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Investigation of the effect of macrophages and other leukocytes on hair cell regeneration in the avian inner ear

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INVESTIGATION OF THE EFFECT OF MACROPHAGES AND OTHER LEUKOCYTES ON HAIR CELL REGENERATION IN THE AVAIN INNER EAR

by

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Abstract

It is known that sensory hair cells regenerate after hair cell death in the avian inner ear after aminoglycoside intoxication. I investigated how the amount of hair cell proliferation was affected by immunosuppression. I hypothesized that the suppression of the ability of leukocytes to secrete peptides will decrease the amount of hair cell regeneration after ototoxic insult in the utricle of the chick. I gave the experimental group of chicks the immunosuppressant dexamethozone. The control group received saline. Half of the chicks from the experimental group (n=10) and half from the control group (n=12) were given a large dose of gentamycin, causing extensive hair cell death in the utricle. The remaining chicks in the experimental group (n=11) and the control group (n=12) received no aminoglycoside. Over the next three days, the immunosuppressed group received further injections of dexamethozone. Bromodeoxyuridine (BrdU) immunohistochemistry was used to label proliferating cells in the utricle. Chicks were sacrificed and the number of proliferating supporting cells in the entire utricle were counted. There was a significant difference ($P = 0.0001$) in the number of BrdU positive supporting cells in the utricles of the immunosuppressed chicks (mean = 138.7 +/- 26.3 S.D.) and the non-immunosuppressed chicks (mean = 185.1 +/- 17.0 S.D.) that received no gentamycin. There was also a significant difference ($P = 0.000016$) between the BrdU positive supporting cells in the utricles of the immunosuppressed chicks (mean = 134.1 +/- 29.0 S.D.) and the non-immunosuppressed chicks (mean = 225.9 +/- 45.4 S.D.) that had been

damaged by the gentamycin. These results suggest that leukocyte activation plays a key role in the molecular processes of hair cell regeneration.

1. Introduction

The sensory epithelium of the chicken utricle is populated by Type I and Type II hair cells, supporting cells, and the unmyelinated endings of the eighth cranial nerve. Type I and Type II hair cells are distinguished by their morphology. Type I hair cells are pear-shaped and enclosed in a single nerve calyx. Type II hair cells are cylinder-shaped and have several button-like nerve endings at their basal end. The supporting cells surround the hair cells and go from the lumen to the basement membrane. The sensory epithelium of the utricle is called the utricular macula. There is a C-shaped area of the utricle called the striola that contains a heavier concentration of the Type I hair cells, which can be easily seen, due to their tall stereocillia (Bhave, 1998).

Unfortunately hair cell death occurs in the cochlea and vestibular organs in vivo after exposure to sound trauma and ototoxic drugs. It is believed that cell death occurs, at least partially, by programmed cell death, or apoptosis. It is known that apoptosis occurs normally during development to reduce the original number of cells produced in various areas of the embryo. Apoptosis sculpts the body by hollowing out cavities and separating digits, for example. The process has been referred to as "cell suicide" (Raff, 1998). The cell literally kills itself by the following process. First, pyknosis occurs, which is the condensation of

nuclear chromatin. Then the DNA is cut into 120 base pair fragments by endonucleases. This is followed by phagocytosis by neighboring cells or resident macrophages envelope, or phagocytize the dead hair cell (Warchol, 1997). Basically the dying cell shrinks, disintegrates, and is swallowed up. Kil et al (1997) found a 58% increase of hair cell death in saccules and a 270% increase in utricles of chicks within 24 hours after exposure to neomycin sulphate, an ototoxic antibiotic. Apoptosis can be suppressed by certain extracellular survival factors and by inhibition of RNA and protein synthesis. How might hair cell apoptosis be brought back to normal during embryonic development, aminoglycoside insult, or acoustic trauma? Could people be given treatments to prevent hair cell apoptosis before being given gentamycin? Further studies must be done to investigate.

Warchol and Corwin (1996) found that the first cells to undergo mitosis after the death of hair cells were the supporting cells in the avian cochlear sensory epithelium. The 10 to 25 hour cell cycle consists of interphase and mitosis. Interphase includes the G1 phase, the S phase, and the G2 phase. In the Gap1 phase RNA and protein are synthesized in the cytoplasm, and the cell grows considerably. During the Synthesis phase the nuclear DNA replicates, resulting in two full sets of chromosomal DNA which is still dispersed throughout the nucleus. In the Gap2 phase cytoplasmic growth continues. Then mitosis occurs in four stages: prophase, metaphase, anaphase, and telophase. In prophase, the nuclear material condenses to form chromosomes, and a spindle forms. In metaphase, the chromosomes migrate toward the center of the spindle, and go to

opposite poles in anaphase. Cytokinesis occurs in telophase as the cell splits and chromosomes decondense (Sheeler and Bianchi, 1983). The supporting cells observed by Warchol and Corwin (1996) entered the S-phase of the cell cycle 16 hours after the hairs cell died.

Warchol et al (1993) demonstrated that human and guinea pig vestibular supporting cells proliferate and show characteristics of hair cells following four weeks of culture exposure to aminoglycoside antibiotics. Before this, it was not known that human hair cells could regenerate.

While the undamaged avian cochlea remains mitotically quiescent, there is ongoing hair cell death and regeneration in the saccule and utricle. Hair cells undergoing apoptosis are scattered randomly across the sensory epithelia of the vestibular organs. These dead cells are then extruded, removed, and replaced. This stimulates mitosis in nearby supporting cells, leading to replacement of the hair cell. In spite of (Kil et al, 1997), the average life of a hair cell in the avian saccule and utricle appears to be about twenty days, in contrast to human cochlear and vestibular hair cells, which last a lifetime.

Three events are necessary for successful hair cell regeneration. First, the precursor cell, which is the supporting cell, must be stimulated to proliferate (undergo mitosis). Second, one or both of the daughter cells must differentiate into a hair cell. Third, there must be an inhibition of further proliferation. BrdU labeling is ideal to follow this process because it gives us a "snapshot" of the cells that have gotten to the S phase of mitosis during the time they were injected. BrdU is a thymidine analogue. It takes the place of thymidine in the cell's DNA

when the cell undergoes mitosis. Most BrdU labeling in the basilar papilla, which is analogous to the human cochlea, will be seen in the area of gentamycin damage. This is in the proximal third of the epithelium. This area corresponds to the basal portion in humans, where high frequencies are perceived (Bhave et al, 1995).

There is a resident population of macrophages in the normal undamaged avian vestibular and auditory sensory epithelia. They also reside in human scala vestibuli and scala tympani, but haven't been found in the Organ of Corti yet (Bhave et al, 1998) until after noise damage. Macrophages are large, highly phagocytic leukocytes found in blood vessels and loose connective tissue. When stimulated by inflammation, however they become free and actively mobile (*Dorland's Illustrated Medical Dictionary*, 1974). Macrophages have several functions. They act as effector cells for the immune system and help develop and maintain body tissues, phagocytize dying cells, and aid in wound healing. They can secrete cytokines, which are leukocyte-signaling molecules that, under some circumstances, can activate the immune system and help neural cell to survive. In other circumstances, however, they can result in increased cell death (Warchol, 1997). Macrophages have been found in increased numbers at the sites of hair cell lesions, so it is possible that they may play a role in stimulating regeneration (Warchol, 1997; Bhave et al, 1998).

The goal of this project is to investigate the role that macrophages play in avian hair cell regeneration. Prior studies have demonstrated that they are recruited to the sites of hair cell lesions in the cochlea. It is possible that they

secrete cytokines, which stimulate the proliferation of supporting cells (Warchol, 1997). I investigated how immunosuppression affects the proliferation of supporting cells in the utricle of the White Leghorn chick (*Gallus domesticus*). If macrophages do play a key role in aiding hair cell regeneration, then one could hypothesize that immunosuppression would retard supporting cell proliferation.

2. Methods and Materials

Drug Injections

White Leghorn chickens (*Gallus domesticus*), age 9-21 days posthatch, were divided into experimental and control groups. The experimental group (n=10) was given a subcutaneous injection of the immunosuppressant dexamethazone (10 mg/kg; Sigma, St .Louis, MO). The control group (n=12) was given a subcutaneous injection of 4 parts 0.9% saline diluted with 1 part ethanol. Immediately after this, chicks in both groups were given a subcutaneous injection of the aminoglycoside antibiotic gentamycin (250 mg/kg; Sigma, St. Louis, MO). Over the next three days, the experimental and control groups received three more injections of the dexamethazone or the saline/ethanol mixture, respectively, at the same dosages and time of day as before. On the third day, two hours after being injected with the immunosuppressant, all chicks were given peritoneal injections of 5-bromo-2-deoxyuridine (BrdU) (100 mg/kg; Sigma, St. Louis, MO), a thymidine analogue used to label cells undergoing the S phase of mitosis. Chicks were euthanized by carbon dioxide asphyxiation and decapitated two hours later.

Tissue Preparation

The skin covering the head and lower jaw was removed. The bone was broken away to expose the middle ear spaces. Right and left utricles were removed with fine forceps and placed in Medium-199 (Life Technologies, Gaithersburg, MD). The otoconia were removed to expose the lumenal surface of the utricular macula. The utricles were then immersion-fixed in 4% paraformaldehyde for 30 minutes.

Immunohistochemistry

The utricles were rinsed five times in phosphate buffered saline, PBS, and treated in 90% methanol with 0.3 % H_2O_2 for 30 minutes to reduce endogenous peroxidase activity and permeablize the cells. The utricles were rinsed five times in PBS and placed in 2N HCl for 30 minutes to denature their DNA. Then they were preblocked for 30 minutes to 1 ½ hours in PBS that contained 2% normal horse serum, 1%BSA (bovine serum albumin) and 2% Tritan X-100. Next, the specimens were incubated overnight in the primary antibody against BrdU [mouse IgG: (Becton-Dickinson, San Jose, CA), diluted 1:50 with PBS and 2% normal horse serum with 0.5% Tritan X-100]. After five rinses in PBS, the utricles were incubated for two hours in the secondary antibody [biotinylated horse anti-mouse IgG: (Vector Labs, Burlingame, CA), diluted 1:150 with PBS and 1 ul normal horse serum and 1 ul Tritan X-100] with slow agitation. After five rinses in PBS, the utricles were treated with streptavadin, a mixture of avidin and biotinylated horseradish peroxidase, and 2.5 ul of Tween 20 (Sigma, St. Louis, MO) for 1 ½ hours with slow agitation. The streptavadin was made with a Vectastain kit

(Vector Labs, Burlingame, CA). After five rinses in PBS, the utricles were reacted for six minutes in diaminobenzidine (DAB), using a nickel intensification procedure (SK 4100 Peroxidase Substrate Kit) (Vector Labs, Burlingame,CA). After five rinses in PBS over a thirty-minute period, the utricles were mounted in PBS/glycerol (1:9) and analyzed as whole mounts.

Control Group

In order to look at the normal rate of proliferation with and without immunosuppression, a second set of chicks were used for the exact same procedure as detailed above, only with no antibiotic injection. The experimental group (n=11) received the dexamethazone injection, while the control group (n=12) received the saline/ethanol .

Data Collection

The darkly stained proliferating cells were visualized using an inverted microscope (Zeiss Axiovert 135). These cells were counted over the entire sensory surface of the utricles. Counts were repeated to double-check the figures. The data reported below were obtained from 45 utricles. The groups were as follows: no gentamycin, no dexamethazone group (n = 12), no gentamycin, dexamethazone group (n = 11), gentamycin, no dexamethazone group (n = 12), gentamycin, dexamethazone group (n = 10). All data are given as means +/- S.D., and statistical analysis were carried out using Student's *t* test (two-tailed).

3. Results

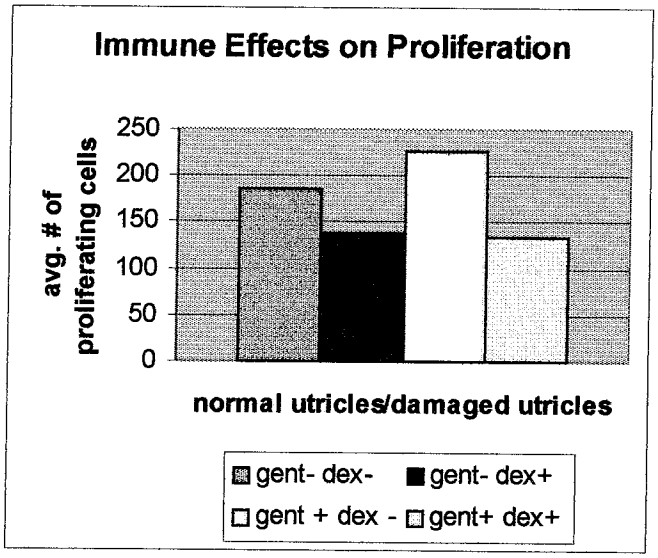
Control Group

The BrdU positive nuclei identify cells that have entered the S phase of the cell cycle, These are easily visualized because they stain dark brown. The number of BrdU labeled nuclei were counted over the entire sensory surface of each utricle. The control group, which received no gentamycin, displayed normal levels of ongoing proliferation. There were significantly fewer proliferating supporting cells in the immunosuppressed utricles (mean = 138.7 +/- 26.3 S.D.) as opposed to the non-immunosuppressed utricles (mean = 185.1 +/- 17.0 S.D.), (P = 0.0001).

Gentamycin Damaged Group

There was extensive hair cell damage in this group, particularly in the striolar region. The Type I hair cell stereocilia could barely be seen in most areas. If they could be seen, they were obviously damaged.

When focusing down to the supporting cell layer it was evident that there was a dramatic difference between the immunosuppressed utricles and those that had normal macrophage function. The immunosuppressed utricles looked basically the same as the control group that had received no gentamycin. There simply was not much supporting cell proliferation when considering all of the damaged hair cells in the layer above. The difference between the immunosuppressed group (mean = 134.1 +/- 29.0 S.D.) and the non-immunosuppressed utricles (mean = 225.9 +/- 45.4 S.D.) was highly significant (P=0.000016).



4. Discussion

All data are suggestive of a significant role for macrophages and other leukocytes in the hair cell regeneration in the avian inner ear. Results support the hypothesis that the suppression of the ability of the leukocytes to secrete peptides will decrease the amount of hair cell regeneration after ototoxic insult in the utricle of the chick. Cotanche et al (1997) describes us a slightly different number of steps for successful hair cell regeneration than that of Bhave et al (1995). First, there has to be damage to the sensory epithelium that results in loss of hair cells. Second, 18 to 24 hours later, the supporting cells in that area must reenter the cell cycle and proliferate. Third, the proliferating cells must differentiate into hair cells and supporting cells. Scanning electron microscopy reveals that the stereociliary bundles on the newly formed hair cells have a very similar morphologic structure to the stereociliary bundles in the earliest stages of hair cell differentiation in the embryonic chick cochlea. Fourth, the new cells must mature. The staircase pattern of stereocilia must be reestablished. Each bundle

must grow to the exact height that is specific to its position on the basilar papilla. The whole bundle must be correctly oriented so that its tallest stereocilia reach the tectorial membrane. Fifth, the mature hair cells and supporting cells must have functional recovery. They must reconnect with both afferent and efferent nerve endings in order to work. Electrophysiologic recordings have confirmed that hearing thresholds do indeed return to normal.

The present results suggest an additional event between Cotanche's first and second step: macrophages must migrate to the area of damage (Warchol, 1997). In addition, the macrophages must not be immunosuppressed, so that they are able to secrete mitogenic growth factors (Bhave, 1998) and other cytokines.

Cotanche et al (1997) go on to describe how the mitotic division in the chick cochlea (in response to damage) parallels that of the embryonic development of the cochlea. This has implications for humans as well. Warchol et al (1993) reported that human vestibular supporting cells in culture can proliferate four weeks after exposure to aminoglycoside antibiotics. This suggests that human cochlear hair cell regeneration could also be possible.

One area for further study is hair cell regeneration in the chick cochlea. Lee et al (1996) have shown that fimbrin, an actin-binding protein, is found only in the hair cells, and not in the supporting cells, hyaline cells, or border cells. It first appears in the damaged areas of the cochlea 96 hours after sound trauma. It is found specifically in the newly forming stereociliary bundles of regenerating hair cells. It appears that the fimbrin helps organize the actin filaments in the cochlear hair cell stereocilia by forming cross-links between them. Not only could fimbrin

be used as a label for the early stages of hair cell regeneration, it could also be involved in the molecular changes that actually facilitate the regeneration.

Another area for further study is the survival of auditory neurons. Park et al (1998) have found that it is not only the cochlear hair cells that are damaged by gentamycin. The brainstem auditory neurons, specifically the nucleus magnocellularis (NM), which is the avian homologue of the antroventral cochlear nucleus in mammals, also showed a significant decrease in the number of neurons five days after a single dose of gentamycin. Chicks sacrificed 70 days post gentamycin, however, had normal numbers of neurons compared to age-matched controls. This suggests that neurons, as well as cochlear hair cells can recover from aminoglycoside damage.

All of these data suggest a possibility for preventative and therapeutic treatments for deafness and balance disorders in humans. Further studies must be done in order to reveal the molecular processes involved in avian hair cell regeneration.

4. References

Bhave, Sujata A., Oesterle, Elizabeth C., and Coltrera, Marc D. (1998). Macrophage and Microglia-Like Cells in the Avian Inner Ear. *The Journal of Comparative Neurology* 398:241-256.

Bhave, Sujata A., Stone, Jennifer S., Rubel, Edwin W., and Coltrera, Marc D. (1995). Cell Cycle Progression in Gentamycin-Damaged Avian Cochleas. *The Journal of Neuroscience* 15:4618-4628.

Cotanche, Douglas A. (1997). Hair Cell Regeneration in the Avian Cochlea. *Annals of Otorhinolaryngology* Supplement 168:9-15.

Kil, J., Warchol, M. E., and Corwin, J.T. (1997). Cell Death, Cell Proliferation, and Estimates of Hair Cell Life Spans in the Vestibular Organs of Chicks. *Hearing Research* 114:117-126.

Lee, Kennth H., and Cotanche, Douglas A. (1996) Localization of the Hair-Cell Specific Protein Fimbrin During Regeneration in the Chicken Cochlea. *Audiology and Neuro-Otology* 1: 41-53.

Park, Debra L., Girod, Douglas A., and Durham, Diane. (1998). Evidence for Loss and Recovery of Chick Brainstem Auditory Neurons During Gentamycin-Induced Cochlear Damage and Regeneration. *Hearing Research* 126:84-98.

Raff, Martin. (1998). Cell Suicide for Beginners. *Nature* 368:119-122.

Press of W. B. Saunders Company. (1974). Dorland's Illustrated Medical Dictionary. U.S.A.: Author.

Sheeler, Philip, and Bianchi, Donald E. (1983). Cell Biology: Structure, Biochemistry, and Function. John Wiley & Sons, Inc. U.S.A..

Warchol, Mark E. (1997). Macrophage Activity in Organ Cultures of the Avian Cochlea: Demonstration of a Resident Population and Recruitment to Sites of Hair Cell Lesions. *Journal of Neurobiology* 33: 724-734.

Warchol, Mark E., and Corwin, Jeffery. (1996). Regenerative Proliferation in Organ Cultures of the Avian Cochlea: Identification of the Initial Progenitors and Determination of the Latency of the Proliferative Response. *The Journal of Neuroscience* 16(17) 5466-5477.

Warchol, Mark E., Lambert, Paul R., Goldstein, Bradley J., Forge, Andrew, and Corwin, Jeffery T. (1993). Regenerative Proliferation in Inner Ear Sensory Epithelia from Adult Guinea Pigs and Humans. *Science* 259:1619-1622.