) corresponding to the upper left corner of each grid square lying over the SE was recorded. SE volume was then estimated by using the following formula:

$$V_{(SE)} = f(\Sigma\, P_i \cdot A_{(point)} \cdot t_{(section)}), \qquad (1)$$

where V is the volume of the SE, f is the fraction of the SE examined, P_i is the sum of all of the points counted on the SE, A is the area of the point on the test grid, and t is the thickness of the section (Gundersen, 1986; Gundersen and Jensen, 1987). The coefficient of error (CE; Gundersen and Jensen, 1987) for each volume calculation was calculated by using the following equation:

$$CE_{(\Sigma Pi)} = \frac{\sqrt{(3A + C - 4B)/12}}{\Sigma P_i}, \quad (2)$$

where P_i is the sum of all the points on the SE, $A = P_i \cdot P_i$, $B = P_i \cdot P_{(i+1)}$, and $C = P_i \cdot P_{(i+2)}$. All of the calculated volume data had CEs ≤ 0.4. Sample calculations are included in footnote 1.[1]

Estimation of number of [³H]thymidine-labeled SE cells. Autoradiographic sections were analyzed to detect the presence of [^{3}H]thymidine-labeled nuclei in the SE. Cells were considered labeled when five or more silver grains overlay the cell nucleus. Two different analysis methods were used to quantify the number of cells synthesizing DNA.

Method 1. Each utricular explant was sampled by using a systematic random sampling procedure with a sampling frequency of one in six sections. One autoradiographic section was randomly chosen from every six serial sections (18 μm intervals) through half of the organ. Sixteen to twenty sections were examined from each organ. Using a ×40 objective and Image 1.45, the length of the vestibular SE was quantified for each section examined. The number of [^{3}H]thymidine-labeled cells in the SE was counted, and the labeled nucleus linear density was computed (labeled nucleus linear density = number of labeled nuclei per 100 μm of SE). Values for individual sections were averaged to yield a mean linear density value for each organ culture. Mean values were determined for each experimental paradigm. In this way, it was possible to determine when the density of [^{3}H]thymidine-labeled cells increased in experimental vs. control explants. This traditional method of counting labeled cells has certain potential biases and fails to yield information about absolute volumes or absolute numbers of labeled cells. Many authors suggest using volumetric measurements, such as those utilized below in method 2 (Gundersen et al., 1988; West and Gundersen, 1990; Pover and Coggeshall, 1991; Tandrup, 1993).

Method 2. Unbiased estimates for 1) the number of [^{3}H]thymidine-labeled cells, 2) the number of unlabeled cells, and 3) the total number of cells (labeled and unlabeled) in the vestibular SE were obtained by using the physical disector principle (Sterio, 1984; Pakkenberg and Gundersen, 1988). The physical disector is a direct estimator of particle number that is unbiased by the size and shape of the particles being counted. A pair of adjacent sections was systematically selected every 60 sections through the vestibular maculae by using a random start in the first interval. Each pair served as a physical disector. By using a ×63 oil-immersion objective, the number of viewing fields that covered the length of the SE in the first section of the disector pair was noted, and a random number generator chose the field within the section to be examined. A rectangular counting frame was drawn over the boundaries of the SE within this field, and it's area, $a_{(fra)}$, was computed by NIH image. The corresponding field in the second section of the disector pair was then identified, and its area was similarly computed. A digitized image of the first section of each disector pair, with the outlined counting frame, was printed on white paper with a LaserWriter II printer. This section was then named the reference section. A digitized image of the second section, with the outlined counting frame, was printed on transparency film with the same printer. This section was then referred to as the "look-up section." The transparency was overlaid on the white paper, and the counting frames were aligned. Nuclear profiles located within the counting frames were then examined in the following manner. Nuclear profiles present in the reference section but not present in the look-up section were quantified. Similarly, nuclear profiles present in the look-up section but not present in the reference section were quantified. The numerical density, NV (NV = number of cells per μm^3 of SE), was estimated by a double disector and the following equation:

$$NV_{(nuclei/\mu m^3)} = \Sigma Q_i^- /(t \cdot \Sigma a_{(fra)}), \quad (3)$$

where ΣQ_i^- = the total number of nuclear profiles observed in the reference section but not present in the look-up section plus the total number of nuclear profiles in the look-up section but not the reference section, t = section thickness (and height of the physical disector), and $\Sigma a_{(fra)}$ = cumulative area of the counting frame from each disector. The numerical density was determined for each disector pair, and an average numerical density was calculated for each explant. Sample calculations are included in footnote 2.[2] By using this approach, the numerical densities of the [^{3}H]thymidine-labeled cells, the unlabeled cells, and the total number of cells (labeled and unlabeled) were determined. In addition, the total volume of the SE in each explant was estimated by using the method of Cavalieri, as described above. The total number of labeled SE cells was estimated by multiplying the total volume of the SE times the numerical density of the labeled cells (see footnote 2 for sample calculation). Similarly, the total number of unlabeled SE cells in each explant was esti-

[1]Sample calculation for estimating the total volume of the vestibular SE in a utricular explant using the Cavalieri method of volume estimation: For explant number 93-4009u1, 411 serial sections were saved and processed for autoradiography (AR). The volume of SE in 14 of these sections, 1/29th of the utricular macula (1/29.4), was quantified, and this was used to estimate the total volume of SE in the explant (see Table 1).

The volume of SE in the utricle was estimated by using Equation 1:

$$V_{(SE)} = f(\Sigma P_i \cdot A_{(point)} \cdot t_{(section)}), \quad (1)$$

where f (the fraction of SE examined) = 1/(14/411) = 29.36, $A_{(point)}$ (the grid square size) = 30 × 30 μm = 900 μm^2, $t_{(section)}$ (section thickness) = 3 μm, $\Sigma P_i = 552$, and $V_{(SE)}$ = (29.36)(552)(900 μm^2)(3 μm) = 43,758,144 μm^3 = 0.044 mm^3. The coefficient of error (CE) for the above volume calculation was computed by using Equation 2:

$$CE_{(\Sigma Pi)} = \frac{\sqrt{(3A + C - 4B)/12}}{\Sigma P_i}, \quad (2)$$

where P_i (the sum of all the points on the SE) = 552, $A = P_i \cdot P_i$ = 25,010, $B = P_i \cdot P_{(i+1)}$ = 24,199, $C = P_i \cdot P_{(i+2)}$ = 22,556, and $CE_{(\Sigma Pi)} = \sqrt{(75{,}030+22{,}556-96{,}796)/12}/552 = 0.01$.

mated by multiplying the total volume of the SE times the numerical density of the unlabeled cells.

Estimation of number of BrdU-labeled SE cells in wholemount preparations. The density of BrdU-labeled SE cells (density = number of BrdU-labeled SE cells per μm^2 of SE) was assessed by light microscopy in wholemount preparations of the utricules. Density distributions of BrdU-positive SE cells were mapped by using Nomarski optics and a squared ocular reticule (10×10) at a final magnification of $\times 400$ ($\times 10$ ocular, $\times 40$ objective). Brown-labeled nuclei within the SE were counted as positive cells. Counts of positive nuclei were made in consecutive squares, completely sampling the entire wholemounted utricular macula. Positively-labeled SE nuclei within the reticule square or touching its upper and left borders were included in the counts in order to avoid double counting nuclei. Data were plotted onto an outline of the wholemounted utricule drawn to scale. The numbers of labeled SE cells were totaled as well as the number of squares containing SE. The number of labeled SE cells per μm^2 of SE was computed for each explant.

Statistics

Significance values were determined with one-way and two-way analyses of variance (ANOVAs) by SUPERANOVA (Abacus Concepts, Berkeley, CA), and two-group comparisons were determined with t tests or with the Mann-Whitney U test (STATVIEW, Abacus Concepts). Posthoc comparisons, where appropriate, used the Duncan New Multiple Range or Tukey-Kramer tests.

RESULTS

In situ topologic and normal intercellular relationships are preserved in the organotypic cultures, allowing easy and definitive identification of sensory epithelial cells (Oesterle et al., 1993). The ability of our culture system to maintain the sensory epithelium of the mature avian inner ear for periods up to 7 days and to support the proliferation and differentiation of hair-cell progenitors has been documented previously (Oesterle et al., 1993). Bombesin, EGF, insulin, IGF-I, and TGF-α were tested for their effects on progenitor-cell division in cultured chicken vestibular SE. Two of the factors tested, IGF-I and insulin, were found to stimulate DNA synthesis in the normal SE. These factors will be examined first, followed by a discussion of the factors that failed to affect proliferation.

IGF-I

IGF-I stimulation of DNA synthesis within the utricular maculae. Figure 1A shows a cross section taken from a control utricle that was grown in culture for 2 days in the absence of any growth factor supplements. [^{3}H]thymidine was added at the start of the culture period to label cells synthesizing DNA, i.e., cells passing through the S phase of the cell cycle. Note that only 1 nucleus (lying in the lower half of the SE) is labeled by the [^{3}H]thymidine. A cross section from a utricle cultured identically to the control utricle, except that it was grown in the presence of 100 ng/ml IGF-I, is shown in Figure 1B. Many cells throughout the SE are labeled by the [^{3}H]thymidine.

The increase in the number of SE cells synthesizing DNA in the IGF-I supplemented vis-à-vis control explants was quantitatively assessed in 54 IGF-I-supplemented and 18 control utricles. The average number of [^{3}H]thymidine-labeled cells per 100 µm of SE (the mean labeled nucleus linear density) was estimated by using method 1. Results from three separate IGF-I experiments are shown in Figure 2A. Effects of GIBCO's IGF-I were examined in experiment 1, and the effects of a second IGF-I, which was purchased from Boehringer Mannheim, were examined in experiments 2 and 3. Boehringer Mannheim's IGF-I, like GIBCO's IGF-I, stimulated DNA synthesis in vestibular sensory epithelial cells in a dose-dependent manner. Values from the three individual experiments were averaged, and, in Figure 2B, they are expressed as a percentage of control cultures. These data, along with other data presented in this report, are expressed as a percentage of controls to facilitate comparisons between different experiments and different growth factors. The raw data for most comparisons are provided in figure captions or in tables, where applicable. One hundred percent represents the control mean. Note that the IGF-I stimulation of DNA synthesis is dose dependent. A threefold (3.3) increase in the number of SE cells synthesizing DNA is produced by the addition of 100 ng/ml of IGF-I. Note that, in this experiment, [^{3}H]thymidine was continuously present. Therefore, labeled cells represent cells that have recently progressed into S phase and those that have progressed through one or more mitotic cycles.

It is conceivable that changes in SE volume resulting from the IGF-I treatment could potentially bias estimates of the number of labeled cells per 100 µm of SE. Hence, the total volume of the vestibular SE in IGF-I-supplemented and control explants was examined. To assess SE volumes of control vs. IGF-I treated utricles, 6 of the 16 IGF-I-supplemented (100 ng/ml) explants and 6 of the 18 control explants were randomly selected. The total volume of the vestibular SE in each explant was estimated by using the Cavalieri method of volume estimation, and an average volume was calculated for each experimental paradigm. The average length of SE per section was also determined for each explant, and a mean value was calculated for each experimental paradigm. The volume of vestibular SE in the control explants and IGF-I-supplemented explants

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[2]Sample calculation for estimating the number of [^{3}H]thymidine-labeled SE cells in an explant by using method 2: For explant number 93-4009u1, 411 serial sections were saved and processed for AR. A pair of sections (two adjacent serial sections) was systemically selected every 60 sections through the vestibular maculae. After a random start, six pairs of sections were examined, each pair serving as a physical disector.

The numerical density of [^{3}H]thymidine-labeled cells, $NV_{(labeled\ nuclei/\mu m^3)}$, was estimated by using Equation 3:

$$NV_{(labeled\ nuclei/\mu m^3)} = \Sigma\, Q_i^- / (t \cdot \Sigma\, a_{(fra)}) , \quad (3)$$

where ΣQ_i^- (total number of [^{3}H]thymidine-labeled nuclear profiles observed in the reference section but not present in the look-up section plus the total number of [^{3}H]thymidine-labeled nuclear profiles observed in the look-up section but not present in the reference section; a double disector) = 2 + 2, t (section thickness; height of physical dissector) = 3 µm, $\Sigma a_{(fra)}$ (cumulative area of the counting frame from each dissector) = 18,517.66 μm^3 + 18,529.29 μm^3 = 37,046.95 μm^3, $NV_{(labeled\ nuclei/\mu m^3)}$ = 4 labeled cells/(3 $\mu m^3 \cdot$ 37,046.95 μm^2) = 3.6×10^{-5} labeled cells/μm^3

The total number of labeled SE cells per utricle was estimated by multiplying the numerical density of the labeled cells ($NV_{(labeled\ nuclei/\mu m^3)}$) by the total volume of SE, as determined by using the above-described Cavalieri method (see footnote 1). The total number of labeled SE cells = (3.6×10^{-5} labeled cells/μm^3)(43,758,144 μm^3) = 1,575.3 labeled cells.

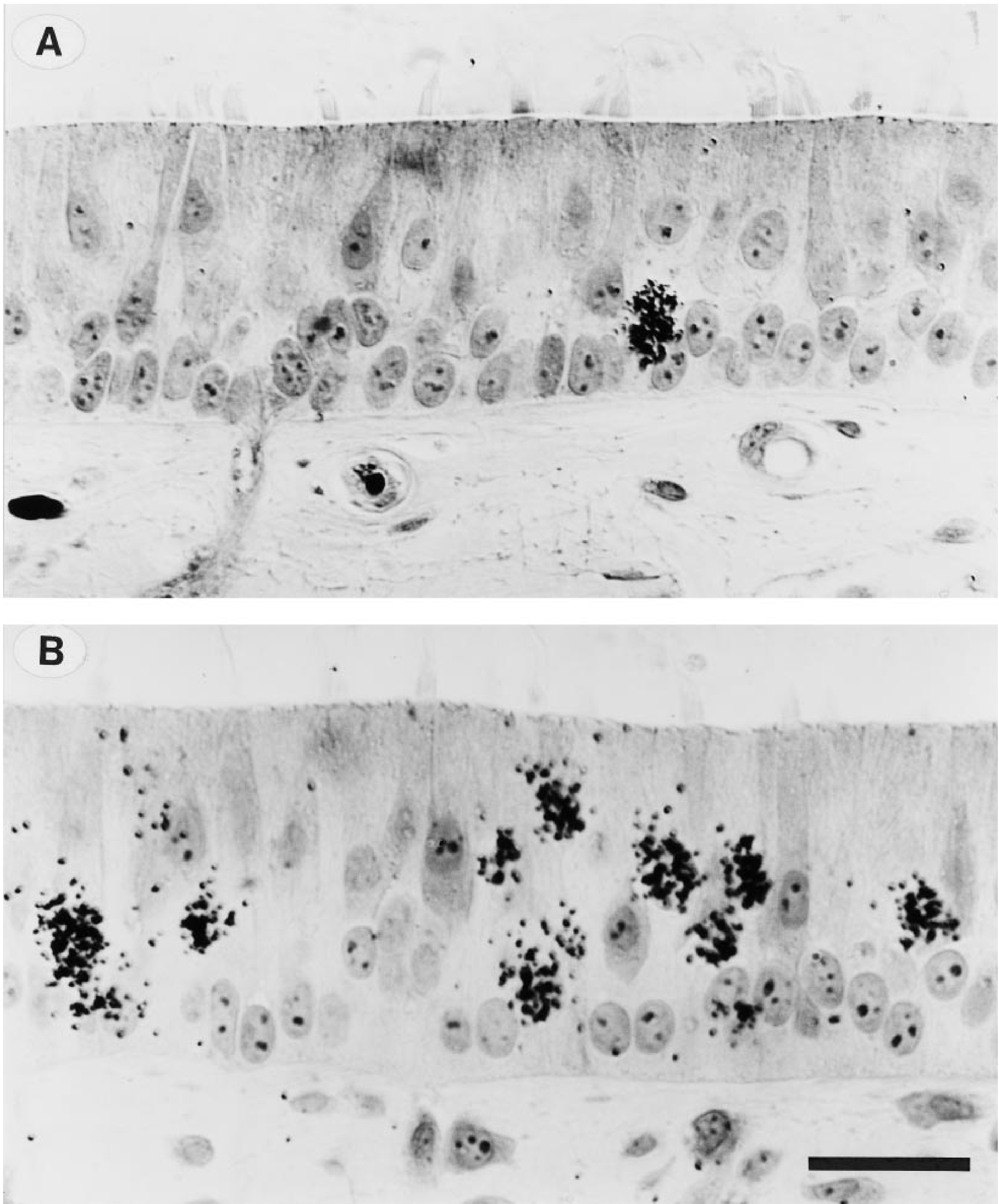

Fig. 1. Photomicrographs of normal chicken utricles grown in culture for 2 days in media alone (**A**) and in media supplemented with 100 ng/ml insulin-like growth factor-I (IGF-I; **B**). Note the markedly greater number of [^{3}H]thymidine-labeled cells in the IGF-I-supplemented explant compared with the control tissue. Scale bar = 20 µm.

averaged 0.038 ± 0.007 mm^3 S.D. and 0.045 ± 0.009 mm^3 S.D., respectively. The nonparametric Mann-Whitney U test indicated that this difference did not approach statistical significance ($P > 0.1$). The length of the SE per section in the control explants and IGF-I-supplemented explants averaged 953.4 μm/section (±100.9 μm S.D.) and 909.1 μm/section (±97.1 μm S.D.), respectively. Again, the Mann-Whitney U test did not approach significance ($P > 0.1$). The addition of IGF-I did not reliably alter total SE volume or estimates of SE length per section. Hence, it was concluded that estimates of the number of labeled cells per 100 μm of SE were unbiased by volumetric changes.

Method 2 was also used to quantitatively assess the proliferative increase in the explants supplemented with 100 ng/ml IGF-I vis-à-vis control explants. This was done to confirm the accuracy of analysis method 1 and to provide additional information regarding absolute numbers of labeled and unlabeled SE cells. Three of the 16 IGF-I-supplemented (100 ng/ml) explants and 3 of the 18 control explants were randomly selected, and a detailed, exhaustive analysis was performed by using both method 1 and method 2 on the same tissue. The results are shown in Table 2. The average number of [^{3}H]thymidine-labeled cells in the IGF-I-supplemented cultures is roughly four times greater than that in concordant control cultures for method 1 (this estimate differs from the 3.3-fold difference reported above because of the difference in sample size) and roughly three times greater than controls for method 2. In view of the agreement between the methods and the absence of evidence for differences in average length of SE per section or total SE volume in IGF-supplemented explants relative to controls, the remainder of the quantitative [^{3}H]thymidine data will be reported by analysis method 1.

Time course of IGF-I effect. The time course of IGF-I stimulation of DNA synthesis in the SE was assessed by using a pulse-fix experimental paradigm. IGF-I-supplemented (100 ng/ml IGF-I) and unsupplemented control cultures were grown in vitro for 4, 24, or 48 hours. Four hours prior to fixation, the cell proliferation marker BrdU was added to the explants to allow a determination of the number of SE cells in the S phase of the cell cycle at the time of fixation. Results are shown in Figure 3. At 4 hours, numbers of S phase SE cells in control cultures do not differ significantly from those in concordant IGF-I-supplemented cultures ($P > 0.1$). At 24 hours, 1.7 times more S-phase cells are present in IGF-I-supplemented cultures than in control cultures ($P < 0.01$). At 48 hours, the difference is much more pronounced, with 3.8 times more S-phase cells being present in IGF-I vis-à-vis control cultures at this time ($P < 0.05$), but the variance was also somewhat greater than at earlier times.

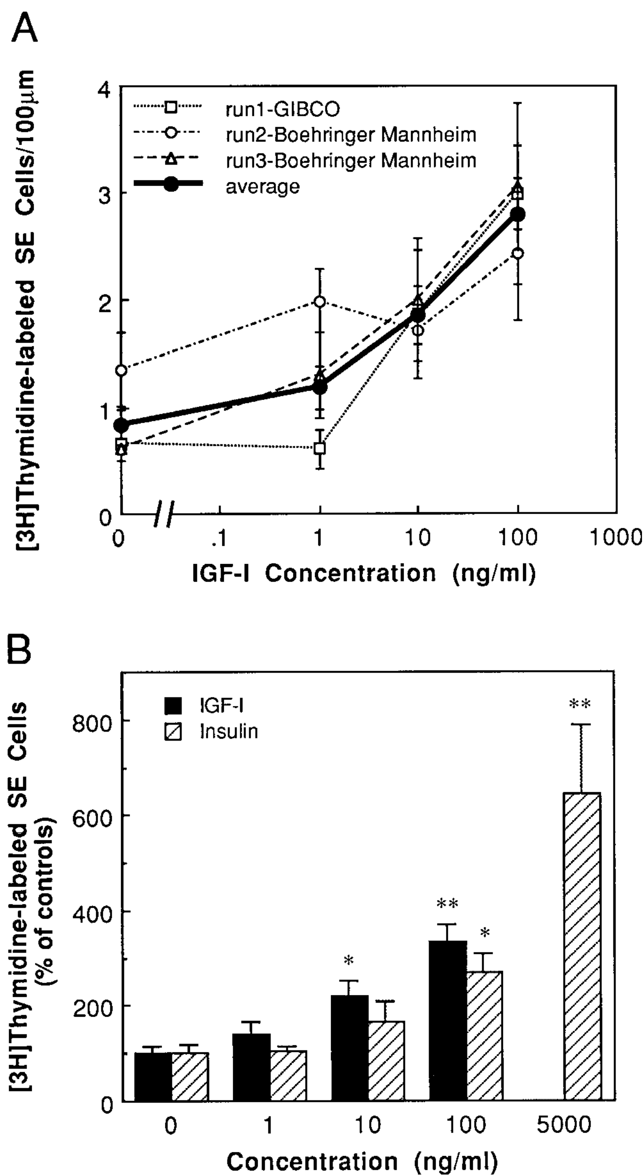

Fig. 2. The effects of IGF-I and insulin on [^{3}H]thymidine incorporation in vestibular sensory epithelial cells cultured for 2 days. [^{3}H]thymidine was present during the entire incubation period. **A:** Effects of IGF-I on [^{3}H]thymidine incorporation. Three separate IGF-I experiments were conducted. In the first experiment, the effects of an IGF-I purchased from GIBCO were examined (open squares), and, in experiments two (open circle) and three (open triangle), effects of Boehringer Mannheim's IGF-I were assessed. Each data value represents the mean number ± S.E.M. of [^{3}H]thymidine-labeled sensory epithelial cells per 100 μm of sensory epithelium. The solid circles represent the mean value ± S.E.M. of all three IGF-I experiments. **B:** Effects of IGF-I and insulin on [^{3}H]thymidine incorporation in vestibular sensory epithelial cells. Each data value for the IGF-I experiments represents the mean value ± S.E.M. of least 16 specimens from three separate replications of the experiments. Each data value for the insulin experiments represents the mean value ± S.E.M. of at least 11 specimens from two independent replications of the experiments. The mean number of [^{3}H]thymidine-labeled cells in the sensory epithelium of the IGF-I- or insulin-supplemented cultures was expressed as a percentage of the concordant control cultures. One hundred percent represents the control mean. Single asterisks indicate a significant difference at the 0.05 level. A double asterisk indicates that the growth factor mean is significantly different from the corresponding control mean at the 0.01 level. In terms of the "raw data," the average number of [^{3}H]thymidine-labeled cells per 100 μm of sensory receptor epithelium (SE) for the IGF-I-supplemented cultures were 0.84 ± 0.53 S.D., 1.18 ± 0.68 S.D., 1.86 ± 1.0 S.D., and 2.8 ± 1.32 S.D. for 0, 1, 10, and 100 ng/ml IGF-I, respectively. The average numbers of [^{3}H]thymidine-labeled cells per 100 μm of SE for the 1, 10, and 100 ng/ml insulin-supplemented cultures were 0.48 ± 0.12 S.D., 0.77 ± 0.51 S.D., and 1.24 ± 0.51 S.D., respectively, and 0.46 ± 0.25 S.D. for the control cultures (0 ng/ml insulin). Mean values for the 5 μg/ml insulin-supplemented cultures and concordant control cultures (0 ng/ml insulin) were 1.68 ± 1.11 S.D. and 0.26 ± 0.11 S.D., respectively.

TABLE 1. Quantification of Sensory Receptor Epithelium (SE) Volume in the Sampled Sections for Explant 93-4009u1[1]

Slide	Section	P_i	$P_i \cdot P_i$	$P_i \cdot P_{(i+1)}$	$P_i \cdot P_{(i+2)}$
1	11	13	169	13 × 24 = 312	13 × 35 = 455
2	15	24	576	840	744
3	10	35	1,225	1,085	1,295
4	4	31	961	1,147	1,550
4	34	37	1,369	1,850	1,924
5	27	50	2,500	2,600	2,850
6	22	52	2,704	2,964	2,860
7	15	57	3,249	3,135	3,363
8	10	55	3,025	3,245	2,695
9	4	59	3,481	2,891	2,655
9	34	49	2,401	2,205	1,715
10	28	45	2,025	1,575	450
11	22	35	1,225	350	
12	18	10	100		
Sum		552	25,010 = A	24,199 = B	22,556 = C

[1]The number of points corresponding to the upper left corner of each grid square that fell on SE, P_i, for the 14 sections examined is listed.

TABLE 2. Comparison of Data Analysis Methods 1 and 2: Number of [^{3}H]Thymidine-Labeled Sensory Receptor Epithelium (SE) Cells

	Method 1 (no. LC/100 μm)[1]	Method 2 (no. LC/μm³)
Control		
Mean	0.8	4.0 × 10⁻⁵
S.E.M.	0.2	0.3 × 10⁻⁵
N	3	3
100 ng/ml IGF-I		
Mean	3.1*	10.1 × 10⁻⁵ *
S.E.M.	1.3	2.2 × 10⁻⁵
N	3	3
IGF-I/control	3.9	2.5

[1]Note that only a subset of the 100 ng/ml insulin-like growth factor-I (IGF-I) supplemented and control utricles was analyzed for these method comparisons. Therefore, the numbers in this table are not equivalent to those in Figure 2. No. LC, number of [^{3}H]thymidine-labeled cells; S.E.M., standard error of the mean.
*IGF-1 mean is significantly different from the control at the 0.05 level.

Survival vs. mitogenic factor. In the organotypic cultures, IGF-I may have enhanced tritiated-thymidine incorporation through a number of mechanisms. IGF-I may have served as a mitogenic factor, stimulating precursors to divide, thereby producing additional numbers of DNA synthesizing cells. Alternatively, it may have provided trophic support, rescuing dividing precursor cells that would otherwise have died in culture by 2 days. To determine whether IGF-I reduces cell death (acts as a survival factor), we quantified the total number of unlabeled SE cells in three control cultures and in three cultures grown in the presence of 100 ng/ml IGF-I. Analyses were carried out by method 2. The results are shown in Table 3. If IGF-I enhances cell survival, then it is reasonable to expect that there would be increased cell numbers in IGF-I-supplemented cultures relative to controls. Note that, instead, numbers of unlabeled sensory epithelial cells in IGF-I-supplemented cultures are somewhat lower than those in control cultures. The Mann-Whitney U test ($P = 0.28$) indicated no evidence for a significant difference in the number of unlabeled SE cells in IGF-I-supplemented and control explants. Thus, these data do not support the idea that IGF-I is rescuing dividing precursor cells that would otherwise have died in culture.

An additional experiment, which was a pulsed [^{3}H]thymidine experiment, was performed to further explore whether IGF-I may have provided trophic support to cells that would otherwise have died in culture by 2 days. Twenty-one utricles were placed into culture medium supplemented with [^{3}H]thymidine. After 5 hours of in vitro growth, six of the explants were fixed. The remaining 15 explants were thoroughly washed with [^{3}H]thymidine-free medium and then grown for an additional 2 days in [^{3}H]thymidine-free medium with (n = 6) or without (n = 9) 100 ng/ml IGF-I. The number of [^{3}H]thymidine-labeled cells per section was quantified in each of the explants, and a mean value was computed for each experimental paradigm. The results are shown in Table 4. Comparison of the three paradigms provides information regarding the ex-

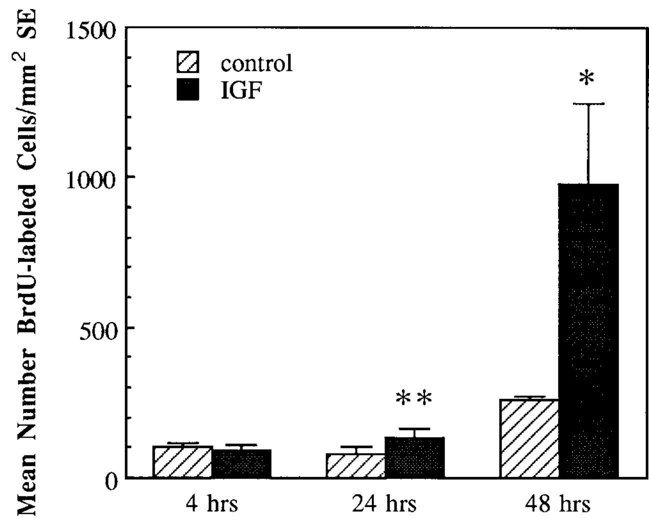

Fig. 3. Graph of the mean number of 5-bromo-2′-deoxyuridine (BrdU)-labeled SE cells in IGF-I-supplemented and control cultures at various time points (4, 24, or 48 hours) after explantation. Error bars represent standard deviation. A single asterisk indicates that the growth factor mean is significantly different from its concordant control mean at the 0.05 level. A double asterisk indicates that the growth factor mean is significantly different from the concordant control mean at the 0.01 level. In terms of the “raw data,” the mean number of BrdU-labeled cells was 169.5, and the mean area of the SE was 1,633,650 μm² for the 4 hour control. For the 24 hour control, the mean number of BrdU-labeled cells was 95.0, and the mean SE area was 1,202,700 μm². For the 48 hour control, the mean number of labeled cells was 401.0, and the mean SE area was 1,537,650 μm². For the 4 hour IGF-I, the mean number of BrdU-labeled cells was 147.3, and the mean area of the SE was 1,589,400 μm². For the 24 hour IGF-I, the mean number of BrdU-labeled cells was 180.8, and the mean SE area was 1,370,100 μm². For the 48 hour IGF-I, the mean number of labeled cells was 1,477.0, and the mean SE area was 1,518,300 μm². A one-way analysis of variance (ANOVA) of the 4 hour, 24 hour, and 48 hour control means revealed significant differences (0.01 level) between the 24 hour and 48 hour control means and between the 4 hour and 48 hour control means. The 4 hour and 24 hour control means did not differ from one another significantly at the 0.05 level. A one-way ANOVA of the 4 hour, 24 hour, and 48 hour IGF-I-supplemented means revealed a significant difference (0.01 level) between the 24 hour and 48 hour IGF-I-supplemented means and between the 4 hour and 48 hour IGF-I-supplemented means. The 4 hour and 24 hour IGF-I-supplemented means did not differ from one another significantly at the 0.05 level.

TABLE 3. Estimates of Cell Number in the Chicken Utricular Maculae[1]

	Total no. labeled cells[2]	Total no. unlabeled cells
Control		
Mean	1,669	46,198
S.E.M.	105	10,471
IGF-I		
Mean	4,121	30,620
S.E.M.	1,260	2,751
IGF-I/control	2.5	0.6

[1]Method 2 was used.
[2][^{3}H]thymidine-labeled cells.

TABLE 4. Pulsed [^{3}H]Thymidine Experimental Results: Number of Labeled SE Cells per Section in Chicken Utricular Macula

Experimental paradigm	No.	Mean no. labeled SE cells/section	S.E.M.
5-Hour pulse, fix[1]	6	0.42	0.09
5-Hour pulse, wash, 2-day growth, fix	6	0.37	0.06
5-Hour pulse, wash, 2-day growth with 100 ng/ml IGF-I, fix	9	0.42	0.07

[1][^{3}H]thymidine.

tent of death of the mitotic cells; if proliferating cells were dying in the cultures, then it is reasonable to expect that the number of [^{3}H]thymidine-labeled cells in the 2-day cultures without any IGF-I supplement will be significantly lower than those seen in the 5 hour cultures. Instead, the mean number of labeled cells for the 5 hour in vitro growth (mean = 0.42 ± 0.09 S.E.M.) paradigm was essentially unchanged from that for cultures grown an additional 2 days in culture (mean = 0.37 ± 0.06 S.E.M.). In addition, the number of labeled cells in the 2-day group supplemented with IGF-I (mean = 0.42 ± 0.07 S.E.M.) was identical to that for the 5 hour group. These results indicate that IGF-I is not rescuing cells that would otherwise have died in culture. They also suggest that the 2 additional days in culture (with or without IGF-I) did not result in another round of division of the labeled cells (see Discussion). Thus, the increased numbers of labeled cells in the IGF-supplemented cultures indicates that IGF-I serves as a mitogenic factor, stimulating SE precursor cells to divide.

Insulin

The effect of insulin on DNA synthesis is illustrated in Figure 2B. Numbers of [^{3}H]thymidine-labeled cells were quantitatively assessed in 36 insulin-supplemented and 9 control utricles. Insulin, like IGF-I, stimulates DNA synthesis in vestibular sensory epithelial cells in vitro in a dose-dependent manner. Insulin concentrations equal to or greater than 100 ng/ml are effective in stimulating an almost threefold (2.7) increase in the number of cells in the mature avian inner-ear epithelium to synthesize DNA.

EGF, bombesin, and TGF-α

The effects of bombesin, EGF, and TGF-α are summarized in Figure 4. Bombesin was tested at concentrations ranging from 10 nM to 100 nM, TGF-α was tested at 50 ng/ml, and EGF was tested at 10–200 ng/ml. These concentrations were chosen because they span the reported biologically active concentrations. Results of the growth factor-supplemented cultures are expressed as a percentage of controls, and the solid line indicates 100% of the control value (Fig. 4). Bombesin failed to significantly affect DNA synthesis in the mature avian vestibular SE. TGF-α also had little effect on DNA synthesis in the SE relative to that seen in control cultures. TGF-α, however, did stimulate DNA synthesis in cells in the extrasensory epithelia of the utricle; the stroma, the squamous epithelial cells that line the aneural portion of the stromal region and face the perilymph of the vestibule, and the membranous labyrinth bilayer that forms the roof over the macula (in the epithelial cells that face the endolymphatic space). EGF had little effect on DNA synthesis in the SE relative to that seen in control cultures, but EGF did stimulate DNA synthesis in an extrasensory epithelia; the squamous epithelial lining cells that face the perilymphatic space.

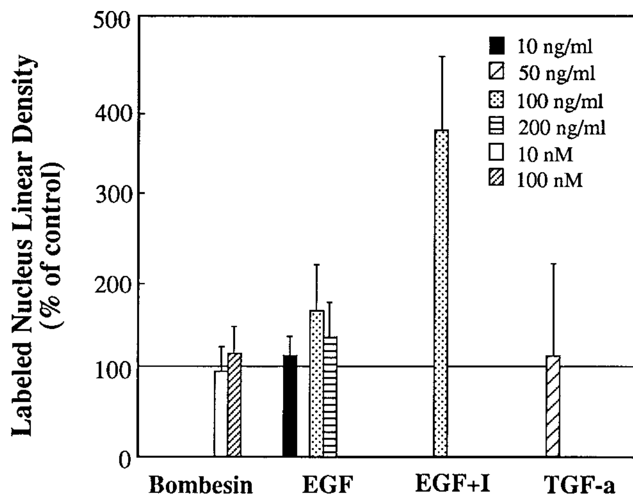

Fig. 4. The effects of bombesin, epidermal growth factor (EGF), and transforming growth factor alpha (TGF-α) on tritiated-thymidine incorporation in vestibular sensory epithelial cells cultured for 2 days. Tritiated thymidine was present during the entire incubation period. Results are mean ± S.E.M. Data are expressed as a percentage of the control cultures, and the solid line represents the control mean.

The addition of 100 ng/ml EGF supplemented with 5 μg/ml insulin stimulated DNA synthesis ($P < 0.05$) within the SE, but these results did not differ significantly from 5 μg/ml insulin alone ($P = 0.48$).

DISCUSSION

This study demonstrates the following: 1) IGF-I and insulin stimulate DNA synthesis in precursor cells in the mature avian vestibular sensory epithelium; and 2) EGF, TGF-α, and bombesin do not appear to stimulate DNA synthesis in the normal avian vestibular sensory epithelium.

Measuring technique comparison

Counts of labeled cells were measured in three ways: 1) by quantifying the number of labeled cells per 100 μm of SE in sectioned tissue, a measure of linear density; 2) by quantifying the number of labeled cells per μm^3 of SE in sectioned tissue using the physical disector; and 3) by quantifying the number of labeled cells per μm^2 SE in wholemount preparations. Comparisons between the results obtained with the three measurement techniques can be made by examining the IGF-I/control ratio. A direct comparison can be made between methods 1 and 2, because the same tissue was analyzed using both methods. Only a "ballpark" comparison can be made between method 3 and the other methods, because a shorter exposure paradigm to the cell proliferation marker (4-hour pulse vs. a continuous 2-day exposure) was used. Table 2 shows that the average number of labeled cells in the 100 ng/ml IGF-I-supplemented tissue is roughly four and three times greater than that in the concordant control cultures for methods 1 and 2, respectively. Thus, there was good agreement between the two methods. Figure 2 shows that the IGF-I/control ratio obtained by method 1 equaled 3.3 when sample size was increased. Data from method 3, the pulsed BrdU experiments where the number of labeled cells in the wholemounts was expressed per μm^2 SE,

suggest good ballpark agreement, in that 1.7 and 3.8 times more labeled cells are present in IGF-I-supplemented tissue than in control tissue at 1 and 2 days, respectively.

Methods 2 and 3 are the most accurate of the methods employed. Method 2, however, is tedious, extremely labor intensive, and, consequently, difficult and impractical to employ in experimental paradigms requiring a large sample size. Method 3 is limited, in that it becomes increasingly difficult to accurately discern individually labeled cells from clumps of label as the number of labeled cells increases. Method 1 provides an accurate and relatively fast estimate of numbers of labeled cells in cases where many cells are labeled; however, it does require that the tissue be serially sectioned.

Pulsed [^{3}H]thymidine experiment

Dividing precursor cells in the vestibular SE were labeled by using a 5-hour pulse of [^{3}H]thymidine at the start of the culture period and were then fixed immediately or fixed at 2 days. Surprisingly, numbers of [^{3}H]thymidine-labeled cells were essentially equivalent in the 5-hour, 2-day control, and 2-day IGF-I-supplemented groups. If the cells that labeled at 5 hours went on to divide, then an increase in the absolute number of [^{3}H]thymidine-labeled cells would have been expected at 2 days. The lack of an increase in the number of labeled cells suggests that 1) cells in S phase at the time of explantation fail to progress through mitosis (i.e., are arrested in G_2 phase); or 2) the time it takes SE cells to pass from S phase to or through M phase (mitosis and cytokinesis) takes longer than 2 days; or 3) some S-phase cells successfully divide, but some of the dividing cells undergo cell death. Explanations 1 and 2 seem unlikely for the following reasons. First, examination of the tissue sections reveals that a greater number of [^{3}H]thymidine-labeled nuclei were located in pairs or triplets (where two or more abutting SE cells were labeled) at 2 days than at 5 hours. Specifically, 4% of the labeled cells were located in pairs at 5 hours, whereas 17% of the labeled SE cells were located in pairs at 2 days, suggesting that some S-phase cells went on to divide and produced daughter cells by 2 days. Second, although the length of the cell cycle has not been characterized for cells in the mature vestibular sensory epithelium, some rudimentary information exists regarding cell cycle times in the regenerating avian auditory sensory epithelium, and a great deal is understood about the temporal properties of the cell cycle in many other types of eukaryotic cells. In the neural tissue of developing chick (Fujita, 1962; Jacobsen, 1991) and in adult chicken erythroblasts (Grosset and Odartchenko, 1975), the S phase lasts 4–8 hours and 6.9 hours, respectively. G_2 lasts 1–4 hours in developing chicken neural tissue (Fuijita, 1962; Jacobsen, 1991) and 1.05 hours in adult chicken erythroblasts (Grosset and Odartchenko, 1975). M-phase durations of 24–70 minutes (developing chicken neural tissue; Fuijita, 1962; Jacobsen, 1991) or 30 minutes (adult chicken erythroblasts; Grosset and Odartchenko, 1975) have been reported. In the mature inner ear, studies of drug-damaged auditory receptor epithelia suggest that hair-cell precursors pass from S phase through M phase in 6–8 hours (Hashino and Salvi, 1993; Stone and Cotanche, 1994). At 5 hours, we observed mitotic structures in cells near the lumenal surface of the SE that were not labeled with [^{3}H]thymidine, suggesting that, in the normal vestibular SE, G_2 through M phase takes at least 5 hours. Taken together, these data suggest that it probably takes vestibular SE cells considerably less than 2 days to pass from S phase to M phase.

Explanation 3 appears most likely—that some S-phase cells successfully divide, but some of the dividing cells immediately undergo cell death. In vivo, new cells are produced on a daily basis in the mature avian vestibular SE (Jørgensen and Mathiesen, 1988; Roberson et al., 1992), but hair-cell numbers do not appear to increase markedly as the bird ages, and the production may be related to a continual hair-cell turnover (Jørgensen, 1981; Jørgensen and Mathiesen, 1988; Weisleder and Rubel, 1993). A measurable level of cell death (apoptosis) in the normal in situ vestibular epithelia was reported recently (Kil et al., 1995). In the mature vestibular SE, the labeling index (fraction of mitotic cells) of the proliferating population may remain unchanged during active division, with cell death balancing the production of new cells. Tissue growth could be achieved by preventing apoptosis of the dividing cells, i.e., by providing trophic support (Holmgren et al., 1995). The lack of a significant increase in the number of labeled cells at 2 days vs. 5 hours is consistent with the idea that some new cells die off to maintain a population balance between mitotic and dying cells. In addition, it should be noted that IGF-I was added to normal, undamaged epithelia to "artificially" stimulate precursor cells to divide. Some of the cell death seen for the labeled cells may be because there was not an increased number of dying hair cells for the artificially stimulated precursor cells to replace. If hair cells were lost, the newly made cells might then go on to become new hair cells. They are not needed as it is, so they are eliminated.

Interestingly, in this experiment, the number of S-phase cells in the 2-day controls did not differ significantly from those seen in the 2-day IGF-I-supplemented cultures. This lack of difference suggests that IGF-I does not play a role in preventing the death of some of these newly formed cells. If IGF-I had played a role in preventing the death of some of the newly formed cells, then the numbers of labeled cells would have been greater in the 2-day IGF-I-supplemented cultures than in the 2-day control cultures.

IGF-I

IGF-I may play a role in controlling cell division and growth during normal development in both the otic vesicle and the cochlear vestibular ganglion (León et al., 1995). IGF-I has been detected in the ventral, lateral, and dorsal aspects of the otic epithelium (the ventral and medial regions are thought to give rise to the sensory receptor epithelium) and throughout the cochlear vestibular ganglion of stage 18 chicken otic vesicles, and IGF-I high-affinity binding sites are present in the otic epithelium and cochlear vestibular ganglion (León et al., 1995). Addition of IGF-I to explant cultures of stage 18 chicken otocysts induced DNA synthesis (fourfold relative to controls) and increased cell number (threefold relative to controls) in the otic vesicle and cochlear vestibular ganglion (León et al., 1995). In the mature ear, findings of the present study suggest that IGF-I plays a role in controlling cell division in the vestibular sensory receptor epithelium. Addition of IGF-I to the explant cultures induced roughly a threefold increase in the number of mitotic cells in the vestibular SE relative to controls. IGF-I did not appear to rescue either "unlabeled" or labeled (mitotic) SE cells from dying in culture; hence, the increased number of mitotic cells in the IGF-I-supplemented cultures suggests that IGF-I acts as a

mitotic factor stimulating cell proliferation within the mature vestibular SE. On the other hand, addition of IGF-I to explant cultures of normal auditory receptor epithelia (basilar papilla) from the chicken ear did not appear to induce proliferation within the sensory epithelium (Oesterle, unpublished observations), and the IGF-I receptor was not detected in cells of the normal or sound-damaged basilar papilla by immunocytochemical techniques (Lee and Cotanche, 1996). Thus, interesting differences in growth-factor-induced proliferation exist between the vestibular and auditory receptor epithelium in the avian inner ear.

Results of our study suggest that it takes 24–48 hours for the IGF-I to stimulate cells not normally proliferating in the vestibular sensory epithelium to reach S phase. In the IGF-I kinetic studies using BrdU labeling in whole-mount preparations of the utricle, BrdU was given for 4 hours before fixing the utricles at 4, 24, and 48 hours of growth. Cultures with and without an IGF-I supplement were studied. At 24 hours, there was a small but significant difference between the IGF-I-supplemented cultures and concordant control cultures, whereas a large difference was seen at 48 hours. Because of the briefness of the BrdU pulse, it is not unreasonable to assume that the majority of BrdU-labeled cells were in S phase, because most cells should not be able to get from S phase to M phase in only 4 hours. In the average eukaryote, S phase lasts approximately 7 hours and G_2 and M phases take about 4 hours to be completed (Baserga, 1985). Thus, these data suggest that it takes 24–48 hours for the IGF-I to stimulate normally nonproliferating vestibular sensory epithelial cells to reach S phase.

In light of the fact that interactions of IGF-I with the IGF-I receptor are typically regulated by a number of high-affinity IGF binding proteins (IGFBPs 1–6; Clemmons, 1990; Ooi, 1990), it is not unreasonable to speculate that IGF-I effects in the inner ear may be regulated by the IGFBPs and by several serum proteases that are known to specifically cleave the IGFBPs (Clemmons et al., 1993). The IGFBPs are genetically related soluble proteins that bind IGFs in extracellular fluids. They control the interaction of the IGF proteins with receptors and generally modulate the actions of the IGF proteins. IGFBPs are synthesized in various different tissues in conjunction with local IGF synthesis (Wood et al., 1990; Lee et al., 1992), and they have complex regulatory roles in local IGF action (Elgin et al., 1987; De Mellow and Baxter, 1988; Jones et al., 1991). IGFBPs demonstrate both inhibition and potentiation of IGF-mediated mitogenesis (Elgin et al., 1987; Burch et al., 1990; De Mellow and Baxter, 1988). Thus, there may be multiple levels of control of IGF function in the inner ear.

Insulin

Mitogenic effects of insulin have been examined previously in cultured chicken embryo otocyst preparations (Represa et al., 1988). In these embryonic preparations, insulin potentiates the mitogenic effects of other growth factors, such as bombesin and epidermal growth factor, but insulin is ineffective if it is used alone at a 5 μg/ml concentration. However, the effects of these factors on the sensory epithelium, per se, of the developing otocyst are unknown. In the mature murine vestibular SE, insulin potentiates the effects of other growth factors (TGF-α and EGF), but insulin alone is not mitogenic. Neither insulin alone nor IGF-I stimulate mitosis in the normal mature murine vestibular SE (Yamashita and Oesterle, 1995). In the mature avian vestibular SE, insulin probably exerts its mitogenic effect by binding to the IGF-I receptors. IGF-I first reliably stimulates mitosis in the avian vestibular sensory epithelium at a concentration of 10 ng/ml, whereas 100 ng/ml of insulin, ten times more, is required to significantly stimulate mitosis. Given the finding that the IGF-I receptor binds IGF-I and insulin with different affinities (Lowe, 1991; Werner et al., 1992), the higher required concentration of insulin suggests that insulin is probably exerting its mitogenic effects in the mature avian inner ear by binding to the IGF-I receptors.

Mammalian vs. avian inner ear

Substantial differences in sensory epithelial cell proliferation exist between avian and mammalian inner ears. Hundreds of new cells are produced daily in the vestibular SE of the normal, undamaged avian inner ear (Jørgensen and Mathiesen, 1988; Roberson et al., 1992; Kil et al., 1995). In contrast, proliferation is a very rare event in the normal mammalian vestibular SE (Warchol et al., 1993; Goldstein et al., 1994; Rubel et al., 1995; Yamashita and Oesterle, 1995). After ototoxic-induced hair-cell damage, the amount of cell mitosis in the vestibular organs of birds is much greater than that seen in mammals (Warchol et al., 1993; Weisleder and Rubel, 1993; Goldstein et al., 1994; Lambert, 1994; Rubel et al., 1995). In addition, TGF-α stimulates cell mitosis in normal murine vestibular SE but not in normal avian vestibular SE, whereas IGF-I stimulates mitosis in normal avian vestibular SE but not in murine vestibular SE. The origin of these differences is likely to be a very fruitful direction for future research.

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LITERATURE CITED

Baird, R.A., M.A. Torres, and N.R Schuff (1993a) Ongoing and gentamicin-induced hair cell regeneration in the bullfrog vestibular otolith organs. Soc. Neurosci. Abstr. *19:*1580.

Baird, R.A., M.A. Torres, and N.R. Schuff (1993b) Hair cell regeneration in the bullfrog vestibular otolith organs following aminoglycoside toxicity. Hearing Res. *65:*164–174.

Balak, K.J., J.T. Corwin, and J.E. Jones (1990) Regenerated hair cells can originate from supporting cell progeny: Evidence from phototoxicity and laser ablation experiments in the lateral line system. J. Neurosci. *10:*2502–2512.

Baserga, R. (1985) The Biology of Cell Reproduction. Cambridge, MA: Harvard University Press.

Baserga, R., and R. Ruben (1993) Cell cycle and growth control. Crit. Rev. Eukaryotic Gene Exp. *3:*47–61.

Burch, W.W., J. Correa, J.E. Shaveley, and D.R. Powell (1990) J. Clin. Endocrinol. Metabol. *70:*173–180.

Cavalieri, B. (1966) Geometria Degli Indivisibili. Torino, Italy: Unione Tipografico-Editrice Publ.

Chardin, S., and R. Romand (1995) Regeneration and mammalian auditory hair cells. Science *267:*707–709.

Clemmons, D.R. (1990) Insulin-like growth factor binding proteins. Trends Endocrinol. Metabol. *1:*412.

Clemmons, D.R., J.I. Jones, W.H. Busby, and G. Wright (1993) Role of insulin-like growth factor binding proteins in modifying IGF actions. In M.K. Raizada and D. LeRoith (eds): The Role of Insulin-Like Growth Factors in the Nervous System. Ann. New York Acad. Sci. *692:*10–21.

Corwin, J.T. (1981) Postembryonic production and aging of inner ear hair cells in sharks. J. Comp. Neurol. *201:*541–553.

Corwin, J.T. (1983) Postembryonic growth of the macula neglecta auditory detector in the ray, *Raja clavata*: Continual increases in hair cell number, neural convergence, and physiological sensitivity. J. Comp. Neurol. *217:*345–356.

Corwin, J.T. (1985) Perpetual production of hair cells and maturational changes in hair cell ultrastructure accompany postembryonic growth in amphibian ear. Proc. Natl. Acad. Sci. USA *82:*3911–3915.

Corwin, J.T., and D.A. Cotanche (1988) Regeneration of sensory hair cells after acoustic trauma. Science *240:*1772–1774.

Cotanche, D.A. (1987) Regeneration of hair cell stereociliary bundles in the chick cochlea following severe acoustic trauma. Hearing Res. *30:*181–196.

Cotanche, D.A., K.H. Lee, J.S. Stone, and D.A. Picard (1994) Hair cell regeneration in the bird cochlea following noise damage or ototoxic drug damage. Anat. Embryol. *189:*1–18.

Cruz, R.M., P.R. Lambert, and E.W Rubel (1987) Light microscopic evidence of hair cell regeneration after gentamicin toxicity in chick cochlea. Arch. Otolaryngol. Head Neck Surg. *113:*1058–1062.

Czech, M.P. (1989) Signal transduction by the insulin-like growth factors. Cell *59:*235–238.

Daughaday, W.H., and P. Rotwein (1989) Insulin-like growth factors I and II: Peptide, messenger ribonucleic acid and gene structures, serum, and tissue concentrations. Endocrinol. Rev. *10:*68–91.

De Mellow, J.S.M., and R.C. Baxter (1988) Growth hormone-dependent insulin-like growth factor differentiation by induction of myogenin gene expression, or (IGF) binding protein both inhibits and potentiates IGF-I stimulated synthesis in human skin fibroblasts. Biochem. Biophys. Res. Commun. *156:*199–204.

Elgin, R.G., W.H. Busby, and D.R. Clemmons (1987) An insulin-like growth factor (IGF) binding protein enhances the biologic response to IGF-I. Proc. Natl. Acad. Sci. USA *84:*3254–3258.

Forge, A., L. Li, J.T. Corwin, and G. Nevill (1993) Ultrastructural evidence for hair cell regeneration in the mammalian inner ear. Science *259:*1616–1622.

Fujita, S. (1962) Kinetics of cellular proliferation. Exp. Cell Res. *28:*52–60.

Girod, D.A., L.G. Duckert, and E.W Rubel (1989) Possible precursors of regenerated hair cells in the avian cochlea following acoustic trauma. Hearing Res. *42:*175–194.

Goldring, M.B., and S.R. Goldring (1991) Cytokines and cell growth control. Crit. Rev. Eukaryotic Gene Exp. *1:*301–326.

Goldstein, B.J., M.Y. Huang, and J.T. Corwin (1994) Regenerative proliferation in the guinea pig utricle in vivo. Assoc. Res. Otolaryngol. Abstr. *17:*131.

Grosset, L., and N. Odartchenko (1975) Duration of mitosis and separate mitotic phases compared to nuclear DNA content in erythroblasts of four vertebrates. Cell Tissue Kinet. *8:*91–96.

Gundersen, H.J.G. (1986) Stereology of arbitrary particles. A review of unbiased number and size estimators and the presentation of some new ones, in memory of William R. Thompson. J. Microsc. *143:*3–45.

Gundersen, H.J.G., and E.B. Jensen (1987) The efficiency of systematic sampling in stereology and its prediction. J. Microsc. *147:*229–263.

Gundersen, H.J.G., T.F. Bendtsen, L. Korbo, N. Marcussen, A. Moller, K. Nielsen, J.R. Nyengaard, B. Pakkenberg, F.B. Sorensen, A. Vesterby, and M.J. West (1988) Some new, simple and efficient stereological methods and their use in pathological research and diagnosis. APMIS *96:*379–394.

Hashino, E., and R.J. Salvi (1993) Changing spatial patterns of DNA replication in the noise-damaged chick cochlea. J. Cell Sci. *105:*23–31.

Holmgren, L., M.S. O'Reilly, and J. Folkman (1995) Nature Med. *1:*149–153.

Jacobsen, M. (1991) Developmental Neurobiology. New York, NY: Plenum Press.

Jones, J.E., and J.T. Corwin (1993) Replacement of lateral line sensory organs during tail regeneration in salamanders: Identification of progenitor cells and analysis of leukocyte activity. J. Neurosci. *13:*1022–1034.

Jones, J.I., A.J. D'Ercole, C. Camacho-Hubner, and D.R. Clemmons (1991) Phosphorylation of IGF-binding protein-1 in cell culture and in vivo: Effects on affinity for IGF-I. Proc. Natl. Acad. Sci. USA *88:*7481–7485.

Jørgensen, J.M. (1981) On a possible hair cell turn-over in the inner ear of the caecilian, *Ichthyophis glutinosus* (Amphibia: Gymnophiona). Acta Zool. *62:*171–186.

Jørgensen, J.M., and C. Mathiesen (1988) The avian inner ear: Continuous production of hair cells in vestibular sensory organs, but not in the auditory papilla. Naturwissenschaften *75:*319–320.

Kil, J., M.E. Warchol, and J.T. Corwin (1995) On-going and aminoglycoside-induced apoptotic cell death in the vestibular epithelia of chicks. Assoc. Res. Otolaryngol. Abstr. *18:*82.

Kuntz, A.L., and E.C. Oesterle (1996) TGFa induces hair cell production in mature mammalian vestibular sensory epithelia in vivo. Assoc. Res. Otolaryngol. Abstr. *19:*198.

Lambert, P.R. (1994) Inner ear hair cell regeneration in a mammal: Identification of a triggering factor. Laryngoscope *104:*701–718.

Lee, K.H., and D.A. Cotanche (1996) Potential role of bFGF and retinoic acid in the regeneration of chicken cochlear hair cells. Hearing Res. *94:*1–13.

Lee, W.-H., J.A. Clemens, and C.A. Bondy (1992) Insulin-like growth factors in the response to cerebral ischemia. Mol. Cell. Neurosci. *3:*36–43.

Lefebvre, P.P., B. Malgrange, H. Staecker, G. Moonen, and T.R. Van de Water (1993) Retinoic acid stimulates regeneration of mammalian auditory hair cells. Science *260:*692–693.

León, Y., E. Vazquez, C. Sanz, J.A. Vega, M.J. Mato, F. Giraldez, J. Represa, and I. Varela-Nieto (1995) Insulin-like growth factor-I regulates cell proliferation in the developing inner ear, activating glycosyl-phosphatidylinositol hydrolysis and fos expression. Endocrinology *136:*3494–3503.

Lewis, M.E., N.T. Neff, P.C. Contreras, D.B. Stong, R.W. Oppenheim, P.E. Grebow, and J.L. Vaught (1993) Insulin-like growth factor-I: Potential for treatment of motor neuronal disorders. Exp. Neurol. *124:*73–88.

Lombarte, A., H.Y. Yan, A.N. Popper, J.S. Chang, and C. Platt (1993) Damage and regeneration of hair cell ciliary bundles in a fish ear following treatment with gentamicin. Hearing Res. *64:*166–174.

Lowe, W.L. (1991) Biological actions of the insulin-like growth factors. In D. LeRoith (ed): Insulin-Like Growth Factors: Molecular and Cellular Aspects. Boca Raton, FL: CRC Press, Inc., pp. 49–86.

Macaulay, V.M. (1992) Insulin-like growth factors and cancer. Br. J. Cancer *65:*311–320.

Oesterle, E.C., and E.W Rubel (1996) Hair cell generation in vestibular sensory receptor epithelia. In S.M. Highstein, B. Cohen, and J.A. Büttner-Ennever (eds): New Directions in Vestibular Research. Ann. New York Acad. Sci. *781:*34–46.

Oesterle, E.C., D.E. Cunningham, and E.W Rubel (1992) Ultrastructure of hyaline, border, and vacuole cells in chick inner ear. J. Comp. Neurol. *318:*64–82.

Oesterle, E.C., T.T. Tsue, T.A. Reh, and E.W Rubel (1993) Hair-cell regeneration in organ cultures of the postnatal chicken inner ear. Hearing Res. *70:*85–108.

Oesterle, E.C., T.T. Tsue, and E.W Rubel (1994) Mitogenic effects of insulin and IGF-I on cultured vestibular sensory epithelium from the avian inner ear. Assoc. Res. Otolaryngol. Abstr. *17:*114.

Ooi, G. (1990) Insulin-like growth factor-binding proteins (IGFBPs): More than just 1,2,3. Mol. Cell. Endocrinol. *71:*C39–C43.

Pakkenberg, B., and H.J.G. Gundersen (1988) Total number of neurons and glial cells in human brain nuclei estimated by the dissector and the fractionator. J. Microsc. *150:*1–20.

Popper, A.N., and B. Hoxter (1984) Growth of a fish ear: I. Quantitative analysis of sensory hair cell and ganglion cell proliferation. Hearing Res. *15:*133–142.

Popper, A.N., and B. Hoxter (1990) Growth of a fish ear II. Locations of newly proliferated sensory hair cells in the saccular epithelium of *Astronotus ocellatus*. Hearing Res. *45:*33–40.

Pover, C.M., and R.E. Coggeshall (1991) Verification of the dissector method for counting neurons, with comments on the empirical method. Anat. Rec. *231:*573–578.

Raphael, Y. (1992) Evidence for supporting cell mitosis in response to acoustic trauma in the avian inner ear. J. Neurocytol. *21:*663–671.

Rechler, M.M., and S.P. Nissley (1990) The nature and regulation of the receptors for insulin-like growth factors. Annu. Rev. Physiol. *47:*425–442.

Represa, J.J., C. Miner, E. Barbosa, and F. Giraldez (1988) Bombesin and other growth factors activate cell proliferation in chick embryo otic vesicles in culture. Development *102*:87–96.

Represa, J., Y. León, C. Miner, and F. Giraldez (1991) The *int*-2 proto-oncogene is responsible for induction of the inner ear. Nature *353*:561–563.

Roberson, D.W., and E.W Rubel (1994) Cell division in the gerbil cochlea after acoustic trauma. Am. J. Otol. *15(1)*:28–34.

Roberson, D., P. Weisleder, P. Bohrer, and E.W Rubel (1992) Ongoing production of sensory cells in the vestibular epithelium of the chick. Hearing Res. *57*:166–174.

Rotwein, P. (1991) Structure, evolution, expression and regulation of insulin-like growth factors I and II. Growth Factors *5*:3.

Rubel, E.W, L.A. Dew, and D.W. Roberson (1995) Mammalian vestibular hair cell regeneration. Science *267*:701–703.

Ryals, B.M., and E.W Rubel (1988) Hair cell regeneration after acoustic trauma in adult *Coturnix* quail. Science *240*:1774–1776.

Sobkowicz, H.M., B.K. August, and S.M. Slapnick (1992) Epithelial repair following mechanical injury of the developing organ of Corti in culture: An electron microscopic and autoradiographic study. Exp. Neurol. *115*:44–49.

Sterio, D.C. (1984) The unbiased estimation of number and sizes of arbitrary particles using the dissector. J. Microsc. *134*:127–136.

Stone, J.S., and D.A. Cotanche (1994) Identification of the timing of S phase and the patterns of cell proliferation during hair cell regeneration in the chick cochlea. J. Comp. Neurol. *341*:50–67.

Stone, L.S. (1933) The development of lateral-line sense organs in amphibians observed in living and vital-stained preparations. J. Comp. Neurol. *57*:507–540.

Stone, L.S. (1937) Further experimental studies of the development of lateral-line sense organs in amphibians observed in living preparations. J. Comp. Neurol. *68*:83–115.

Tandrup, T. (1993) A method for unbiased and efficient estimation of number and mean volume of specified neuron subtypes in rat dorsal root ganglion. J. Comp. Neurol. *329*:269–276.

Tanyeri, H., I. Lopez, and V. Honrubia (1995) Histological evidence for hair cell regeneration after ototoxic cell destruction with local application of gentamicin in the chinchilla crista ampullaris. Hearing Res. *89*:194–202.

Tester, A.L., and J.I. Kendall (1969) Morphology of the lateralis canal system in the shark genus *Carcharhinus*. Pac. Sci. *23*:1–16.

Tsue, T.T., D.L. Watling, P. Weisleder, M.D. Coltrera, and E.W Rubel (1994) Identification of hair cell progenitors and intermitotic migration of their nuclei in the normal and regenerating avian inner ear. J. Neurosci. *14(1)*:140–152.

Warchol, M.E., P.R. Lambert, B.J. Goldstein, A. Forge, and J.T. Corwin (1993) Regenerative proliferation in inner ear sensory epithelia from adult guinea pigs and humans. Science *259*:1619–1622.

Weisleder, P., and E.W Rubel (1993) Hair cell regeneration after streptomycin toxicity in the avian vestibular epithelium. J. Comp. Neurol. *331*:97–110.

Werner, H., C.T. Roberts, Jr., M.K. Raizada, C.A. Bondy, M. Adamo, and D. LeRoith (1992) In I.S. Zagon and P.J. McLaughlin (eds): Receptors in the Developing Nervous System. London: Chapman and Hall Ltd., pp. 109–127.

West, M.J., and H.J.G. Gundersen (1990) Unbiased stereological estimation of the number of neurons in the human hippocampus. J. Comp. Neurol. *296*:1–22.

Wood, T.L., A.L. Brown, M.M. Rechler, and J.E. Pintar (1990) The expression pattern of an insulin-like growth factor (IGF)-binding protein gene is distinct from IGF-II in the midgestational rat embryo. Mol. Endocrinol. *4*:1257–1263.

Yamashita, H., and E.C. Oesterle (1994) Mitogenic effects of EGF and TGF-alpha on cultured vestibular sensory epithelium from the mammalian inner ear. Assoc. Res. Otolaryngol. Abstr. *17*:131.

Yamashita, H., and E.C. Oesterle (1995) Induction of cell proliferation in mammalian inner-ear sensory epithelial by transforming growth factor α and epidermal growth factor. Proc. Natl. Acad. Sci. USA *92*:3152–3155.