Apafl-dependent programmed cell death is required for inner ear morphogenesis and growth

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Introduction

The normal patterning and morphological development requires precise coordination of cell proliferation, differentiation, and programmed cell death. Several apoptotic pathways are active during development, the mitochondrial pathway being the best defined. This pathway is initiated when, upon receipt of a death signal, cytochrome c is released through the outer mitochondrial membrane to the cytoplasm where it interacts with the apoptotic protease activating factor 1 (Apaf1). Apaf1 activates caspase 9 in the context of a multiprotein complex termed the apoptosome. The apoptosome in turn activates downstream caspases, mainly caspase 3, involved in the proteolytic destruction of the cell (reviewed by Sanders and Wride, 1995; Cecconi and Gruss, 2001). Members of the Bcl2 family are known to either promote or inhibit apoptosis in response to a variety of cell death signals. The anti-apoptotic isoform produced from the Bcl2-like (Bcl2l) gene (formerly previously known as BclIX), Bcl-XL and Bcl2 can inhibit apoptosis, at least in part, by blocking cytochrome c release, thus preventing apoptosis formation (Kluck et al., 1997; Yang et al., 1997). Bcl2 can also regulate the activation of caspase 2 independently of the apoptosome (Marsden et al., 2002). Pro-apoptotic factors such as Bax, Bad and Bak are known to block the anti-apoptotic effects of Bcl-XL and Bcl2 through heterodimerization (reviewed by Yuan and Yankner, 2000). To add to the complexity, Bcl2l may be alternatively spliced to produce a pro-apoptotic isoform, Bcl-XX, and several anti-apoptotic Bcl2 family members are known to be modulated by protease cleavage that produces C-terminal peptides with pro-apoptotic activity (Basañez et al., 2001; Clem et al., 1998; Fujita et al., 1998). Although the major players in the cytochrome c-dependent apoptotic cascade have been identified, the individual contribution of each component to the development of different organs is largely unknown.

The mammalian inner ear includes specialized vestibular sensory organs for balance and a coiled cochlea for hearing. The early development of the inner ear involves invagination
of the ectodermal placode and its closure to form the otic vesicle. Soon after closure, the otic epithelium undergoes a sequence of complex morphogenetic events to form the membranous labyrinth. The endolympathic duct extends dorsally and the cochlear duct grows ventrally. The utricle and saccule are separated from each other and from the cochlear duct through deepening constrictions in the ventral vestibular part, whereas the dorsal portion develops into three semicircular ducts through a multi-step process. These ducts first emerge as bilayered epithelial outpocketings. The two opposing layers then approach each other and form a so-called fusion plate, in which the cells first detach from the underlying basement membrane, intercalate and then disappear, creating a hollow duct which further grows out to obtain its adult form and size (Sher, 1971; Martin and Swanson, 1993). The periotic mesenchyme develops in close contact with the otic epithelium and forms the bony otic capsule to surround the membranous labyrinth (Sher, 1971; Martin and Swanson, 1993).

Apoptotic cells have been detected in restricted areas of the developing vertebrate inner ear. These areas include the epithelial cells that transiently connect the surface ectoderm and the otic vesicle during and after its closure, the base of the outgrowing endolympathic duct, a ventral area above the primordium of the vestibulocochlear ganglion and the ventromedial wall of the growing cochlear duct (Marovitz et al., 1976; Marovitz et al., 1977; Represa et al., 1990; Fekete et al., 1997; Nikolic et al., 2000). In chicken embryos, apoptosis has also been detected in the fusion plates of the semicircular ducts (Fekete et al., 1997). Apoptosis at the fusion plates has not been detected in mouse or human embryos (Martin and Swanson, 1993; Nishikori et al., 1999).

Although the anatomic distribution of apoptotic cells in the inner ear has been described, the molecular pathways regulating programmed cell death in this structure have not been studied in detail. The Apaf1 mutant mice die usually perinatally (Cecconi et al., 1998; Yoshida et al., 1998). A few postnatal survivors show a hyperactive behavior that was suspected to be a consequence of inner ear defects, because the brain of these mice appeared completely normal (Honarpour et al., 2000). Postnatal defects have been reported in the inner ear of caspase 3 mutant mice (Takahashi et al., 1993). Here we undertook an analysis of the generation and genotyping of Apaf1 mutant mice in order to gain insight into the molecular mechanisms controlling cell death during inner ear morphogenesis. We observed that the majority of the cell death in the inner ear was Apaf1-dependent and most probably occurred through the Apaf1/caspase 9 apoptosome pathway. The lack of apoptosis in Apaf1 mutant mouse embryos led to widespread defects in epithelial morphogenesis and outgrowth. In contrast, increased apoptosis in Bcl2l1 mutant embryos had no major impact on morphogenesis. However, developmental defects with the latter mutants co-localized with a decrease of apoptosis.

Materials and methods

Mouse stocks and strains
The generation and genotyping of Apaf1+/−, Bcl2l1+/− and caspase 9−/− mice has been described previously (Motoyama et al., 1995; Kuida et al., 1998; Cecconi et al., 1998; Salminen et al., 1998). The Apaf1 locus contains a promoterless IRES-lacZ cassette and thus the expression of the lacZ gene is under the control of Apaf1 transcription regulatory elements. All mouse lines were backcrossed into the C57BL/6 genetic background for at least 12 generations. To generate embryos deficient in both Apaf1 and Bcl2l1, double heterozygous animals were interbred. The embryos were taken from timed matings and the day on which a vaginal plug was detected was assigned as E0.5.

Histological analysis and immunohistochemistry
For histological analysis serial paraffin sections through the inner ear were stained with Hematoxylin and Eosin or Giemsa. Immunohistochemical detection of activated caspase 3 was performed with the rabbit anti-CM1 antibody as before (Cecconi et al., 1998). For Bcl-XL immunostaining with mouse anti-Bcl-XL (H-5) antibody (Santa Cruz), Boun’s-fixed paraffin sections were deparaffinized and boiled for 20 minutes in 10 mM Citrate buffer (pH 6). After secondary antibody (donkey anti-mouse HRP IgG) treatment, the signal was amplified using the TSA Cyanine 3 System Kit (PerkinElmer Life Science Products). BrdU intraperitoneal injection and detection in proliferating cells were performed as before (Salminen et al., 2000). Anti-class III β-tubulin (TUJ1, Babco) was used as a control antibody. Three-dimensional reconstructions from histological sections of inner ears at stage E13.5 were constituted from serial paraffin cross-sections of 10 μm thickness. One ear from each genotype was used for reconstruction and the embryos all came from the same litter of a mating between two Apaf1/Bcl2l1 double heterozygous animals.

RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR) assay
Total RNA was isolated with Trizol Reagent (Life Technologies) from wild-type E9.5 and E11.5 mouse embryos. To detect the different splice variants produced from the Bcl2l1 gene, 2 μg of the isolated RNA was subjected to RT reaction using the RT-primer 5′-GACTGAAGAGTGAGCCCAGATC-3′. The forward and reverse primers for PCR were 5′-CAGTGAAGCAAGCGCTGAGAG-3′ and 5′-CGTCAGAAAACCCACGGTTGAAG-3′, respectively.

RNA in situ hybridization and staining for β-galactosidase activity
In situ hybridization and preparation of the radioactive antisense and sense Netrin1 (Serafini et al., 1996), Pax2 (Dressler et al., 1990) and Dlx5 (Acampora et al., 1999) probes were performed as described in Salminen et al. (Salminen et al., 2000). Whole-mount lacZ staining for E12.5 Apaf1+/− and −/− embryos was conducted as described in Hogan et al. (Hogan et al., 1994).

Detection of apoptosis
Detection of apoptosis was performed with the in situ DNA-end labeling technique, the TUNEL method, that detects DNA fragmentation as an indicator of ongoing apoptosis. TUNEL analysis was performed on 10 μm-thick paraffin sections with the ApopTag Fluorescein In Situ Apoptosis Detection Kit (Intergen). The proportion of TUNEL-positive cells from all otic vesicle cells was calculated on serial sections of 2 or 3 E9.5, E10.5 or E12.5 embryos.

Results
Expression of Apaf1 and Bcl2l1 genes during inner ear development
The expression of Apaf1 and Bcl2l1 was analyzed at various stages during the early development. Apaf1/lacZ is widely expressed in developing embryos heterozygous for the lacZ-containing allele (Fig. 1L). A rather weak expression could be detected at E9.5-10.5 all through the otic vesicle and in the periotic mesenchyme developing into the otic capsule (Fig. 1A,B). At E12.5 highest levels of Apaf1/lacZ gene expression were observed in the
endolymphatic duct epithelium. Expression could also be detected in the periotic mesenchyme and it was slightly elevated in the tips of the semicircular ducts as well as in the cochlear duct compared with the rest of the otic epithelium (Fig. 1D,E).

The Bcl2l gene has been shown to produce several protein isoforms through alternative splicing (Bcl-XS) and cleavage of the major anti-apoptotic Bcl-XL isoform (Clem et al., 1998; Fujita et al., 1998). We detected the Bcl-XL isoform in the developing inner ear with an isoform-specific antibody and studied the expression of the alternatively spliced Bcl-XS isoform with RT-PCR assay. To our knowledge, antibodies specific for cleaved Bcl-XL protein isoforms are unavailable.

Bcl-XL immunoreactivity was found all over the otic epithelium at E10.5-12.5, but no protein could be detected in the periotic mesenchyme (Fig. 1G-I). To analyze the expression of the alternatively spliced Bcl-XS isoform with RT-PCR assay. To our knowledge, antibodies specific for cleaved Bcl-XL protein isoforms are unavailable.

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Many TUNEL-positive cells were detected at the base of the wild-type endolymphatic duct at E10.5 (Fig. 2F). In the posterior part of the otic vesicle apoptotic cells were spread over a broad area extending dorsally (Fig. 2G). Anti-caspase 3

**Changes in apoptotic profiles at E9.5-10.5 in the Apaf1 and Bcl2l mutant inner ears**

We compared the pattern of programmed cell death in Apaf1 and Bcl2l mutant inner ears with the corresponding wild-type littermates. In an early otic vesicle, at E9.5-10.0, a TUNEL-positive cell cluster localized to the ventro-posterior area (arrowhead Fig. 2B) and a few scattered dying cells could be observed more dorsally. In Apaf1 mutant otic vesicles, no apoptotic cells were detected at E9.5-10.5 (Fig. 2C and data not shown). In Bcl2l mutant otic epithelium, a significant 3-fold increase in the proportion of TUNEL-positive cells could be observed (Fig. 2D, Table 1).

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Table 1. Increase in cell death in Bcl2l mutant otic epithelium

<table>
<thead>
<tr>
<th>Stage</th>
<th>Wild type (%)</th>
<th>Bcl2l−/− (%)</th>
<th>P</th>
</tr>
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<tbody>
<tr>
<td>E9.5 ov</td>
<td>14.4±5.4</td>
<td>41.2±10.9</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>E10.5 ed</td>
<td>25.3±11.9</td>
<td>77.2±8.9</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>E10.5 ov w/o ed</td>
<td>16.9±6.5</td>
<td>29.9±5.9</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>E12.5 ssd</td>
<td>25.8±6.7</td>
<td>52.0±8.6</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

The proportion of TUNEL-positive cells from all otic vesicle cells was calculated on serial sections covering the whole vesicle at E9.5 or E10.5 (two embryos each genotype at each stage). In case of E10.5 the otic vesicle (ov) was divided in two areas: the endolymphatic duct (ed) and the rest of the epithelium. At E12.5, the proportion of TUNEL-positive cells was calculated in the tip of the outgrowing superior semicircular duct (ssd). The calculated mean values are given with standard deviation (±s.d.). The differences in the tip of the outgrowing superior semicircular duct (ssd). The calculated mean values are given with standard deviation (±s.d.). The differences between wild-type and Bcl2l mutant values was analyzed using the two-tailed Student’s t-test.

staining showed a very similar spatial pattern of dying cells to TUNEL (data not shown) but revealed far fewer cells. This discrepancy may be because of the fact that activated caspase 3 is present only transiently in dying cells, whereas the DNA breaks detected by the TUNEL assay persist for a longer period.

A marked 3-fold increase in the ratio of TUNEL-positive cells could be detected in the endolymphatic duct epithelium of the Bcl2l mutant embryos at E10.5 (Table 1). In addition, the distribution of dying cells in Bcl2l mutant ears extended more ventrally (Fig. 2H). In the rest of the otic epithelium, a 2-fold increase of TUNEL-positive cells could be observed (Table 1). A cluster of apoptotic cells could be observed in the ventro-posterior area of the Bcl2l mutant otic epithelium, but the broad area of apoptotic cells observed more dorsally in the wild-type otic vesicle (Fig. 2G) was not detected (Fig. 2H). Extensive cell movement occurs in the otic epithelium after its closure (Brigande et al., 2000) and thus, the lack of dying cells in the dorsal wall of the Bcl2l mutant otic vesicle could be because of premature death of these cells while still in a more ventro-posterior location (Fig. 2B,D).

Apopotosis has been thought to be responsible for the complete detachment of the mammalian otic vesicle from the surface ectoderm by removing the connecting epithelial stalk after the closure (Marovitz et al., 1976; Represa et al., 1990; Lang et al., 2000). We observed a cluster of apoptotic cells in the region between the surface ectoderm and the endolympathic duct epithelium in wild-type embryos at E9.5 and E10.5 (Fig. 2F and data not shown). In the Apaf1 mutant embryos (n=3), this apoptotic group of cells could not be observed but the otic vesicles had nevertheless closed normally (Fig. 2C). In Bcl2l mutant embryos (n=3) no clear cluster of apoptotic cells but instead a few scattered cells could be observed in the closure region (Fig. 2H).

Very little or no apoptosis could be observed in the periotic mesenchyme of the wild-type embryos (Fig. 2F,G). Apoptosis could, however, be detected elsewhere in the head mesenchyme (Fig. 2B,F). Also, no apoptosis could be observed in the periotic mesenchyme of either Apaf1 or Bcl2l mutant embryos (Fig. 2C,D,H).

Taken together, our results demonstrate that the normal developmental apoptotic pattern had changed in both Apaf1 and Bcl2l mutant inner ears. The almost complete lack of apoptotic cells in the Apaf1 mutant ears indicates that the majority of the apoptosis detected in the wild-type otic epithelium at E9.5-10.5 is Apaf1-dependent. Inactivation of the Bcl2l gene led to a general increase of cell death in the expected apoptotic areas of the otic epithelium. This result is probably because of the lack of the anti-apoptotic Bcl2l isoform. However, at the closure site, a decrease of apoptotic cell numbers was observed, suggesting that pro-apoptotic Bcl2l isoforms might be active there.

Changes in the apoptotic pattern at E12.5

In wild-type embryos at E12.5, TUNEL-positive cells were detected in the endolympathic duct epithelium (Fig. 3A), the wall of the cochlear duct (Fig. 3A-D), the distal edge of the three semicircular duct epithelium (shown for the superior duct in Fig. 3A-D, arrow in A) and the areas where the utricle, saccule and the cochlear duct will be separated by deepening...
constrictions (Fig. 3B, arrowheads). In addition, some apoptotic cells were observed in the superior and posterior semicircular duct epithelium prior to the fusion in the area that is destined to form the fusion plate. A few dying cells were also detected in the immediately adjacent periotic mesenchyme (Fig. 3A,G, arrowheads). In accordance with previous observations (Martin and Swanson, 1993), we could not detect any dying cells when the two epithelial cell layers were opposed and fused (data not shown). However, a few TUNEL-positive cells were observed in the mesenchyme lining the small empty hole after the fusion plate had been cleared (arrowheads in Fig. 3C,D). In general, no or very little apoptosis occurred in other areas of the periotic mesenchyme at this stage.

Very few or no TUNEL-positive cells could be observed in the Apaf1 mutant (n=6) otic epithelium or the periotic mesenchyme. Further away from the ear, in the head mesenchyme, apoptosis could, however, be detected as in the wild-type embryos, suggesting an Apaf1-independent mechanism for the removal of these cells (Fig. 3E). In the Bcl2l mutant (n=6) ears, an overall increase of TUNEL-labeled cells could be detected in the normal locations of the otic epithelium (Fig. 3F). In the tips of the outgrowing superior semicircular duct a 2-fold increase was observed (Table 1). However, a local lack of apoptosis could be observed in the future fusion plate a 2-fold increase was observed (Table 1). However, a local lack of apoptosis could be observed in the future fusion plate epithelium and the adjacent periotic mesenchyme of the posterior semicircular duct as compared with the wild-type embryos (compare Fig. 3G,H, arrowheads). No TUNEL-positive cells could be detected in the Bcl2l mutant periotic mesenchyme (Fig. 3F,H).

In summary, our results suggest that very little or no Apaf1-independent apoptosis occurs in the otic epithelium at E12.5. In the Bcl2l mutant embryos a general increase of apoptosis was observed as at earlier stages, except for the fusion plate area of the posterior semicircular duct where a lack of apoptotic cells was observed. Very little apoptosis can be detected in the periotic mesenchyme, suggesting that apoptosis is not critical for the formation of the otic capsule.

Three-dimensional reconstructions of the mutant inner ears reveal specific morphogenetic defects and reduced growth

The morphogenetic architecture of the membranous labyrinth has been established by E13.5. To examine the morphology of the epithelial labyrinths of the embryos at this stage we reconstructed three-dimensional images from serial sections. The reconstructions showed that both Apaf1 and Bcl2l mutant ears were smaller than the wild-type ears and were reduced to 54% and 70%, respectively (Fig. 4A-C). The diameter of the corresponding mutant heads were 86% and 77% of that of the wild-type heads, respectively (data not shown), which could explain the reduction in membranous labyrinth size in the case of Bcl2l mutants but not in the case of Apaf1 mutants.

The lack of apoptosis in the Apaf1 mutant inner ear epithelium led to severe abnormalities in morphogenesis (Fig. 4B). The inner ear epithelium appeared misshapen and there were no clear borders between the different compartments. The endolymphatic duct was not properly elongated and instead, an enlarged sac-like structure could be seen. At later stages (E16.5-18.5), dorsal extension of the endolymphatic duct could be observed, but the diameter of the duct always remains larger than in the wild-type embryos, and therefore no sac structure could be distinguished (Fig. 5A-C and data not shown). The two semicircular duct outpocketings that form first, the superior and posterior, show a small clearing of the fusion plate at E13.5 as seen also in Fig. 4E for the posterior duct. At this stage, the lateral semicircular duct remained as a very small outpocketing without visible cleared area in the middle. The
cochlear duct was much shorter and more immature in shape compared with the wild-type duct.

The morphology of the Bcl2l mutant inner ear was rather normal except for the posterior semicircular duct that does not form at all. Instead, an outpocketing without epithelial fusion or clearing in the middle could be observed (Fig. 4C,F). In all nine Bcl2l mutant embryos analyzed at E13.0-13.5 the posterior duct failed to form.

The three-dimensional reconstructions demonstrated that the decrease of apoptosis in Apaf1 mutant embryos led to a growth retardation and to defects in shaping or ‘sculpting’ the membranous labyrinth. Bcl2l inactivation led only to a restricted phenotype concerning the posterior semicircular duct.

Superior semicircular duct formation is most severely affected in Apaf1 mutant embryos

Some variation in the severity of the phenotype of the Apaf1 mutant ears could be observed when sections through the ears of 16 E12.5-18.5 embryos were analyzed. General variability in Apaf1 mutant phenotypes has been observed before. In the case of brain, variation ranges from severely overgrown nervous tissue to a completely normal-looking tissue (Cecconi et al., 1998; Honarpour et al., 2000). We obtained embryos at a ratio of 10/6 for severe/mild phenotypes judged according to the brain overgrowth. The severity of the inner ear morphological phenotype seemed to correlate to some extent with that in the brain. The Apaf1 mutant inner ear in Fig. 4B,E comes from an embryo with a highly overgrown and open brain structure, whereas Fig. 5B,C shows the two ears from an embryo with no obvious brain defects. In embryos in which brain overgrowth was extensive, the ear was often aberrantly positioned directly ventrally from the neural tube instead of its normal lateral position (Fig. 4E). In addition, the ear had turned in a more horizontal position. However, irrespective of the severity of the brain phenotype or ear position, the inner ear epithelium was always reduced except for the endolymphatic duct which was always enlarged (Fig. 4B, Fig. 5B,C). In addition, the superior semicircular duct was always markedly reduced (Fig. 5B) and sometimes completely absent (41%) even in embryos with no detectable brain phenotype (asterisk in Fig. 5C). The other two semicircular ducts always formed and a
clearing of the fusion plates could be detected by E14.5 (Fig. 4E, Fig. 5B,C).

The periotic mesenchyme seems to develop normally as judged from those Apaf1 mutant embryos that lived until E16.5-18.5 (n=5). The mesenchymal cells closest to the otic epithelium are destined to undergo cell death once the main morphogenetic events have occurred to generate the perilymphatic space between the membranous and bony labyrinths. Interestingly, this late cell death seems to occur normally in the absence of Apaf1 (Fig. 5A-C).

The inner ear phenotype in the caspase 9 mutant embryos closely resembled that of Apaf1 mutants

The results from the analyses of Apaf1 mutant animals emphasized the importance of Apaf1 for the developmental apoptosis that occurs during otic epithelium morphogenesis. To determine whether this cell death involves the apoptosome or occurs through an apoptosome-independent pathway (Marsden et al., 2002), we analyzed the inner ear phenotype in six caspase 9 mutant embryos at E13.5-17.5.

The inner ear phenotype in caspase 9 mutant embryos including morphogenetic changes (enlarged endolymphatic duct), smaller size, reduced semicircular ducts and variation in phenotype severity was very similar to that observed in Apaf1 mutant embryos (Fig. 5D-F and data not shown). As for other organs affected, the caspase 9 phenotype appeared slightly milder than the Apaf1 phenotype (Cecconi et al., 1998; Yoshida et al., 1998; Kuida et al., 1998; Hakem et al., 1998). These results support the idea that most of the cell death in the developing inner ear occurs through the activation of the apoptosome complex.

Dorso-ventral compartmentalization occurred normally in Apaf1 and Bcl2l mutant otic epithelium

To verify whether the regionalization of the inner ear epithelium occurred normally in Apaf1 and Bcl2l mutant inner ears, we performed in situ hybridization analyses with marker genes known to be specific for either dorsal or ventral parts of the inner ear. The Dlx5 gene was expressed normally in the dorsal vestibular part and in the endolymphatic duct of both mutants (shown for wild-type and Apaf1 mutant in Fig. 6A,B). Pax2 expression was detected ventrally along the utricular wall and in the developing cochlear duct (shown for wild-type and Bcl2l mutant in Fig. 6C,D). Netrin1 is specifically expressed in the semicircular duct fusion-plate-forming epithelium and after duct formation it remains expressed at the inner edge of the duct epithelium (Salminen et al., 2000). In both Apaf1 and Bcl2l mutant ears Netrin1 was expressed in the fusion plate-forming cells as well as the newly formed ducts (Fig. 4D-F, Fig. 6E-F). These results demonstrate that in spite of the changes in apoptotic patterns, abnormal cell positional identities could not be detected in the mutants.

Cell proliferation changes in Apaf1 and Bcl2l mutant otic epithelium

In many cases, the Apaf1 membranous labyrinth was reduced to almost half of the normal size at E13.5 (Fig. 4A,B). To verify whether cell proliferation was affected to the same extent in different parts of the epithelium, we performed BrdU injections into pregnant females and stained sections from the obtained embryos with an anti-BrdU antibody. We chose to analyze two stages, E11.5 and E12.5, in which the epithelial outgrowth is most active. To calculate the proliferation indexes we analyzed sections from two wild-type and two mutant embryos at each stage.

In the severely affected Apaf1 mutant embryos, the overall size of the otic epithelium was clearly diminished already at E11.5 (Fig. 7A,B). In these ears, 22.9%±5.5% of the cells were proliferating in comparison with the wild-type ears in which the proliferation index was 33.4%±4.9% (P<0.001, Student’s t-test). At E12.5 the defect in the semicircular duct outgrowth became evident (compare superior and lateral ducts in Fig. 7F,G). However, no clear difference in the proliferation index could no longer be detected here (data not shown). The consequence of a decrease in the proliferation index at an earlier stage could be observed as a strong reduction in the number of cells in the mutant duct area at later stages. At E12.5 the proliferation index in the cochlear duct was 40.4%±10.3% in the Apaf1 mutant and 68.8%±13.6% (P<0.001) in the wild-type (examples in Fig. 7G,H). Moreover, an almost complete lack of proliferating cells was observed in the outer wall of the turning cochlear duct (arrowheads in Fig. 7G,H). No clear
change in the local cell proliferation could be observed in the endolymphatic duct epithelium between wild-type and Apaf1 mutant embryos at E11.5 (data not shown).

Interestingly, the dramatic increase of apoptosis in the otic epithelium of the Bcl2l mutant embryos did not seem to have much of an effect on its final size at E13.5, suggesting that the increased apoptosis stimulated a compensatory increase in cell proliferation. To verify this we compared cell proliferation in wild-type (46.1±10.2%) and Bcl2l mutant otic vesicles (55.7±9.3%) at E10.5 and observed a significant increase in proliferation (P<0.0001) in Bcl2l mutant inner ears (examples in Fig. 7D,E). At this stage, no clear difference (p=0.42) in the total number of otic epithelium cells could be observed between wild-type (189.7±19.7 average number of cells/section±s.d.) and Bcl2l mutant (184.5±15.5) embryos (n=2). At later stages, no significant difference could be observed in proliferation indexes, for example, in the tips of the outgrowing semicircular ducts (data not shown). At E13.5 the mutant semicircular ducts appeared slightly thinner (Fig. 4A,C) and the number of cells (24.6±3.1) in cross-sections through the superior duct of the Bcl2l mutant ears (n=2) was 26% smaller than in wild-type ones (33.2±3.9, P<0.0001).

Taken together, our results suggest that Apaf1-dependent apoptosis might be required to achieve the normal level of cell proliferation in the developing otic epithelium excluding the area of the endolymphatic duct. Lack of apoptosis seemed to especially affect the outgrowth of the semicircular and the cochlear ducts. Furthermore, an increase in apoptosis in Bcl2l mutant inner ears seems to result in an increase of proliferation at early stages that could at least partially compensate for the important loss of cells through excess cell death.

Otic vesicle closure failed to occur in Apaf1/Bcl2l double mutant mice

In order to check whether the anti-apoptotic Bcl2l and the pro-apoptotic Apaf1 operated independently of each other or through a common pathway during inner ear development, we generated double mutant embryos. These embryos died at E12.5-13.0, similar to the Bcl2l single mutants, and suffered from a hematopoietic failure indicating that the inactivation of Apaf1 cannot inhibit the excessive cell death occurring in the hematopoietic system when the Bcl2l gene is inactivated (Motoyama et al., 1995). Apaf1 deficiency did, however, prevent the increased cell death observed in immature neurons throughout the embryonic Bcl2l-deficient nervous system (Motoyama et al., 1995) (data not shown). The same observations have been reported by Yoshida et al. (Yoshida et al., 2002).

We analyzed the inner ear phenotype in three double mutant embryos at E11.5. In none of the three cases did the otic epithelium undergo massive apoptosis as

in Bcl2l mutants. Instead, no apoptosis could be detected, a phenotype very similar to that of Apaf1 mutant inner ears (Fig. 8A-B). This result suggests that in contrast to the hematopoietic system, the excess of cell death that results from Bcl2l inactivation requires Apaf1 in the inner ear.

The epithelial morphogenesis of the inner ear in the double mutant embryos occurred in a very similar way to that in Apaf1 mutants. However, the otic vesicle failed to close completely in all the six mutant ears that were analyzed (Fig. 8B,D). The imperfect closure may have led to the lack of any morphologically detectable endolymphatic duct formation, because normally the endolymphatic duct forms close to the site where the closure has taken place. In other areas of the otic vesicle the regionalization occurred normally and Netrin1, Dlx5 and Pax2 expression could be detected in anticipated areas (Fig. 8F-G and data not shown).

Discussion

Programmed cell death is required to shape the membranous labyrinth

In Apaf1 mutant embryos, none or very few dying cells could be observed in the otic epithelium leading to a malformed membranous labyrinth in which different compartments were not clearly defined. In addition, the endolymphatic duct remained enlarged and no sac structure could be distinguished. Therefore, the localized Apaf1-dependent apoptosis that normally removes cells at the constriction sites and at the base of the endolymphatic duct is required for the correct shaping.

**Fig. 7.** Cell proliferation in Apaf1 and Bcl2l mutant otic epithelium. Proliferating cells were detected with an anti-BrdU antibody staining of the developing inner ear at E11.5 (A-C), at E10.5 (D-E) and E12.5 (F-H) in wild-type (A,D,F,H), Apaf1 (B,G) and Bcl2l (E) mutant otic epithelium. Control staining with secondary antibody only (C). Scale bar: 100 μm.
of the organ. The inner ear phenotype of the caspase 9 mutant embryos closely resembled that of Apaf1 mutants, strongly suggesting that Apaf1 affects inner ear morphogenesis through the apoptosome complex.

The morphogenesis of the three semicircular ducts is a complex procedure, the main steps of which seem to be common for all of them. However, targeted mutations of several genes in mouse have shown selective defects in duct formation and suggested that the formation of each duct might be independently controlled by specific sets of genes (Fekete, 1999). Here we show that the inactivation of the Bcl2l gene selectively affects the formation of the posterior semicircular duct. This phenotype could be because of the observed lack of local apoptosis at the fusion plate-forming epithelium and/or in the adjacent periotic mesenchyme, suggesting that pro-apoptotic Bcl2 isoforms may have a developmental role there. Fekete et al. (Fekete et al., 1997) have shown that overexpression of the Bcl2 gene in chicken embryos leads to a block of cell death that most severely affects the posterior duct. These observations suggest a special regulatory role for Bcl2 family members concerning the development of the posterior duct of the two species. Interestingly, Apaf1 also seems to be required especially for one of the semicircular ducts; the growth of the superior duct is most severely affected in Apaf1 mutant embryos.

Some variation of the Apaf1 mutant inner ear development was observed and the severity of the phenotype correlated with that observed in the brain. Therefore, it is possible that a severely overgrown hindbrain enhances the aberrant ear phenotype. However, we observed that the principal structural and growth defects were present also in Apaf1 mutant embryos where no brain phenotype could be detected.

**Apoptosis and outgrowth of the inner ear epithelium**

Unneeded cells are removed by apoptosis in many organs during development. The phenomenon is most striking in brain where in some areas nearly 50% of the cells are eliminated (reviewed by D'Mello, 1998). In Apaf1 mutant mice, there is a decrease in apoptosis in many neuronal populations including neural precursors. This results in an increased amount of cells undergoing cell division and to overgrowth of the brain mass (Cecconi et al., 1998; Yoshida et al., 1998).

A balance between cell proliferation and apoptosis also seems to play a critical role during inner ear development. There are regional differences in the proliferative activity in the otic vesicle resulting in differential outgrowth of the epithelium (Lang et al., 2000). In some areas where cells undergo active proliferation, dying cells are abundant. These areas include the distal edges of the semicircular ducts and the wall of the cochlear duct (Fekete et al., 1997; Nicolic et al., 2000). In Apaf1 mutant embryos, a surprising size reduction of the membranous labyrinth was already observed at E11.5 and cell proliferation had diminished by 10-30% depending on the area and stage analyzed. There was an especially clear reduction in the elongation of the cochlear duct and the outgrowth of the semicircular ducts. Therefore, the morphogenetic defects observed in the cochlea and in the semicircular ducts seem to be a consequence of decreased proliferation together with decreased apoptosis.

How does the lack of apoptosis affect cell proliferation in these outgrowing areas of the otic epithelium? Apoptosis might stimulate proliferation through increasing locally the amount of growth-promoting factors released to the environment. This kind of passive release of functional factors into the environment from dying cells has been described (Scaffidi et al., 2002). Fibroblast growth factors (FGFs) are potential candidates to promote otic growth because several of them are expressed in the otic epithelium during its outgrowth period (reviewed by Noramly and Craigher, 2002). Interestingly, a subset of extracellular FGFs do not have any signal peptide and it has been proposed that they could be released passively from damaged cells (reviewed by Ornitz and Itoh, 2001).

In Bcl2l mutant inner ears a dramatic increase of apoptosis was observed at certain locations at E9.5-10.5. The excess of cell death seems to be at some extent compensated by an increase in cell proliferation because the ear is not smaller than in the wild-type littermates at this early stage. In mice with a

![Fig. 8. Analysis of the inner ear in Apaf1+/+ Bcl2l+/+ double mutant embryos. Many TUNEL-positive cells could be observed in the otic epithelium of Bcl2l−/− embryos (A) at E11.5. No apoptosis could be observed in the Apaf1+/+ Bcl2l+/+ double mutant otic epithelium (B). In the wild-type embryo, the superior semicircular duct has already formed (C), whereas more immature morphogenesis can be observed in the Apaf1−/− Bcl2l+/+ double mutant otic epithelium (D–E). The arrows in B and D point to the closure defect in the double mutant otic vesicles. In situ hybridization analysis shows Dlx5 (F) and Netrin1 (G) expression in the double mutant superior semicircular duct outpocketing. Scale bar: 100 μm.](image-url)
targeted mutation in the Bcl2 gene an increase in apoptosis can be observed during development of many organs. This leads to a dramatic decrease in the overall size of some organs such as spleen, kidney and thymus. However, some cell types in the developing kidney, such as the epithelium and interstitium, hyperproliferated, leading to increased numbers of these cell types (Veis et al., 1993). Thus, it seems that in some organs and cell types increased apoptosis may lead to overproliferation and decreased apoptosis to a growth delay.

More direct molecular links between proliferation and cell death have been proposed in several cell culture studies involving caspases (reviewed by Guo and Hay, 1999; Los et al., 2001; Mendelsohn et al., 2002) and Bcl2 family members (Los et al., 2001). How Apaf1 and apoptosis affect cell proliferation in the otic epithelium remains to be clarified. Another open question is why the phenotypes in brain and ear are so different. It may be because of the fact that the neuroectodermal cells continue to proliferate by default in the absence of cell death, whereas the ectodermal cells of placodal origin do not seem to proliferate by default and might require stimuli and signals from the neighboring dying cells in order to proliferate normally.

**Closure of the otic vesicle does not occur in the absence of both Apaf1 and Bcl2l gene products**

Apoptotic cells have been detected in the area of otic vesicle closure in normal embryos. However, no defects related to the closure were observed in Apaf1 or Bcl2l single mutant embryos. Interestingly, when Apaf1 mutation was introduced into the Bcl2l-deficient background, the otic vesicle closure was incomplete in 100% of the mutants. These observations suggest that Apaf1 and pro-apoptotic Bcl2l gene product(s) may have redundant functions in controlling cell death at the closure site. The function of pro-apoptotic Bcl2l gene product(s) at the otic vesicle closure site is further supported by the fact that we observed a reduction, rather than an increase, in cell death at the otic vesicle closure site in Bcl2l mutant embryos. The Apaf1+/−/Bcl2l−/− phenotype is the first example of an otic vesicle closure defect in mouse mutants. Interestingly, the otic epithelium compartmentalization seems to occur despite its incomplete closure.

In conclusion, we show that Apaf1-dependent and Bcl-Xl-regulated apoptosis is critical for the normal morphological development and the outgrowth of the otic epithelium. Furthermore, we propose that, together with Apaf1, pro-apoptotic Bcl-Xl isofrom(s) produced from cleavage of Bcl-Xl could play a role in otic vesicle closure and in the formation of the posterior semicircular duct.

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