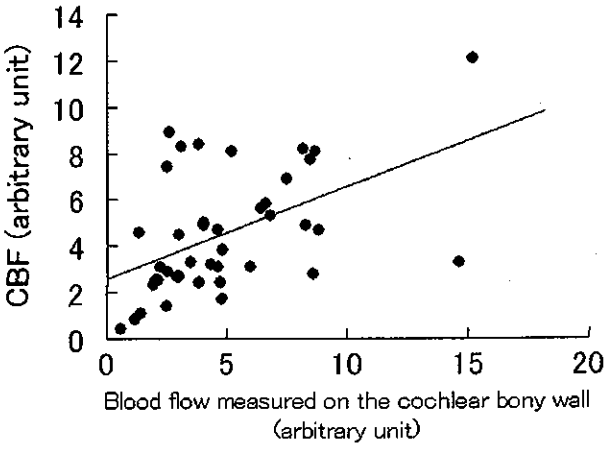

Fig 4. Relationship between blood flow measured on cochlear bony wall and CBF.

the probe was inside the perilymph and those measured when one half of the bone was drilled or when the bone had been drilled down to the membrane surrounding the perilymph ($p < .01$).

DISCUSSION

Among the patients with congenital hearing loss of unknown cause, there was 1 patient who was born from a cryopreserved and thawed embryo after in vitro fertilization. It has been demonstrated that infants conceived by in vitro fertilization have higher risks of major birth defects than do naturally conceived infants.[3] To our knowledge, this is the first report of profound sensorineural hearing loss in a child born from a cryopreserved and thawed embryo, although conductive hearing losses have been reported in such children.[4]

Among the patients with IPSNHL, the CBFs were significantly lower in those more than 40 years of age. Two age groupings have been observed in patients with IPSNHL, suggesting a juvenile-type IPSNHL (patients 10 to 19 years of age) and an adult-type IPSNHL (patients 40 to 69 years of age).[5] The cause of IPSNHL may therefore differ between the juvenile and adult types. Our results suggest that a disturbance of CBF may be closely associated with hearing loss in patients with adult-type IPSNHL.

Cochlear ossification is a common occurrence in patients with postmeningitic deafness,[6-8] and temporal bone studies have revealed some relationships between cochlear ossification and vascular disturbance.[9] In animal experiments, cessation of CBF resulted in cochlear ossification.[10] These reports and our present findings strongly suggest that fibrous and bony occlusion of the perilymphatic space, which occasionally makes cochlear implantation difficult, may be associated with the impairment of CBF. However, it seemed that a decrease in the perilymphatic space might not always reduce the CBF, because the CBF was not low in a patient whose perilymphatic space was partially occluded by fibrous tissue after typhoid fever. The mechanisms leading to deafness after typhoid fever[11] may therefore differ from those following meningitis.

The CBF was low in a patient with narrow internal auditory canals, perhaps because the labyrinthine artery running through the internal auditory canal was poorly developed. The patient with a mitochondrial DNA *1555 point* mutation was unique, because this case was associated with intrauterine aminoglycoside ototoxicity.[12,13] The CBF in this patient was slightly lower than average. Because temporal bone histopathologic analysis in patients with mitochondrial point mutations has revealed atrophy of the stria vascularis,[14] it is likely that CBF may be reduced in the pathological cochleas of patients with such a mutation.

The possibility of disturbed CBF has been described in patients with sudden deafness,[15] Meniere's disease,[1,16] and LVA syndrome.[17] In the present study, the CBF was lower in patients with sudden deafness or LVA syndrome, and larger than average in the patient with Meniere's disease. Further measurements in more cases are necessary to evaluate the CBF pattern in patients with such diseases.

There were correlations between the laser-Doppler outputs measured when the tip of the probe was inside the perilymph and those measured when the tip of the probe was attached to other positions. Because blood flow in the bone surrounding the cochlea does not come from the anterior inferior cerebellar artery,[18] which usually gives off the labyrinthine artery, it is most appropriate to measure the CBF when the tip of the probe is inserted into the perilymph. However, the correlation indicated that it is possible to evaluate the CBF by placing the tip of the laser-Doppler probe on the mucous membrane or on the surface of the bone surrounding the cochlea.[19]

In patients with congenital hearing loss of unknown cause, the degree of language acquisition after cochlear implantation may vary depending on individual age, intelligence, rehabilitation method, social environment, state of the cochlear implant, and number of remaining spiral ganglion cells. In a temporal bone study on postmeningitic deafness, there was a strong negative correlation between the degree of cochlear bony occlusion and the normality of the spiral ganglion cell count.[20] Because the remaining spiral ganglion cells are supplied by the CBF, we plan to follow up the progress of language acquisition in these patients, including the presence or ab-

sence of a relationship between language acquisition and CBF.

CONCLUSIONS

We evaluated the CBF in patients with profound deafness by placing the tip of a laser-Doppler probe at various positions during cochlear implantation. Reduction of the CBF was recognized in patients with cochlear ossification following meningitis and in patients with adult-type IPSNHL. Reduction of the CBF was also recognized in some patients with profound deafness of unknown cause. Evaluation of the CBF thus promises to provide important information on the cause of deafness.

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急性低音障害型感音難聴再発例の平成12，13年全国疫学調査

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要旨：急性低音障害型感音難聴再発例の全国個人調査票を作成し，平成12年度，13年度の症例を収集し解析した。2年間に96例101耳の症例を得た。再発例は，初発例に比べると約1/4の症例数であった。全体の聴力予後は，急性高度難聴調査研究班で提唱している聴力の予後判定基準に従うと，治癒41耳（43%），改善24耳（25%）で治癒と改善をあわせると全体の68%であった。再発例では，早期受診例で予後良好であったが，年齢，再発時聴力レベル，再発までの期間で予後に差はなかった。再発例の特徴として，再発時も急性低音障害型の診断基準をクリアするものは再発例の約60%に過ぎなかった。

－キーワード－
低音障害型感音難聴，再発例，全国疫学調査

はじめに

急性低音障害型感音難聴を発症する症例の特徴として，一度症状が消失した後に同様の症状を繰り返すことがある。比較的短期間で繰り返すものは，再発とすべきか，1回の発作が完全に治療していないと考えるか難しい。そこで急性高度難聴調査研究班では，初回発作が治癒後，6カ月以上経過して同様の症状を呈するものを再発例と定めた。そこで今回，この再発例の疫学的な特徴をつかむために，全国個人調査票により平成12年度，13年度の再発例について症例を収集し解析した。なお，平成12年度の初発例については佐藤ら[1]によりすでに報告されている。

方　法

厚生労働省急性高度難聴調査研究班により，平成11年度に作成された診断基準[2]に従い，平成12年度に急性低音障害型感音難聴再発例の全国個人調査票を作成し，全国12大学（北海道大，岩手医大，東京医歯大，慶応大，北里大，信州大，浜松医大，名古屋大，兵庫医大，愛媛大，岡山大，宮崎医大）から症例を収集した。表1に，今回用いた診断基準を示す。その解析の結果を検討し，平成12年度末に調査票[3]を一部改訂した。改訂版調査票により，平成13年度の症例を収集し解析した。

改訂点として，発作回数は平成12年度では調査方法にあいまいな点があり，回数の計測に不統一が見られた。そのため，平成13年度は初回を1回目，第1回目の再発時を2回目として計数するよう指示した。再発例では必ず2回以上になるはずだが，0回，1回との回答が1例ずつあった。しかし，全体として記入ミスは著減した。平成13年度の調査からみると，発作回数は2回がもっとも多く，つまり初回の後，1回繰り返したものがもっとも多いという結果であった。再発例を再発時聴力が診断基準に合致する例に限局しても同様の傾向であった。

ここで採用した再発例は，治癒後6カ月以上経過して原則として初回発作と同様の症状をきたした例とした。

聴力予後の判定には表2の基準を用いた。

表1 急性低音障害型感音難聴の診断基準（試案）

主症状
1．急性あるいは突発性に蝸牛症状（耳閉塞感、耳鳴、難聴など）が発症する。
2．難聴は低音障害型感音難聴である。
3．難聴の原因は不明または不確実である。
4．めまいを伴わない。

参考事項
1．難聴に関しては以下の基準による。
（1）オージオグラム低音域3周波数（125, 250, 500Hz）の聴力レベルの合計が70dB以上。
（2）同様に高音域3周波数（2000, 4000, 8000Hz）の聴力レベルの合計が60dB以下。
2．蝸牛症状が反復する例がある。
3．メニエール病に移行する例がある。
4．まれに両側性の例がある。
5．上気道炎、ストレス、過労が先行することがある。

表2 聴力予後の判定

（1）治癒：低音3周波数（125, 250, 500Hz）の聴力レベルがいずれも20 dB以内に戻ったもの。あるいは健側聴力と同程度まで回復したとき。
（2）改善：低音3周波数の聴力レベルの平均が10 dB以上回復し、かつ治癒に至らないもの。
（3）不変：低音３周波数の聴力レベルの平均が10dB未満の変化。
（4）悪化：（1）（2）（3）以外のもの

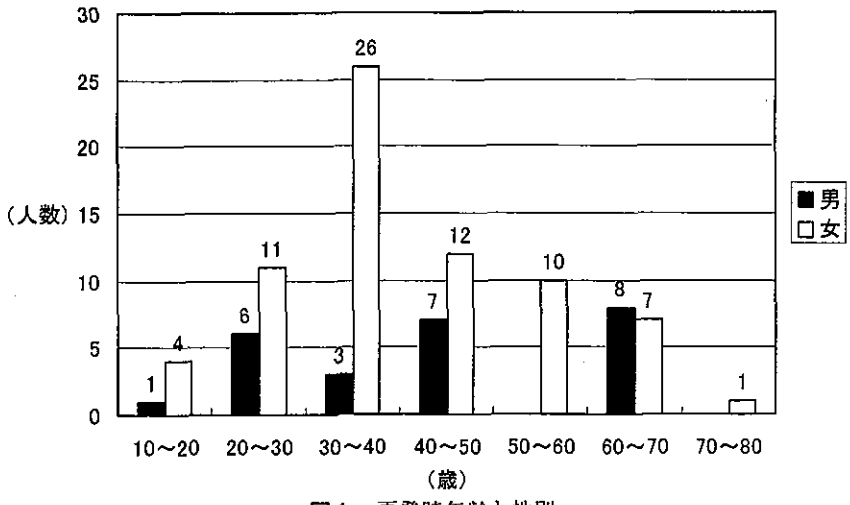

図1 再発時年齢と性別

1） 年齢，性差，2） 自覚症状，3） 合併症，4）治療，5） 予後，6） 予後と各因子（①性差，②年齢，③再発から受診までの期間，④再発時聴力レベル，⑤再発の期間）との相関について検討した。統計学的有意差の検定には，χ^2検定を用い，危険率５％未満（$p<0.05$）を有意差ありとした。

結　果

1）年齢，性差

２年間に96例101耳の症例を得た。年齢は17歳から75歳で，全体の平均年齢は初回時40.6歳(89例)，再発時42.3歳（96例）であった。男性25例，女性71例と女性に多い傾向を示した。再発時の平均年齢は男

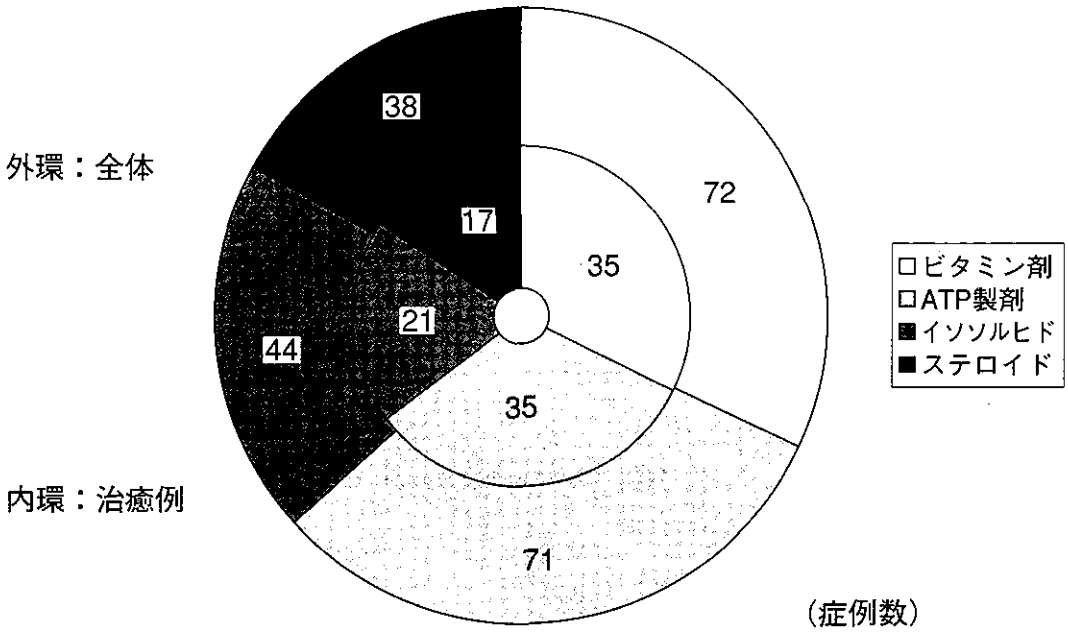

図2　治療薬の割合

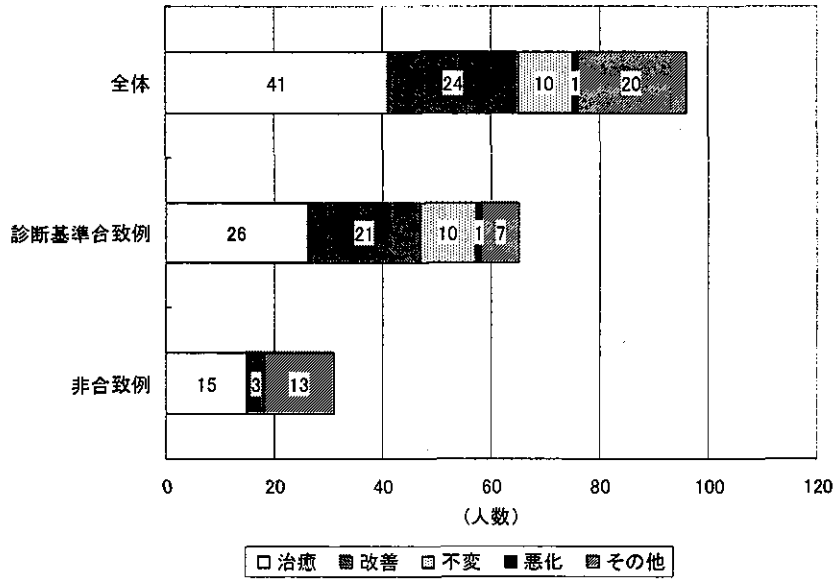

図3　診断基準と予後

性45.7歳，女性42.0歳であった(図1)。初回発作時年齢不詳の6例を除くと発作間隔は平均2.6年であった。

2）自覚症状

主訴は多い順に耳閉塞感（36例），難聴（28例），耳鳴（23例）であった。主訴を含めた随伴症状は難聴(68例)，耳閉塞感(65例)，耳鳴(62例)，めまい(19例)，聴覚過敏(18例)，自声強調(16例)の順であった。

3）既往歴または合併症

既往歴，合併症として最も多かったのは高血圧(11例）であり，ついで，ムンプス，アレルギー疾患，自己免疫疾患，糖尿病，心疾患，ヘルペスであった。逆に，低血圧は1例もなかった。

4）治療

治療としては，複合治療されているものが多く，多い順にビタミン剤72例，ATP製剤71例，イソソルビド44例，ステロイド38例であった。治癒例だけを見てもその割合に変化はなかった。

5）予後

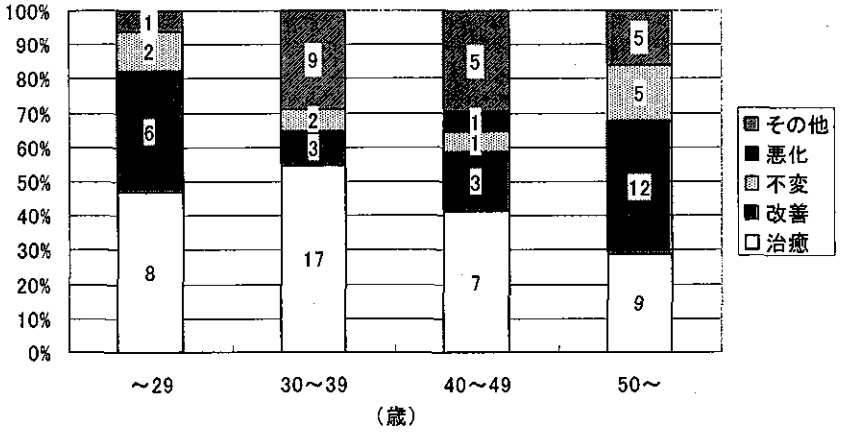

図4　再発時年齢と予後

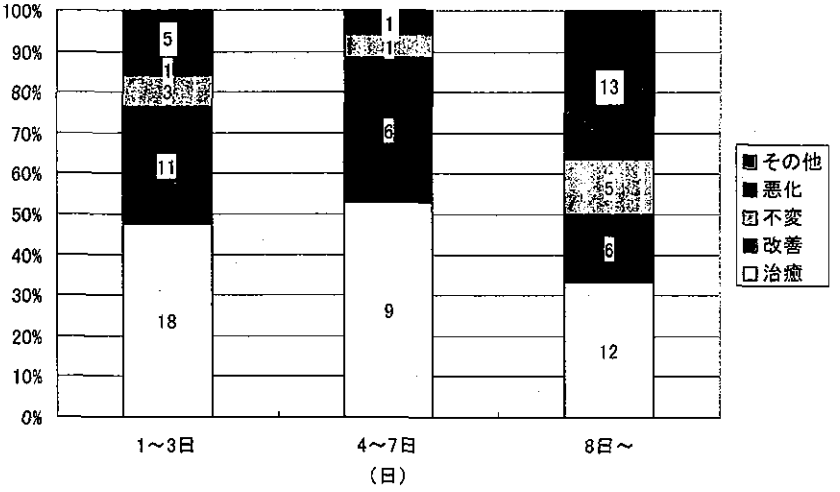

図5　再発後初診までの日数と予後

全体の聴力予後は，急性高度難聴調査研究班で提唱している聴力の予後判定基準に従うと，治癒41例(43%)，改善24例（25%）で，治癒と改善をあわせると全体の68%であった。全体96例の中で再発時にも診断基準に合致した例は65例であった。その予後は，治癒・改善率あわせると72%で，全体との差はなかった。診断基準に合致しなかった症例（非合致例）では31例であり，治癒・改善率は58%であった(図3)。

また，経過中メニエール病に移行したと考えられた症例は6例あり全体の6%を占めた。

6）予後と各因子との相関

①　性差と予後

男性の予後は，25例中治癒14例，改善4例，不変3例，悪化0例，その他4例であり，女性の予後は，70例中治癒26例，改善20例，不変7例，悪化1例，その他16例であった。治癒・改善率は男性72%，女性65.7%で互いに有意差は認めなかった。

②　再発時年齢と予後

29歳以下(17例)，30歳代(31例)，40歳代(17例)，50歳以上（31例）と4群に分けて予後を比較した。初発例では，29歳以下の群と50歳以上の群で有意差があったとの報告[1]があるが，再発例では，その治癒・改善率に有意差は認められなかった（図4）。

③　再発後初診までの日数と予後

再発後3日以内に受診した例(38例)，4～7日に受診した例（17例），8日以後に受診した例（36例）で3群に分けて予後を比較した。

それぞれについて治癒・改善率を検討した。初発例と同様，再発後8日以後に受診した例は，他の群に比べ有意に予後不良であった($p<0.05$)(図5)。

④　低音3周波数聴力レベルの合計と予後

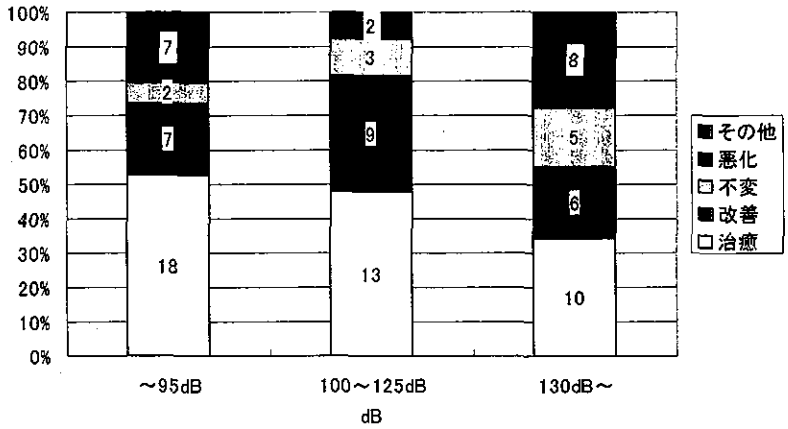

図6　低音3周波数聴力レベルと予後

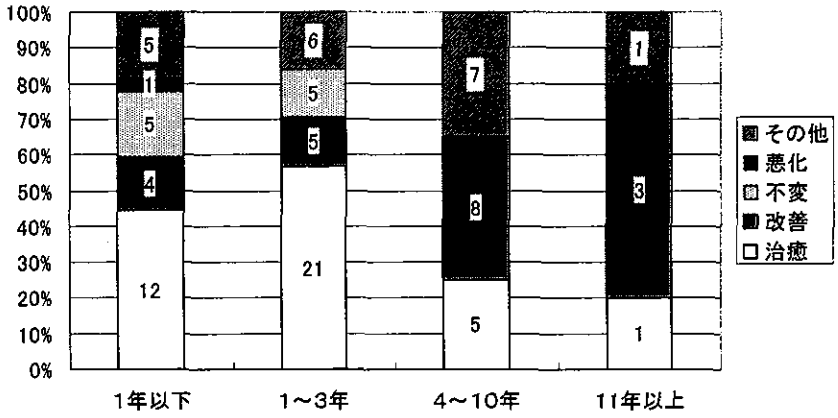

図7　再発期間と予後

再発時低音3周波数聴力レベルの合計が，95dB以下（34例），100～125dB（27例），130dB以上（29例）で3群に分けて予後を比較した。それぞれについて治癒・改善率を検討したが，有意差は認められなかった（図6）。

⑤　再発期間と予後

初発時から再発までの期間が，1年以下（27例），1～3年（37例），4～10年（20例），11年以上（5例）の4群で予後を検討したが，有意差は認められなかった（図7）。

考　　察

1）年齢，性差

平成12年度のみで比較すると，再発例は，初発例[1]に比べ，約1/4の症例数であった。再発時の発症年齢は，20歳～40歳に多く，また女性に多く認められた。この結果は，初発例[1]やこれまでの報告[4~7]と同様であり，基本的には同一または同様の疾患であることが示唆された。

2）自覚症状

主訴も，初発例[1]やこれまでの報告[4~15]と同様，耳閉塞感が最多を占めたが，随伴症状に難聴が多く含まれていた。これは，再発例ということで，以前初発時に難聴を指摘されており，その後の同様の症状で再受診しているという背景を予想すると，その症状として難聴を多く訴えるのは自然なことと思われた。

3）合併症

合併症・既往歴で低血圧は1例もなかったのが特徴的であった。岡本ら[11]によると急性低音障害型感音難聴では低血圧による循環障害が考えられると述べており，この差が，問診上の問題なのか，シェロンテストや血圧測定を施行していないことによるのかについては，この点を主眼に調査を継続してみる

必要があると考えられる。合併症・既往歴の質問項では不明や記載なしも多いので，全国調査を行うに際してはこの点を考慮する必要があると思われる。

4）治療

治療では複合治療が多く，個々の治療法を評価できないが，突発性難聴と比べてイソソルビドの使用例が多いのは特徴の一つといえる。治療的診断という方法があるように，治療から病態を推理すると，急性低音障害型感音難聴は，一部の病態として内リンパ水腫が存在する可能性が考えられ，突発性難聴とは異なる病態が含まれると推察された[9,11]。

5）予後

再発例全体の聴力予後は，治癒と改善をあわせると全体の68%と良好な結果になった。初発例での治癒・改善率は，78.1%との報告[1]があり，再発例の治癒・改善率を単純に比較してみて大きな開きは認められない。疫学上は，再発例についても予後良好な疾患であると考えられた。

6）予後と各因子との相関

予後と性別，再発時年齢，再発時聴力レベル，再発期間との間に相関は認められなかった。初発例[1]やこれまでの報告[4~15]では，29歳以下の群と50歳以上の群で有意差があるとの報告[6,7]があるが，今回の再発例の検討では，その治癒・改善率に有意差は認められなかった。

再発後初診までの日数と予後との相関では，再発後8日以後に受診した例は，他の群に比べ有意に予後不良であった。これは，初発例[1]や一部の報告[6~8]の結果と一致する。この点では，再発例でも突発性難聴に類似した特徴を有すると考えられた。

7）診断基準の考察

全体の聴力予後の中で，再発時も急性低音障害型の診断基準に合致するものは再発例の約2/3（64.5%）に過ぎなかった。そのため，再発時に聴力型が変化したものも本疾患と捉えるのか，再発時は別な疾患に移行したとするか，あるいはまた初回に戻って全体を別疾患とすべきか，などの問題があるが，現時点では解決されていない。

また，再発例の経過中メニエール病に移行したと考えられた症例が，6例あり全体の6%と無視できない割合を占めた。メニエール病とした症例は聴力変動のほかにめまい症状の出現やその反復が生じた症例である。今回の再発例の中で，さらに経過を追っていった場合にメニエール病に移行する症例の割合はさらに増加する可能性がある。しかし，現時点で臨床的には，両者の明確な区別は困難であった。このように初発例，再発例含め本疾患の一部にメニエール病が含まれていることは確実であり，特に再発例はその可能性が高くなると考えられる。今後メニエール病の診断基準も含め，再考する必要があると思われた。

固定時聴力についても，別の判定基準が必要と思われる。診断基準に合致しない症例では，突発性難聴の「8 kHzを除く5周波数で20dB以内を治癒とする」という診断基準の適用が一案として考えられるが，著明回復では平均30dB以上の回復となり，5周波数では該当例はほとんどなくなる。125Hzをどうするかの問題もある。一方で再発時聴力についても，診断基準に合致しない例の中には，診断基準に合致しないほど軽いものも含まれており，その取り扱いも検討課題と考えられる。この場合の予後判定基準も問題として残されている。

まとめ

1．2年間に96例101耳の症例を得た。全体の平均年齢は初回時40.6歳（89例），再発時42.3歳（96例）で，発作間隔は平均2.6年であった。

2．合併症・既往歴で低血圧は1例もなかった。

3．治療では複合治療が多いが，突発性難聴と比べてイソソルビドの使用例が多いのは特徴の一つといえる。

4．再発例全体の聴力予後は，治癒と改善をあわせると全体の68%と良好な結果になった。

5．再発例の経過中メニエール病に移行したと考えられた症例は，6例あり全体の6%と無視できない割合を占めた。

6．予後と性別，再発時年齢，再発時聴力レベル，再発期間との間に相関は認められなかったが，再発後初診までの日数が，再発後8日以後に受診した例は，7日以内に受診した群に比べ有意に予後不良であった。

本論文の要旨は第47回日本聴覚医学会学術講演会（平成14年10月3日，仙台市）で口演した。

Results of Nationwide Epidemiological Surveys in 2000-2001 on Recurrent Cases of Acute Low-Tone Sensorineural Hearing Loss

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New criteria have been proposed for acute low-tone sensorineural hearing loss (ALHL) by the Research Committee on Acute Profound Deafness of the Ministry of Health, Labor and Welfare of Japan, and nationwide epidemiological surveys based on these criteria were conducted in 2000-2001. We especially investigated recurrent cases of ALHL. The results in 96 cases, including 5 cases of bilateral hearing loss were analyzed for two years. All curative percentage of recurrent cases about full recovery and better recovery in regard to the criteria is 68 %. The outcome of hearing was better in patients who consulted a physician within a week after the onset of the recurrence. No significant differences were noted in other factors, i. e., sex, age, severity of hearing loss at the time of the examination for recurrence or recurrent time during the first attack. The specification of recurrent cases is that the percentage of cases cleared for the criteria at recurrent attack (cleared at first attack) are about 60 % in recurrent cases.

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Transient Cochlear Ischemia Causes Delayed Cell Death in the Organ of Corti: An Experimental Study in Gerbils

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ABSTRACT

To elucidate whether ischemia–reperfusion can cause delayed cell death in the cochlea, the effects of transient cochlear ischemia on hearing and on neuronal structures in the cochlea were studied in Mongolian gerbils. Ischemia was induced by bilaterally occluding the vertebral arteries for 5 minutes in gerbils, which lack posterior cerebral communicating arteries. In gerbils, the labyrinthine arteries are fed solely by the vertebral arteries. Occlusion of the vertebral arteries caused a remarkable increase in the threshold of compound action potentials (CAPs), which recovered over the following day. However, 7 days after the onset of reperfusion, the threshold began to increase again. Morphologic changes in the hair cell stereocilia were revealed by electron microscopy. The number of nuclear collapses was counted in cells stained for DNA and F-actin to evaluate the degree of cell death in the organ of Corti. Changes in spiral ganglion cell (SGC) neuron number were detected, whether or not progressive neuronal death occurred in the SGC. These studies showed that sporadic fusion of hair cells and the disappearance of hair cell stereocilia did not begin until 4 days after ischemia. On subsequent days, the loss of hair cells, especially inner hair cells (IHCs), and the degeneration of SGC neurons became apparent. Ten days after ischemia, the mean percentage cell loss of IHCs was 6.4% in the basal turn, 6.4% in the second turn, and 0.8% in the apical turn, respectively, and the number of SGC neurons had decreased to 89% of preischemic status. These results indicate that transient ischemia causes delayed hearing loss and cell death in the cochlea by day 7 after ischemia. J. Comp. Neurol. 456: 105–111, 2003.

Indexing terms: compound action potentials; inner hair cells; delayed hearing loss; rhodamine-phalloidin; Hoechst 33342

Ischemic injury to the cochlea is considered one of the major causes of acute sensorineural hearing loss (Schuknecht and Donovan, 1986; Shikowitz, 1991). Interruption of the blood supply to the cochlea is known to cause immediate loss of function and to produce characteristic damage to the inner ear (Billett et al., 1989; Kimura and Perlman, 1958; Perlman et al., 1959). It has been reported that anoxia of the cochlea causes an increase in the compound action potential (CAP) threshold (Konishi et al., 1961) and that the CAP threshold recovers completely when the duration of anoxia is shorter than 10 minutes. (Kusakari et al., 1981). Permanent cochlear ischemia in the lateral wall was induced by a photochemical reaction (Iwasaki et al., 1997), and a histologic study revealed thrombus formation in the stria vascularis, spiral ligament, and modiolus (Saito et al., 2001). Further-

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more, cochlear vessel obstruction was induced by a ferromagnetic thrombosis method (Gieble et al., 1985). By using this method, Schweinfurth and Cacace (2000) showed a correlation between the absence of distortion-product otoacoustic emissions and ischemic cochlear injury. Recently, the ability of chemical asphyxiants (cyanide and carbon monoxide) to disrupt the CAP and endocochlear potential was also investigated (Tawackoli et al., 2001). However, the details of the effects of transient cochlear ischemia on the cochlea are unclear, because of the difficulty in temporarily interrupting the blood supply to the cochlea, and animal experimentation seems unfeasible. Nevertheless, by using a technique called experimental hindbrain ischemia (Hata et al., 1993), we successfully made a chronic animal model of transient cochlear ischemia in Mongolian gerbils. Mongolian gerbils lack the posterior cerebral communicating arteries; the labyrinthine arteries are fed solely by the vertebral arteries.

This study was designed to determine whether the hearing of ischemic animals changed over a follow-up period of 10 days and to determine what histologic changes occurred in the organ of Corti and the spiral ganglion cells (SGCs). We report here the first evidence that delayed cell death occurs in the cochlea after transient ischemia.

MATERIALS AND METHODS

Ischemia of the cochlea

The following experiments were conducted in accordance with the Guide for Animal Experimentation at Ehime University School of Medicine. Transient cochlear ischemia was induced by the technique called experimental hindbrain ischemia (Hata et al., 1993) in Mongolian gerbils. Mongolian gerbils, each weighing 60–80 g, were anesthetized with a mixture of nitrous oxide/oxygen (7:3) gas and 3% halothane. The vertebral arteries were exposed bilaterally, just before their entry into the transverse foramina of the cervical vertebra, through a ventral midline incision of the neck. They were dissected free from surrounding connective tissue, and 4-0 silk sutures were loosely looped around each artery. The animals were administered artificial respiration through a ventilation tube inserted into the mouth. Cochlear ischemia was induced bilaterally by pulling the sutures with the force of a 5-g weight. After a 5-minute ischemic load, the sutures were removed to allow recirculation. Recirculation was confirmed by direct observation with an operating microscope. The animals were kept in incubators until they had completely recovered from anesthesia.

Measurement of CAPs

Sixteen animals were used in this study. These animals were divided into two groups (n = 8, ischemia group; n = 8, sham operation group). After exposure of the otic bulla, a platinum electrode coated with epoxy resin was placed into the stylomastoid foramen. The electrode was then fixed to the bony bulla with dental cement, and a wire was fed under the scalp to the outside of the skin at the vertex. This was the reference electrode. A second electrode made of a stainless-steel needle was placed in the ipsilateral mastoid muscle to measure CAPs. Cochlear function before and after occlusion of the vertebral arteries was evaluated by using a signal processor (NEC Synax 1200, Tokyo, Japan) to record CAPs in response to 8,000 Hz, 4,000 Hz, and 500 Hz tone bursts of 1.25 msec rise/fall time and 10-msec duration. The responses were processed through a 50 to 31 kHz band-pass filter and averaged 300 times. Sound pressure in front of the tympanic membrane was monitored by using a small microphone incorporated in the conduction tube of a sound stimulator. The threshold of the CAPs was obtained by applying acoustic stimuli in 10-dB steps. Near the threshold, the acoustic stimuli were applied in 5-dB steps. For the sham operation experiments, the same procedure was performed without bilateral occlusion of the vertebral arteries.

Scanning electron microscopic study

Twelve animals were used in this study. Animals were subjected 1, 4, 7, or 10 days after the 5-minute ischemia (n = 3 for each group). After intraperitoneal administration of an excessive dose of pentobarbital sodium (50 mg/kg), the otic bullae were removed, and an intrascalar perfusion of the cochlea with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) was performed. The cochleas were immersed overnight at 4°C in the same fixative. After removal of the bony capsule, they were post-fixed with 2% osmium tetroxide. The specimens were then sequentially dehydrated in ethanol, mounted on a metal base, and coated with gold. The cochleas were observed with a scanning electron microscope (Hitachi S-800, Tokyo, Japan).

Counting hair cell loss

Filamentous actin (F-actin), which is the primary structural filament of hair cells, can be stained by using rhodamine-phalloidin, a fluorescent marker for filamentous actin, and morphologic changes in the nuclei of hair cells can be evaluated by DNA staining using Hoechst 33342. In this study, the two staining procedures were combined to simultaneously observe the stereocilia and the nuclei of hair cells, so that the loss of hair cells in the cochlea could be precisely detected.

Thirty-two animals were used in this study. Animals were subjected to either ischemia (killed 1, 4, 7, or 10 days after 5-minute ischemia, n = 4 for each group) or a sham operation (killed 1, 4, 7, or 10 days after sham operation, n = 4 for each group). Immediately after the removal of the bilateral otic bullae after deep pentobarbital anesthesia (50 mg/kg), intrascalar perfusion of the cochlea with 4% paraformaldehyde (in 0.1 M phosphate buffer, pH 7.4) was performed. The cochleas were post-fixed for 2 hours with the same fixative at 4°C. The cochleas were immersed in phosphate buffered saline (PBS), pH 7.4, and all parts of the organ of Corti were dissected through all of the turns. The specimens were then stained for 30 minutes at room temperature with rhodamine-phalloidin (Molecular Probes, Eugene, OR), which was diluted 1:250 in PBS containing 0.25% Triton-X-100 and 1% bovine serum albumin. After rinsing in PBS, the specimens were stained with Hoechst 33342 in PBS (20 μg/ml; Calbiochem-Novabiochem Corporation, La Jolla, CA) in the dark for 1 hour. The specimens were rinsed again in PBS and mounted in carbonate-buffered glycerol (1 part 0.5 M carbonate buffer, pH 9.5, 9 parts glycerol) containing 2.5% 1,4-diazabicyclo-(2,2,2,)-octane to retard bleaching of the fluorescent signal. Fluorescence was detected by using a Zeiss Axiophot FL microscope with a green filter

(BP 546, FT 580, LP 590) and a UV filter (BP 365, FT 395, LP 397). Because the structure of hair cells is three-dimensional, we adjusted the focus to observe the stereocilia and nuclei of inner hair cells (IHCs; 300 cells in the basal turn, 200 cells in the second turn, and 100 cells in the apical turn) and outer hair cells (OHCs; 900 cells in the basal turn, 600 cells in the second turn, and 300 cells in the apical turn) in each cochlea. We counted the numbers of hair cells lost and intact, and the percentage of hair cell loss was calculated.

Population of spiral ganglion neurons

Twenty animals were used in this study. Animals were subjected preischemia or 1, 4, 7, or 10 days after 5-minute ischemia (n = 4 for each time point). After intraperitoneal administration of an excessive dose of pentobarbital sodium (50 mg/kg), transcardial perfusion with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) was performed. After removing the cochleas bilaterally, they were fixed overnight in the same fixative at 4°C and decalcified in 10% ethylenediaminetetraacetic acid for 2 weeks. The specimens were embedded in paraffin, serially sectioned in 5-μm slices, and stained with hematoxylin and eosin. Every tenth section was examined by light microscopy (Olympus BX60, Tokyo, Japan), and the neurons in Rosenthal's canal with a distinct nucleus more than 1 μm in size were counted. The raw neuronal cell density count for each slide was corrected for double counting by using the formula of Abercrombie (1946): $Hi = hi \times t/(t + d)$, where, Hi is the corrected density of spiral ganglion neuronal cells, hi is the counted density of spiral ganglion neuronal cells, t is the section thickness (5 μm), and d is the diameter of the nucleus. The mean nucleus diameter determined by measuring 50 spiral ganglion neurons was 2.25 μm (±0.25). The total Hi was multiplied by 10 to give the total population of spiral ganglion neurons.

Statistical evaluation

Individual differences between experimental groups for the average increase in CAPs, the percentage of hair cell death, and the population of spiral ganglion neurons were evaluated by one-way or two-way analyses of variance followed by Scheffe's multiple comparison test.

Photomicrograph production

Photographic film was scanned by a film scanner (Coolscan III, Nikon, Tokyo, Japan) into a personal computer (Power Mac G4, Apple, Cupertino, CA). The contrast and brightness of a photograph was adjusted by using Adobe Photoshop 5.02 software (Adobe Systems Incorporated, San Jose, CA) and then printed (PICTROGRAPHY 3000, Fujifilm, Tokyo, Japan).

RESULTS

Hearing sequences after ischemic insult

Sequential changes in the CAP thresholds are shown in Figure 1. In the sham operation group, there were no significant changes in the CAP threshold at any frequency. Bilateral occlusion of the vertebral arteries caused a remarkable increase in the CAP threshold by more than 65 dB, which was the maximal output intensity of our measuring system. The responses recovered after reperfusion. Hearing recovered completely during the following day at all frequencies. However, after the 7th day, the threshold for 8,000 Hz and 4,000 Hz increased again. The average increase in CAP threshold 10 days after ischemia was 11.3 dB and 10.6 dB for 8,000 Hz and 4,000 Hz, respectively.

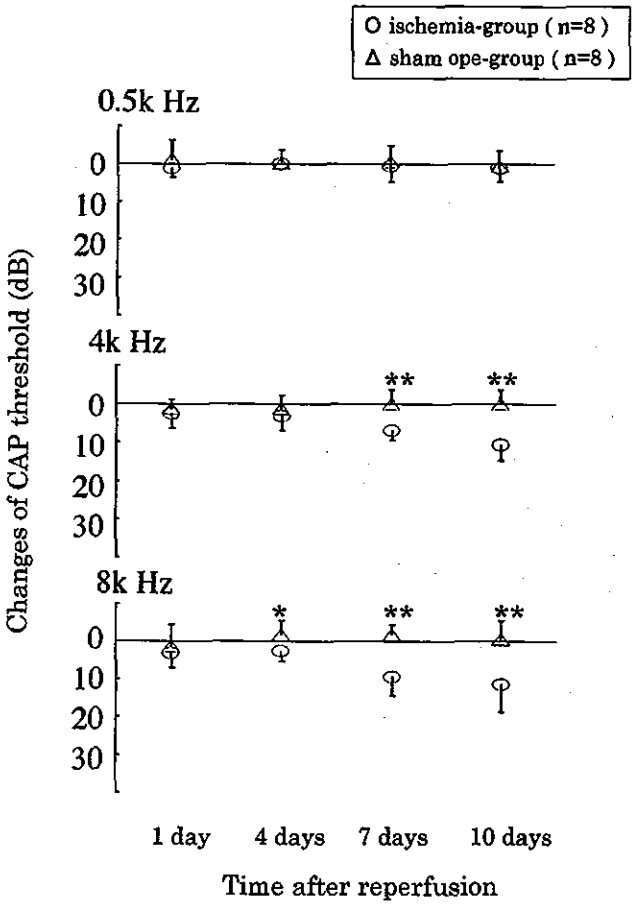

Fig. 1. Sequential changes in the threshold of compound action potentials (CAPs) in response to 8,000 Hz, 4,000 Hz, and 500 Hz after 5-minute ischemia. Each point shows the mean value of the threshold after the occlusion of bilateral vertebral arteries 1, 4, 7, and 10 days after reperfusion. The CAP threshold before occlusion was defined as 0 dB. Error bars depict standard deviation. After the seventh day, the CAP threshold for 8,000 Hz and 4,000 Hz increased. Individual differences between ischemia group and sham operation group for the average increase in CAPs were evaluated by two-way analyses of variance followed by Scheffe's multiple comparison test. $*P < 0.05$, $**P < 0.01$.

Scanning electron microscopic study

The IHCs, OHCs, and supporting cells remained intact until 4 days after ischemia (Fig. 2A). Seven days after ischemia, hair cell stereocilia were found to have sporadically fused or disappeared in IHCs more frequently than in OHCs (Fig. 2B). Ten days after ischemia, such findings were obvious in all specimens (Fig. 2C).

Quantifying hair cell loss

The stereocilia and nuclei of hair cells generally remained intact until 4 days after ischemia (Fig. 3A,B). However, by 7 days after ischemia, they had sporadically

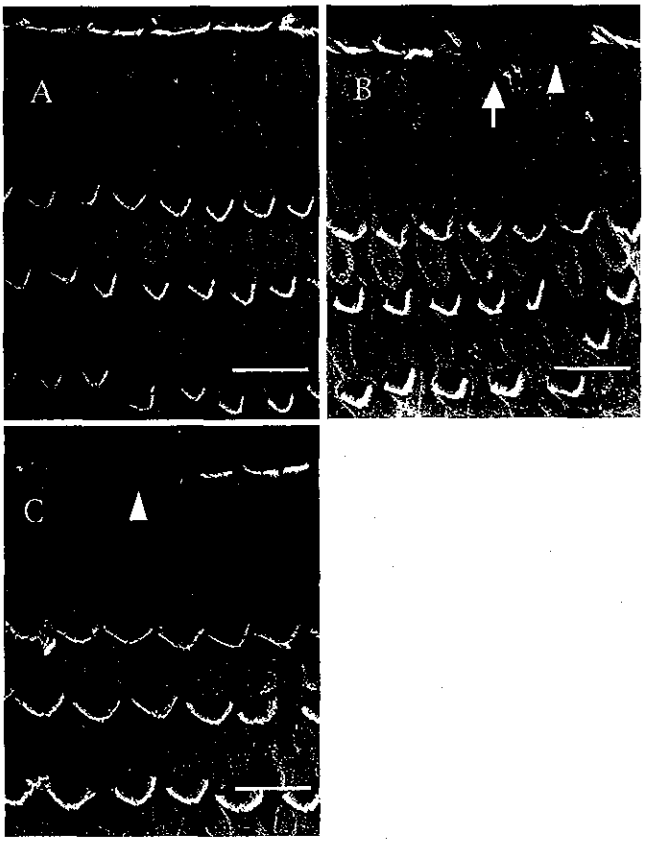

Fig. 2. Scanning electron photomicrographs showing an organ of Corti 4 days (**A**), 7 days (**B**), and 10 days (**C**) after 5-minute ischemia. No degenerative changes are seen in inner hair cells (IHCs), outer hair cells (OHCs), and supporting cells 4 days after ischemic insult. The stereocilia of IHCs had sporadically fused (arrow) or disappeared (arrowheads) 7 and 10 days after the ischemic insult, while OHCs remained almost intact. Scale bars = 10 μm in A–C.

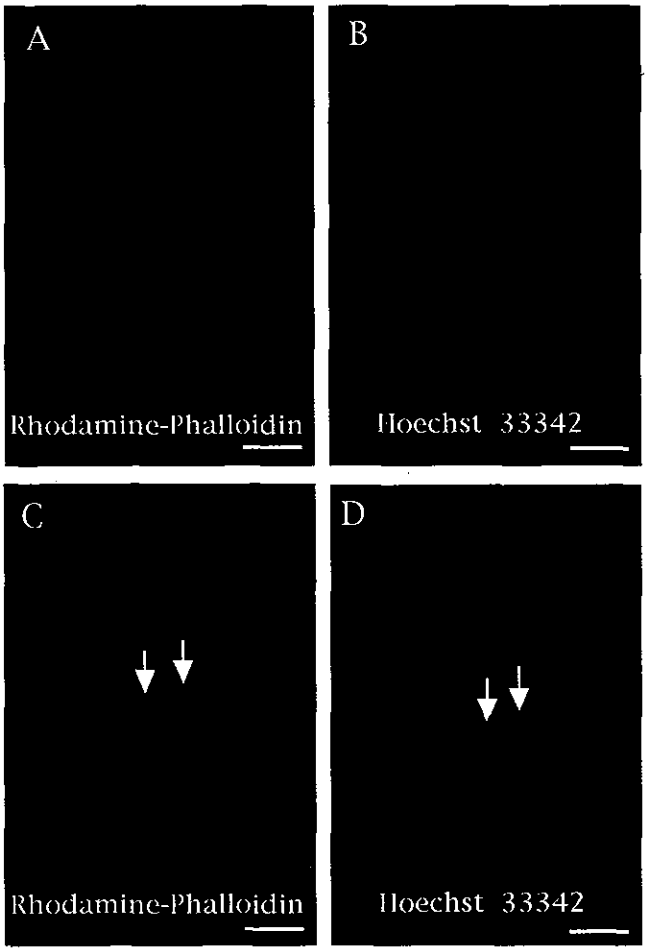

Fig. 3. The prepared surface of organ of Corti stained with rhodamine-phalloidin and with Hoechst 33342. Photomicrographs of the inner hair cells (IHCs) 4 days (**A,B**) and 7 days (**C,D**) after 5-minute ischemia. The stereocilia and nuclei were almost intact 4 days after ischemia. However, the loss of IHC, in which both stereocilia and nuclei had disappeared (arrows), was observed 7 days after ischemic insult. Scale bars =10 μm in A–D.

disappeared (Fig. 3C,D). The percentages of hair cell loss are summarized in Figure 4. Ten days after ischemia, the mean percentages of cell loss in the IHCs and OHCs, respectively, were 0.8 ± 2.0% and 0.1 ± 0.2% in the apical turn, 6.4 ± 2.6% and 0.5 ± 0.6% in the second turn, and 6.4 ± 2.5% and 0.2 ± 0.2% in the basal turn. These results show that IHCs were more vulnerable than OHCs. Furthermore, hair cells in the basal and second turn were more vulnerable than were those in the apical turn.

Population of spiral ganglion neurons

The populations of spiral ganglion neurons before and after ischemia are summarized in Figure 5. The average number of spiral ganglion neurons before ischemia was 15,762 ± 729. On days 1 and 4 after ischemia, the numbers were 16,221 ± 453 and 15,874 ± 1,199, respectively. These numbers are in the same range as those obtained for the preischemia group. However, by 7 days after ischemia, the number had decreased. The numbers at 7 and 10 days after ischemia were 15,103 ± 845 and 14,010 ± 957, respectively. This indicates that approximately 11% of the spiral ganglion neurons had been lost by 10 days after transient ischemia.

DISCUSSION

Morphologic changes of IHCs

The degeneration and sporadic fusion of stereocilia were obviously observed by scanning electron microscopy more frequently in IHCs than in OHCs 4 days after transient ischemia. In our previous study, we demonstrated that the ischemia induced obvious swelling of afferent dendrites of the auditory nerve in contact with IHCs 5 minutes after reperfusion. In contrast, the efferent nerves ending in synaptic contact with OHCs appeared to be intact 5 minutes after reperfusion (Hakuba, 1998). The fluorescence microscopic study further demonstrated that the stereocilia and nuclei had begun to disappear 7 days after ischemia in IHCs, whereas OHCs remained almost intact. These results suggest that delayed ischemic damage affects IHCs much more than OHCs.

Transient ischemia of the forebrain causes delayed neuronal death, especially in the CA1 field of the hippocampus. This area of the brain is known to have glutamate

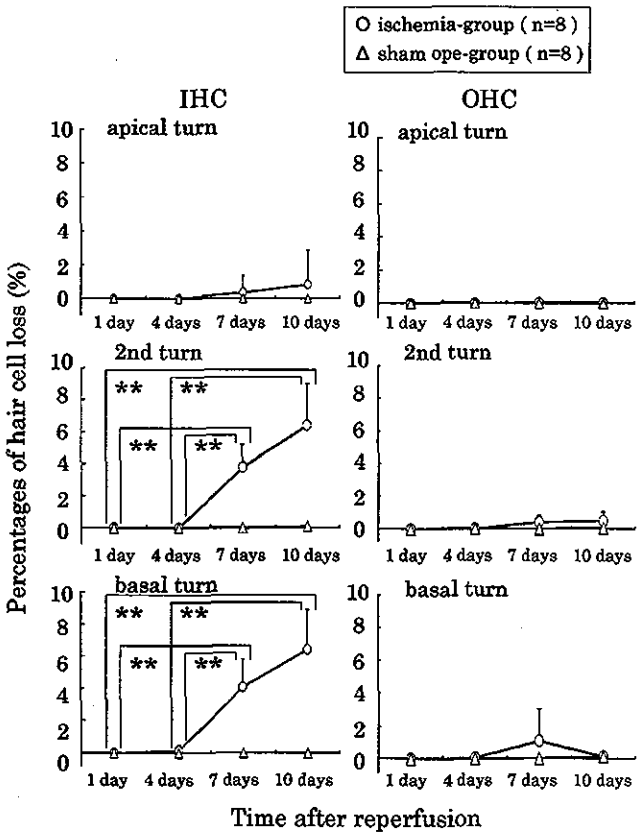

Fig. 4. The percentages of cell loss of inner hair cells (IHCs) and outer hair cells (OHCs) in each cochlear turn. Each point shows the mean value of the percentages of cell death 1, 4, 7, and 10 days after 5-minute ischemia. Error bars depict standard deviation. Circles, IHCs; triangles, OHCs. The percentage of cell death of IHCs increased significantly 7 days after the ischemic insult in the second and basal turns. Individual differences between each time point for the average increase in the percentage of hair cell death were evaluated by one-way analyses of variance followed by Scheffe's multiple comparison test. $^{**}P < 0.01$.

receptors (Kirino, 1982). The exact mechanism of this phenomenon is still controversial, but is proposed to be mainly caused by excessive release of glutamate, which normally works as an excitatory neurotransmitter (Benveniste et al., 1984). Matsubara et al. (1996) observed that glutamate receptors are present on synapses between IHCs and afferent dendrites, but not on those of OHCs. In the ear, glutamate is known to be a major neurotransmitter in auditory hair cells (Eybalin, 1993). In a previous study using the same ischemic model in gerbils, we demonstrated that glutamate levels in the perilymph increased dramatically after ischemic insult (Hakuba et al., 2000) and that hypothermia prevented the release of glutamate into the perilymph (Hyodo et al., 2001), as well as progressive hair cell loss in the cochlea after transient ischemia (Watanabe et al., 2000). These findings suggest that delayed cell death in the IHCs after ischemia is in part caused by an increased glutamate concentration in the perilymph. This process is similar to what occurs in the hippocampus after brain ischemia.

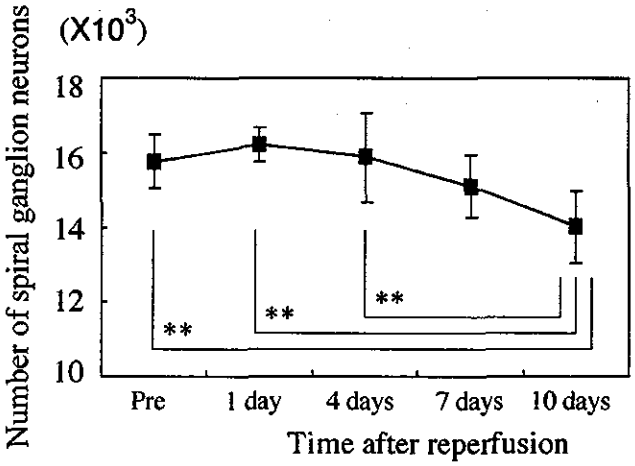

Fig. 5. The number of spiral ganglion neurons, after transient ischemia. Each point shows the mean value of the number of spiral ganglion neurons preischemia and 1, 4, 7, and 10 days after ischemia. Error bars depict standard deviation. The number of neurons did not change significantly for 4 days after ischemia. However, 10 days after ischemia, there was an evident neuronal loss. Individual differences between each time point for the average increase in the population of spiral ganglion neurons were evaluated by one-way analyses of variance followed by Scheffe's multiple comparison test. $^{**}P < 0.01$.

Ischemic damage to SGC neurons

By 10 days after ischemia, the number of SGC neurons had decreased to 11% of the number in the preischemic condition. The percentage IHC loss and SGC decrease after the injection of carboplatin into chinchillas has been studied (Takeno et al., 1998). Of interest, the rate of IHC impairment was very high (75.5%), but the total SGC decrease was only 11.8% in the previous study. The reason for this difference may be that the loss of SGC neurons after carboplatin injection is caused by a loss of IHCs, rather than by a direct effect of drug toxicity on ganglion cells (Bichler et al., 1983; Leake and Hradek, 1988; Dupont et al., 1993). In contrast, the degree of SGC degeneration that we observed was approximately twice that of the total IHC impairment rate (4.5%). In this study, ischemic insult was induced by the total hindbrain ischemia method (Hata et al., 1993), which might cause immediate damage to SGC neurons. As far as we know, the SGC neuronal degeneration caused by cochlear ischemia has not been studied to date. It can be assumed that the damage was caused by the loss of IHCs to some extent, but it may also have been partly caused by direct impairment of blood supply and energy. Therefore, it is suggested that cochlear damage after transient ischemia induces IHC loss and SGC neuronal degeneration. Both were apparently involved in increasing the CAP threshold in our study.

Sekiya et al. (2001) induced loss of SGC by mechanical injury, i.e., direct compression of the cochlear nerve, and showed almost 70% degeneration of SGC neurons 2 weeks after compression, compared with a noncompression group. We suggest that compression injury to the cochlear nerve has a much greater effect on SGC neuronal degeneration than does damage to the cochlear blood supply.

Correlation between physiological dysfunction and morphologic changes

In the morphologic examination, hair cells and SGC neurons were almost unchanged 4 days after ischemia. However, the loss of hair cells and degeneration of SGC neurons appeared later, at a time corresponding to the CAP findings. The auditory brainstem response (ABR) threshold shift in response to IHC loss and SGC degeneration for different acoustic stimuli were previously studied in chinchillas after injection of carboplatin (Takeno et al., 1998). Our results are similar to those for chinchillas. Our highest CAP threshold (10 days after ischemia) was in the 8 kHz region, with an 11.3 dB threshold shift. Furthermore, we observed a 6.4% loss of IHCs and an 11% decrease in SGCs. This damage is very mild compared with carboplatin-induced IHC impairment, which resulted in a 49-dB increase in ABR threshold with a 67% loss of IHCs in response to 8 kHz and an 11.8% decrease in SGCs (Takeno et al., 1998). The difference in the ratio of IHC loss and SGC degeneration in the two models makes it very difficult to compare changes in physiological dysfunction and morphology. However, according to our results, the degeneration of SGCs seems to be more responsible for the rise in the hearing threshold than does the loss of IHCs. By comparing the models in more detail, we believe that it will become clearer to what degree the damage to IHCs and/or SGC neurons is responsible for the functional hearing disorder.

Vulnerability to ischemic damage at corresponding frequency locations

This study also demonstrated that the basal and second turns are more vulnerable to transient ischemia than the apical turn and showed that an increase in the CAP threshold occurred at 4,000 and 8,000 Hz but not at 500 Hz. The threshold shift was more pronounced in the high-frequency regions. Mizukoshi and Daly (1967) demonstrated that the rate of oxygen consumption in the basal turn is approximately 2.5 times that of the apical turn. It may be that the basal turn is more vulnerable to anoxia than the apical turn, because the aerobic metabolism of the hair cells is more active in the basal turn than in the apical turn. Fechter et al. (1987) used electrophysiological techniques to show that CAP thresholds at high frequencies are more susceptible to hypoxia than those at low frequencies, reflecting the basal turn's greater vulnerability to hypoxia. Their findings support the results of this study.

In conclusion, we have demonstrated that delayed cell death occurs in hair cells and SGC neurons after transient cochlear ischemia. It remains unknown what damage transient ischemia causes to human Corti. However, these results indicate that transient ischemia may be one of the causes of progressive hearing loss that is resistant to conventional treatments. Glutamate neurotoxicity might be one of the major causes. In addition, Seidman and Quirk (1991) found that an inhibitor of lipid peroxidation was otoprotective against ischemic/reperfusion injury in the inner ear. Oxygen-derived free radicals (Yamane et al., 1995) and nitric oxide (NO; Tabuchi et al., 1999; Takumida and Anniko, 2002) are reported to play an important role in cochlear anoxia/ischemia re-perfusion injury. In fact, increased production of hydroxyl radicals (Ohlemillaer and Dugan, 1999) and NO (Ruan et al., 2001) were measured in the cochlear perilymphatic spaces after ischemic injury. Huang et al. (2000) suggested that ischemia/hypoxia is linked to oxidative stress and induces apoptosis in both auditory hair cells and the neurons of the spiral ganglions. Further study is needed to clarify the cause of ischemia-induced hearing loss and to learn how to prevent or relieve damage to the cells involved.

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ORIGINAL ARTICLE

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Clinical features of patients with *GJB2* (connexin 26) mutations: severity of hearing loss is correlated with genotypes and protein expression patterns

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Abstract Mutations in the *GJB2* (connexin 26, Cx26) gene are the major cause of nonsyndromic hearing impairment in many populations. Genetic testing offers opportunities to determine the cause of deafness and predict the course of hearing, enabling the prognostication of language development. In the current study, we compared severity of hearing impairment in 60 patients associated with biallelic *GJB2* mutations and assessed the correlation of genotypes and phenotypes. Within a spectrum of *GJB2* mutations found in the Japanese population, the phenotype of the most prevalent mutation, 235delC, was found to show more severe hearing impairment than that of V37I, which is the second most frequent mutation. The results of the present study, taken together with phenotypes caused by other types of mutations, support the general rule that phenotypes caused by the truncating *GJB2* mutations are more severe than those caused by missense mutations. The present in vitro study further confirmed that differences in phenotypes could be explained by the protein expression pattern.

Keywords Connexin 26 · *GJB2* · 235delC · V37I · Deafness · Phenotype · Genotype

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Introduction

Mutations of the *GJB2* (connexin 26, Cx26) gene have recently drawn much attention because they have been recognized as the most prevalent genetic cause of congenital hearing loss. A broad range of phenotypes, from mild to profound hearing loss, is associated with *GJB2* mutations (Cryns et al. 2004), and more than 90 different *GJB2* mutations are associated with recessive forms of nonsyndromic hearing loss (The Connexins-deafness Homepage: http://www.crg.es/deafness). Universal neonatal hearing screening programs are the current trend and have become popular in many countries (Govaerts et al. 2001; Joint Committee on Infant Hearing 2000; Mehl and Thomson 2002; National Institutes of Health 1993), because it is thought that optimum language development requires early identification of hearing loss and early intervention (Yoshinaga-Itano et al. 1998). Cochlear implantation has resulted in remarkable

improvement in auditory skills and development of speech production for patients with profound hearing loss associated with *GJB2* mutations (Fukushima et al. 2002; Matsushiro et al. 2002). It is clear that genetic testing to determine the cause of deafness facilitates prediction of the course of hearing loss and prognostication of language development. There is, however, some controversy regarding genotype/phenotype correlation (Cohn et al. 1999; Cryns et al. 2004; Denoyelle et al. 1999; Estivill et al. 1998; Murgia et al. 1999; Orzan et al. 1999). For example, prediction of the degree of hearing loss was difficult, and environmental factors as well as modifier genes may have been involved (Cohn et al. 1999; Murgia et al. 1999; Orzan et al. 1999). On the other hand, a series of reports have indicated that certain phenotypes are dependent on certain genotypes (Denoyelle et al. 1999; Estivill et al. 1998). A recent report of a multi-center-based study in Europe and the United States suggested that inactivating mutations, which include stop or frameshift mutations, show significantly severer phenotypes than those caused by noninactivating mutations (missense mutations) (Cryns et al. 2004).

We have recently shown that mutation spectrums are quite different between the Japanese population and populations with European ancestry and emphasized the importance of specific population-based genetic databases for genetic testing (Ohtsuka et al. 2003). In Japanese (who are one example of Asian populations), the most common mutation was an inactivating mutation, 235delC, which is comparative to the 35delG mutation known as the most prevalent mutation in those with European ancestry. Interestingly, the second most common mutation was the V37I mutation, which has recently been reported as a mild phenotype causative genotype (Cryns et al. 2004). Given this background, we attempted to: (1) compare the differences in phenotypes caused by the 235delC and V37I mutations, (2) test a hypothetical general rule that inactivating mutations show more severe phenotypes than those caused by noninactivating mutations, and (3) test whether the differences in phenotype could be explained by protein expression study.

Materials and methods

Subjects and clinical evaluation

Pure-tone audiometry results were available for 60 individuals from independent families in whom biallelic *GJB2* mutations were identified. These patients were from seven university hospitals (Hirosaki, Iwate, Gunma, Shinshu, Kokusai Iryoufukushi, Hamamatsu, and Kyushu) located in different regions in Japan. The age when the patients/parents noticed hearing impairment was from 0 to 49 (mean 8.00, SD 12.51) years of age. None of these patients had any other associated neurological signs. All subjects gave prior informed consent for participation in the project, which was approved by the ethical committee of each hospital.

Severity was classified by using a pure-tone average over 500, 1,000, 2,000, and 4,000 Hz in the better-hearing ear. Hearing impairment was classified as follows: normal hearing, < 20 dB; mild hearing loss, 21–40 dB; moderate hearing loss, 41–70 dB; severe hearing loss, 71–95 dB; and profound hearing loss, greater than 95 dB. When the threshold exceeded the output limits of the audiometer, it was recorded as the output limit for air-conducted sounds plus 10 dBHL; i.e., if the output limit of the audiometer was120 dBHL, the threshold was described as 130 dBHL.

Mutation Analysis

To identify *GJB2* mutations, a DNA fragment containing the entire coding region was amplified using the primer pair Cx48U/Cx1040L, as described elsewhere (Abe et al. 2000). Polymerase chain reaction (PCR) products were sequenced and analyzed with an ABI sequencer 377XL (Perkin-Elmer, Wellesley, MA, USA). DNA samples from 147 unrelated Japanese who had normal hearing were used as controls.

Reverse transcription (RT)-PCR analysis

Total RNA was extracted from NCTC2544 cells with the Catrimox-14 RNA Isolation Kit Ver.2.11 (Iowa Biotechnology, Urbandale, IA, USA). The yield of total RNA was determined by Agilent 2100 Bioanalyzer RNA 6000 Nano Assay (Agilent Technologies, Palo Alto, CA, USA). Reverse transcription (RT)-PCR assay was performed with the aid of an RNA PCR kit (Takara, Tokyo, Japan). The primers for human *GJB2* and the specific sites of restriction enzymes were added with the amplification step. The primers were sense *Xho* I-Cx26 5′-cccctcgaggatggattggggcacgctgcagacgatcctggg-3′ and antisense Cx26-*EcoR* I 5′-cccgaattcgttaaactggctttttgacttcccagaac-3′. These primers yield oligomer products of a distinctive size: 712 bp. PCR steps were denaturing at 94°C for 2 min, followed by 30 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min, and then processing with a final extension at 72°C for 5 min. After amplification, expected sizes of PCR products were confirmed on 2% agarose gel, and the bands were visualized by ethidium bromide upon exposure to an ultraviolet transilluminator.

Transformation

Wild-type Cx26 PCR products were inserted into a pEGFP-C2 vector (Clontech, Palo Alto, CA, USA). The PCR products and vector were digested with *EcoR* I and *Xho* I. Prepared PCR products were inserted into vector. Ligation reactants were transformed into Escherichia coli DH5α. Positive colonies were incubated in Luria-Bertani (LB) liquid medium containing kanamycin. A QIAprep spin miniprep kit (Qiagen, Valencia, CA,

USA) was used for purification of plasmid DNA according to the manufacturer's protocol. Plasmid DNA was identified by restriction enzyme analysis. Selected constructs were sequenced and analyzed with an ABI sequencer 377XL (Perkin-Elmer).

Mutagenesis of the *GJB2* gene

The following primers were used to produce the *GJB2* mutations: V27I sense 5′-ctggctcaccatcctcttcatt-3′, V37I sense 5′-tatgatcctcattgtggctgcaa-3′, and 235delC sense 5′-ccggctatgggcctgcagctgatct-3′. First, PCR reactions (100 µl) were prepared containing 4 µg of the plasmid DNA (see above), 1.0 µM of mutation primer, 1.0 µM of Cx26-*EcoR* I primer, 2.5 U of Takara Ex taq Hot Start Version (Takara, Tokyo, Japan), and Ex-taq buffer (10x) consisting of 100 mM Tris-HCl (pH 8.3), 500 mM KCl, 15 mM MgCl2, and 1 mM deoxynucleoside triphosphate mixture. These PCR reactions were denatured at 94°C for 2 min, followed by 30 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min, and then processed with a final extension at 72°C for 5 min. Second PCR reactions (100 µl) were prepared containing 10 µl of first PCR products, 1.0 µM of *Xho* I-Cx26 primer, 2.5 U of Takara Ex taq Hot Start Version (Takara), and Ex-taq buffer (10x) consisting of 100 mM Tris-HCl (pH 8.3), 500 mM KCl, 15 mM MgCl2, and 1 mM deoxynucleoside triphosphate mixture. Second PCR conditions were the same as above. These PCR products were inserted into a pEGFP-C2 vector with the same techniques as transformation (see above). The plasmid DNA containing Cx26 mutations were sequenced and analyzed with a sequencer and identified by restriction enzyme analysis.

Transfection and visualization

COS-7 cells grown on glass cover slips were transfected with the cloned plasmid vectors using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Forty-eight hours after the transfection, cells were fixed by 4% formaldehyde and stained by DAPI and TRITC-conjugated phalloidin (Chemicon, Temecula, CA, USA). Cover slips were mounted onto glass slides and visualized under a Leica confocal microscope TCS SP2 AOBS (Leica Microsystems, Wetzlar, Germany).

Results

Mutation spectrums

Among thirteen mutations that have been reported in Japanese (Ohtsuka et al. 2003), 11 were identified in our 60 biallelic patients. These biallelic mutations were found to be either four different homozygous or 14 different compound heterozygous mutations. These included five inactivating mutations and six missense mutations. The five inactivating mutations were one stop mutation (Y136X), three deletion frameshift mutations (235delC, 176-191del16, 299-300delAT), and one insertion frameshift mutation (605ins46). The six missense mutations were V37I (109G → A), G45E (134G → A), T86R (257C → G), T123N (368C → A), R143W (427C → T), and F191L (570T → C). T123N and F191L were categorized as changes with unknown relation to disease (The Connexin-deafness Homepage: *http://www.crg.es/deafness*); however, we included both mutations as missense mutations in the present report because both were found among the hearing-loss patients in either a homozygous or compound heterozygous state. The nonsense mutation, Y136X (408C → A), converts a tyrosine residue (TAC) at codon 136 to a stop codon (TAA). Three deletion frameshift mutations, 235delC, 176-191del16, and 299-300delAT, and one insertion frameshift mutation, 605ins46, were found. The 235delC mutation causes a frameshift at codon 79 resulting in a truncated polypeptide and was found in two of the 147 controls (294 alleles). The 176-191del16 mutation, present in four subjects, causes a frameshift leading to an altered amino-acid sequence from codon 59 followed by a stop at codon 76. The 299-300delAT deletion, seen in two subjects, causes a frameshift leading to an altered amino-acid sequence from codon 100 followed by a stop at codon 113. The 605ins46 mutation has a tandem repeat of 46 nucleotides (corresponding to the positions 559–604 of the Cx26 DNA sequence) at the position 605. A stop codon (TGA) is produced at the 202nd amino acid, leading to the premature truncation in the series of polypeptide synthesis. Three previously described common sequence changes, V27I (79G → A), E114G (341A → G), and I203T (608T → C), which were thought to be nonpathological polymorphic changes (Abe et al. 2000), were frequently found in patients as well as controls.

Audiometric evaluation of the patients with biallelic *GJB2* mutations

Audiometric results were obtained from 60 patients with biallelic *GJB2* mutations. Fig. 1 shows a collection of overlapping audiograms from subjects bearing 18 combinations of mutations. Although the severity of hearing impairment in individuals varied according to the combinations of mutations, there seemed to be certain phenotypes determined by each combination. First, the hearing levels of the patients homozygous for 235delC mutations were comparatively severe to profound (Fig. 1). In addition, 235delC/299-300ATdel, G45E/G45E/Y136X/Y136X, G45E/Y136X/R143W, 235delC/R143W, and R143W/T86R also showed severe hearing impairment. In contrast, the patients homozygous for V37I had significantly mild-to-moderate hearing impairment (Fig. 1). Similarly, relatively milder phenotypes were found in the patients with 235delC/V37I, V37I/R143W, F191L/F191L, T123N/176-191del16, and