ANTI-APOPTOTIC FACTOR z-Val-Ala-Asp-FLUOROMETHYLKETONE PROMOTES THE SURVIVAL OF COCHLEAR HAIR CELLS IN A MOUSE MODEL FOR HUMAN DEAFNESS

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Abstract—A major challenge in the inner ear research field is to restore hearing loss of both non-genetic and genetic origin. A large effort is being made to protect hair cells from cell death after exposure to noise or drugs that can cause hearing loss. Our research focused on protecting hair cells from cell death occurring in a genetic model for human deafness. Pou4f3 is a transcription factor associated with human hearing impairment. Pou4f3 knockout mice (Pou4f3−/−) have no cochlear hair cells, resulting in complete deafness. Although the hair cells appear to form properly, they progressively degenerate via apoptosis. In order to rescue the hair cells in the knockout mice, we produced explant cultures from mouse cochleae at an early embryonic stage and treated the cells with z-Val-Ala-Asp-fluoromethylketone (z-VAD-fmk), a general caspase inhibitor. Hair cell numbers in the knockout mice treated with z-VAD-fmk were significantly higher than in the untreated mice. We found that the time window that z-VAD-fmk has a protective effect is between E14.5 (P = 0.001) to E16.5 (P = 0.03), but not after E16.5. The source of the surviving hair cells is not due to proliferation, as measured by 5-bromo-2-deoxyuridine (BrdU) labeling, or to supporting cell transdifferentiation to hair cells, since there was no change in supporting cell numbers. Instead, the survival appears to be a direct effect of the anti-apoptotic agent on the dying hair cells with an early developmental window. These results help towards providing a comprehensive understanding of the molecular mechanisms of hair cell death, which might lead to the development of new therapeutic anti-apoptotic agents to alleviate hereditary hearing loss (HL). © 2010 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: transcription factor, Pou4f3, hearing loss, cell death, inner ear.

Hearing loss (HL) is a clinically heterogeneous disorder, with differences in age of onset, severity and site of lesion. Hearing impairment is the most common disabling sensory defect in humans with severe to profound HL affecting 1 in 500 newborns and 30% of persons older than 65 years (US National Institute on Deafness and Other Communication Disorders, http://www.nidcd.nih.gov/health/statistics/hearing.asp). Over half of HL cases have a genetic basis and a significant proportion of genetic-based HL is non-syndromic, with no other complications (Nance, 2003). Over 50 deafness-causing genes have been identified thus far, making this an extremely genetically heterogeneous disease (Hereditary Hearing Loss Homepage; http://webh01.ua.ac.be/hhh/). These genes encode a large variety of proteins, and studies of their cellular localization and functional analysis have provided a great deal of insight into mechanisms of inner ear function (Dror and Avraham, 2009). However, one of the greatest challenges for hereditary HL remains: how can hearing be restored following genetic insults?

Hearing is enabled by the hair cells of the inner ear cochlea, specialized sensory cells embedded in the sensory epithelium of the organ of Corti. The hair cells cease to differentiate during embryonic development and become post-mitotic, so that the number of cells we are born with, ~16,000, is our lifetime supply (Wright et al., 1987). Therefore, hair cell loss is irreversible and leads to HL. Hair cell death can be caused either by genetic factors, including age-related changes, or by environmental factors, such as viral exposure, ototoxic drugs or acoustic trauma. Morphological evidence from many vertebrate species suggests that the loss of hair cells occurs, at least in part, by programmed cell death (PCD), or specifically, apoptosis (Matsui and Cotanche, 2004; Cheng et al., 2005; Cotanche, 2008). While apoptosis has a physiological role during normal development, trauma or disease can also induce apoptosis, leading to pathological consequences (Conradt, 2009). Indeed, caspase 3, a member of the cysteine-aspartic acid protease (caspase) family, mediates apoptosis in the cochlea and appears to play a key role in hair cell degeneration. Together with Bcl-2 and Bak, caspase 3 is involved in age-related HL (Alam et al., 2001; Someya et al., 2009). Moreover, caspase inhibitors were used to promote hair cell survival after treatment with ototoxic drugs (Liu et al., 1998; Matsui et al., 2002, 2003) and acoustic trauma (Nicotera et al., 2003). However, the expression of caspase 3 during cochlea development is crucial for the development of functional and vital hair cells, and caspase 3 knockout mice suffer from postnatal loss of hair cells and HL (Morishita et al., 2001; Takahashi et al., 2001).

The transcription factor Pou4f3 (previously named Brn3c and Brm3.1) has a critical role in the inner ear, as Pou4f3 mutations are associated with hereditary HL in humans (Vahava et al., 1998; Collin et al., 2008). In the mouse, this transcription factor is involved in survival and maintenance of hair cells (Erkman et al., 1996; Xiang et al., 1997). Deletion of Pou4f3 by gene-targeted mutagenesis...
leads to progressive hair cell degeneration caused by apoptosis (Xiang et al., 1998). In this report, we treated cells of cochlear explants derived from the Pou4f3−/− mice with an anti-apoptotic factor in order to inhibit or delay cell death. These results help towards providing a comprehensive understanding of the molecular mechanisms of hair cell death, which might lead to the development of new therapeutic anti-apoptotic agents to alleviate hereditary HL.

EXPERIMENTAL PROCEDURES

Animals

All procedures involving animals were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and have been approved by the Animal Care and Use Committee of Tel Aviv University (M-07-061). Pou4f3 knockout mice (Pou4f3−/−) were maintained by crossing heterozygote mice (Xiang et al., 1997). To generate homozygous embryos, pregnant females were euthanized at the desired gestational stage. The appearance of a copulation plug was considered embryonic day 0 (E0). Genotyping was performed by a multiplex PCR reaction using specific primers (available upon request).

Organotypic explant cochlear cultures

Pregnant mice were euthanized at E14.5. When dissecting heterozygote females, embryos’ tails were taken for DNA extraction with normal donkey serum and incubated with mouse monoclonal antibodies overnight at 4 °C or 2 h at 37 °C, appropriate secondary antibodies were used (Molecular Probes). For whole mount preparation of cochlear explants, the cultures were fixed in 4% paraformaldehyde. Samples were rinsed in PBS and incubated in 10% normal goat serum or normal donkey serum in PBS with 0.5% Tween-20. The cells were labeled with one of the following primary antibodies: rabbit anti-myosin VI (Proteus Biosciences, Ramona, CA, USA) and goat anti-Prox1 (R&D, Minneapolis, MN, USA). After incubation with the primary antibodies overnight at 4 °C or 2 h at 37 °C, appropriate secondary antibodies, specified above, were used (Molecular Probes).

Immunohistochemistry

Immunohistochemistry was performed on paraffin sections and whole mount preparations of cochlea explants. For paraffin sections, immunostaining was performed as described previously (Hertzano et al., 2007). For protein detection, samples were incubated with goat anti-myosin VI antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and rabbit anti caspase 3 antibody (Cell Signaling, Beverly, MA, USA). The nuclei were stained with 4’,6-diamidino-2-phenylindole dihydrochloride (Dapi; Sigma, St. Louis, MO, USA). Alexa Fluor 594-conjugated donkey anti-goat and 488-conjugated donkey anti-rabbit (Molecular Probes, Eugene, OR, USA) were used as secondary antibodies.

Caspase inhibition

To inhibit apoptosis in vitro, cultures were maintained in the presence of the general caspase inhibitor, z-VAD-fmk (R&D Systems) at a concentration of 100 μM for the entire time in culture (Matsui et al., 2002, 2003). The medium was replaced every 48 h with fresh z-VAD-fmk.

Hair cell counts

Following immunohistochemistry, the number of hair cells in the cultures was counted, using a Zeiss LSM510 confocal microscope with an X63 objective. Myosin VI-positive hair cells were counted in a 100 μm area along the entire length of the cochlea. At least three cultures were observed for each experimental condition.

BrdU labeling

For immunocytochemical detection, 5-bromo-2’-deoxyuridine (BrdU, 3 μg/mL, Sigma) was added to cultures for 5 DIV. Cultures were fixed in 4% paraformaldehyde for 30 min, washed with PBS, incubated with 1.5 N HCl for 20 min and neutralized with 0.1 M sodium borate pH 8.5 for 10 min. The cultures were washed with PBS, blocked for 1 h with normal donkey serum and incubated with mouse monoclonal antibody against BrdU (Chemicon, Temecula, CA, USA) and rabbit
polyclonal antibody against myosin VI (Proteus Biosciences). Alexa Fluor 488 donkey-anti mouse and Alexa Fluor 568 donkey anti-rabbit (Molecular Probes) were used as secondary antibodies.

**Statistical analysis**

In experiments that have at least three independent results, the values presented in the graphs are mean ± SD. Differences among the different groups were evaluated using two-tailed paired Student’s t-test and a P-value of <0.05 was considered significant.

**RESULTS**

Caspase 3 is activated during hair cell death in cochlear sensory epithelia of *Pou4f3* knockout mice

Caspase 3 is considered to be the main downstream effector caspase of many apoptotic processes (Taylor et al., 2008). As a result, cleaved caspase-3 antibody, used to detect activated caspase-3, a product of apoptosis, is indicative of apoptosis in cell culture. We tested whether there is increased apoptotic cell death in *Pou4f3*^−/−^ sensory epithelia by staining the cleaved caspase-3 from E16.5 to postnatal day 0 (P0). By immunostaining of paraffin sections of cochlea at E16.5, E17.5, E18.5 and P0, we found that activation of caspase 3 in auditory hair cells of *Pou4f3*^−/−^ began at E18.5 and continued to be expressed at P0. No caspase 3 staining was observed in the heterozygote mice (Fig. 1A). In addition, fragmented nuclei were observed in the knockout hair cells (Fig. 1B).

z-VAD-fmk preserves hair cells in *Pou4f3* knockout mice in vitro

A general caspase inhibitor, z-VAD-fmk, was used in order to test whether inhibition of caspases will have an effect on

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**Fig. 2.** z-VAD-fmk inhibits hair cell death of *Pou4f3* knockout mice in vitro. Cochlear explants from *Pou4f3*^−/−^ (A, C, E, G, I, K) and *Pou4f3*^−/−^ (B, D, F, H, J, L) mice were cultured from E14.5 mice for 6 DIV. DMSO (control) (A, B, E, F, I, J) or z-VAD-fmk (C, D, G, H, K, L) was added to the culture at E14.5 (A–D), E16.5 (E–H) or E18.5 (I–L). All cultures were stained with myosin VI (green). Scale bar in (A) is 20 μm applies to (B–L). Zoom-out images of the entire cochlea (z-VAD-fmk added at E16.5) are presented in (M–P). Scale bar in (M) is 200 μm; applies to (N–P). Graph presented in (Q) concludes myosin VI positive cells counting in a 100 μm cochlea length. In each experiment n = 3. * P = 0.03, *** P = 0.001. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.
rescuing hair cells from Pou4f3−/− ears. Following dissection of E14.5 embryos, we treated the cells for different time intervals with z-VAD-fmk, beginning from E14.5, E16.5 or E18.5. One dissected ear was treated with medium containing 100 μM z-VAD-fmk, while the other ear was treated with control medium containing DMSO (the agent vehicle). After 6 days the cultures were immunostained with myosin VI, a hair cell marker. Fig. 2 demonstrates that there is no change in cell number and morphology in Pou4f3 heterozygote ears upon treatment with z-VAD-fmk. Pou4f3 knockout ears that were treated with z-VAD-fmk showed a partial rescue of hair cells as compared with untreated ears. However, although the hair cells survived, they were not organized in the typical shape and arrangement of inner and outer hair cells. Nevertheless, a count of the hair cells revealed that the Pou4f3−/− hair cells were significantly protected from cell death. We observed that there was no significant difference between the hair cell numbers in the Pou4f3 heterozygote culture (for E14.5: P=0.74) (Fig. 2A, C), while the mutant culture had doubled its cell number (for E14.5: P=0.001) (Fig. 2B, D, Q). In order to narrow down the time window in which the protection is effective, we cultured the sensory epithelia of each embryo at E14.5 and added z-VAD-fmk at E16.5 (Fig. 2E–H) or at E18.5 (Fig. 2I–L) to one out of the two ears. The other ear was treated with DMSO. When z-VAD-fmk was added at E16.5 for only 4 DIV, the hair cells were protected from cell death (P=0.03, Fig. 2Q). However, when z-VAD-fmk was added at E18.5, there was no significant rescue of the hair cells in the culture (P=0.22, Fig. 2Q). The wide effect of the z-VAD-fmk treatment on the Pou4f3−/− culture are presented in zoom-out images that capture the entire sensory epithelia that were cultured (Fig. 2M–P).

No proliferation or transdifferentiation occurs during the rescue

We tested the proliferation status in the explant cultures by adding BrdU to the culture at day E14.5+1 DIV for 5 DIV. At E14.5+6 DIV, we double-labeled the culture with antibodies against BrdU and myosin VI. As shown in Fig. 3, no BrdU labeled cells were observed after 5 DIV, suggesting that the relatively larger number of hair cells that survive are not due to proliferation. There is no double-labeling of hair cells with both myosin VI and BrdU, although each can be seen separately (Fig. 3D). BrdU labeling is not detected in the focal plane of myosin VI-positive cells and some BrdU-positive cells are seen in the Hensen cell and possibly outer sulcus or mesothelial cell layer.

Since BrdU positive hair cells were not seen among the hair cells, we wanted to determine whether the new hair cells that arise are a result of direct transdifferentiation from supporting cells. Prox1 is a nuclear supporting cell marker that is expressed in both Deiters’ and pillar cells. We stained the cultures after 6 DIV with both anti-Prox1 and anti-myosin VI antibodies and looked for cells that express both markers in the Pou4f3−/− treated cultures. At E14.5+6 DIV no cells that express both Prox1 and myosin VI were observed (Fig. 4A–D). Hair cells (green) and supporting cells (red) reside in a different focal plane. In addition, there was a significant change (P<0.05) in supporting cell numbers between Pou4f3 heterozygote cultures and Pou4f3−/− cultures, regardless of the treatment (Fig. 4E).

**DISCUSSION**

PCD is a vital and necessary process in order to rid the tissue of excess cells formed during development (Conradt, 2009). Apoptosis, one form of PCD, which is activated at the molecular level by caspases, often mediates degeneration of inner ear sensory hair cells (Cheng et al., 2005). Our goal in this study was to delay or prevent the death of hair cells in a mouse model for human deafness. Apoptosis
POU4F3, a member of the POU IV subfamily of transcription factors, is a bipartite DNA-binding protein that contains two conserved regions of homology that are joined by a variable linker (Aurora and Herr, 1992). The two conserved domains are referred to as the POU-specific (POU_s) domain and the POU-homeodomain (POU_HD), and both are required for high affinity binding to DNA. Pou4f3 knock-out mice (Erkman et al., 1996; Xiang et al., 1997) serve as a mouse model for human hereditary deafness linked with DFNA15 (Vahava et al., 1998), the locus that contains the POU4F3 gene in humans. Mutations in the POU4F3 gene are associated with dominantly-inherited hearing loss in three extended families reported thus far: an Israeli family with a deletion in the POU_HD domain (Vahava et al., 1998), and Dutch families with missense mutations in the POU_HD and the POU_s DNA-binding domains of the protein (Collin et al., 2008; Pauw et al., 2008). Only a few of the targets of the Pou4f3 transcription factor are known (Hertzano et al., 2004), but the protein encoded by one of them, Gfi1, is known to have an anti-apoptotic role in T cells by repressing the pro-apoptotic proteins Bax and Bak (Grimes et al., 1996). A microarray analysis performed on T cells derived from Gfi1 knock-out mice found that the death receptor CD95, Bad, Apaf1 and the cell cycle inhibitor p21 are upregulated, while Bcl2 is downregulated (Pargmann et al., 2007). In our laboratory, we identified Lhx3 as another target of Pou4f3 in the auditory system, but not the vestibular system (Hertzano et al., 2007). Lhx3 null mice exhibit activation of caspase 3 and TUNEL staining in the pituitary glands, both indicators of apoptosis (Zhao et al., 2006; Elsworth et al., 2008). Recently, LHX3 mutations have been also linked with syndromic hearing loss in humans, accompanied by hypopituitarism (Rajab et al., 2008). In addition, Pou4f3 binds to the promoters of the neurotrophic factors BDNF and NT-3 (Clough et al., 2004), both known to act as survival factors of different sensory neurons in the inner ear (Emfors et al., 1995; Liebl et al., 1997). Thus, Pou4f3 increases the transcription of several anti-apoptotic proteins. Actually, several members of the POU homeodomain family of transcriptional regulators (Pou4f1, Pou4f2, Oct1, Oct2) have been shown to act as anti-apoptotic factors and their lack of expression causes cell death (Budhram-Mahadeo et al., 1999; Gan et al., 1999; Smith et al., 2001a,b; Hudson et al., 2005, 2008; Heckman et al., 2006). For example, the Pou4f1/Bm-3a transcription factor blocks p53-mediated activation of the proapoptotic target genes Noxa and Bax (Hudson et al., 2005). Together with the involvement of Pou4f3 targets in the cell cycle and apoptosis, our data suggest that Pou4f3 regulates, directly or indirectly, the expression of genes that play a part in cell death and survival pathways. When Pou4f3 is removed specifically from cochlear hair cells, the equilibrium between cell death and survival pathways is disturbed, forcing the cell to choose the cell death pathway. Moreover, our data suggest that reconstruction of this equilibrium by inhibition of caspases, even without directly addressing the reason for this apoptosis, may be sufficient to prevent apoptosis of hair cells. Therefore, the results may suggest that a rela-
tively straightforward therapy may help to prevent hair cell apoptosis, even when the reason for the apoptosis is unknown or more complex.

**Pou4f3** is normally expressed in the mouse cochlear hair cells only from E14.5 (Xiang et al., 1998). While the anti-apoptotic agent z-VAD-fmk promoted hair cell survival in **Pou4f3**/−/− cochlear cultures derived from E14.5 mice if it was added to the cultures after 0–2 DIV (E14.5–E16.5), this effect was no longer demonstrated upon addition of z-VAD-fmk to the cultures after 4 DIV (E18.5). These results suggest that a general anti-apoptotic agent may be effective only if the therapy is given as soon as possible after the beginning of the insult that is responsible for the hair cell apoptosis. In our model, 4 days later, the protective effect of z-VAD-fmk was lost.

We have shown that the cochlear hair cells from **Pou4f3**/−/− mice, treated with z-VAD-fmk, are not proliferating and supporting cells do not appear to transdifferentiate to become new hair cells. There were, however, significantly less supporting cells in the cultures derived from **Pou4f3**/−/− mice, regardless of the treatment. This secondary effect of the death of supporting cells in **Pou4f3**/−/− mice was reported previously (Xiang et al., 1997), perhaps due to exposure to substances that are released from the dying hair cells. Our data demonstrate that the supporting cells in **Pou4f3**/−/− cochlear cultures are not influenced by z-VAD-fmk and their number does not increase significantly after treatment.

To preserve the ability to hear, it is not enough to prevent apoptosis of hair cells. The strict organizations of hair cells in the organ of Corti, as well as their interaction with other cells and nerve terminals, should also be preserved. At the next stage, in vivo experiments are required to evaluate whether z-VAD-fmk can not only delay or prevent hair cell apoptosis, but can also rescue the hearing of the **Pou4f3**/−/− mice. Furthermore, can this approach be applied to other models for human hearing loss? Defining the pathways impeded in hair cell loss may provide the key to identifying appropriate therapeutic targets.

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