Fig. 3. Projection images of statistical parametric maps, showing activation with word stimuli in normal subjects (a) and CI users (b). The activated areas were thresholded at uncorrected p < 0.05, since a word stimulus evoked wide activation without very steep peaks. SFM = superior frontomedian cortex; for other abbreviations of brain areas, see text.

In CI users (fig. 1b), the pattern of activation was different. Strong and broad activation was observed in the right primary auditory cortex, ipsilateral to the sound stimuli. Two distinct peaks were found, one in the anterolateroinferior part (Rprm1) and the other in the posteromediosuperior part (Rprm2). Rprm1 had a symmetrical position to Lprm in normal subjects. Two foci with weaker activation than on the right side were observed in the left temporal lobe (Lprm' and Lasc). Lasc was situated in the left association auditory cortex (Lasc, superior or middle temporal gyri). Lprm' was located almost symmetrical to, but somewhat more lateral than, Rprm2 and corresponded to the border area between Brodmann's areas 41 (transverse gyrus) and 42 (superior gyrus) by reference to the standardized stereotaxic atlas [Talairach and Tournoux, 1988]. Figure 2 shows Rprm1, Rprm2 and Lprm' in CI users and Lprm in normal subjects, superimposed on the typical MRI image. All these 4 activated foci were located along and/or adjacent to the transverse temporal gyrus,

confirming activation in the primary auditory area because both cytoarchitectonic subdivision study [Brodmann, 1909] and functional study [Lauter et al., 1985] located the human primary auditory cortex on and adjacent to the transverse gyrus. However, Lprm' in CI users seems to involve the adjacent association area more strongly than the other 3. In reality, an activation in the temporal lobe has been considered to be in the primary cortex if it is near the transverse gyrus and is evoked by pure tones [Bilecen et al., 2000], because the activations of 2 distinct but adjacent areas, such as areas 41 and 42, cannot be distinguished on their border by the current resolution of imaging and because the morphological localization determined by gyri does not perfectly correspond to the cytoarchitectonic configuration (Brodmann's areas). Therefore, we regard Lprm' in CI users as an activation in the primary area, rather than an extraordinary plasticity occurring in the association cortex.
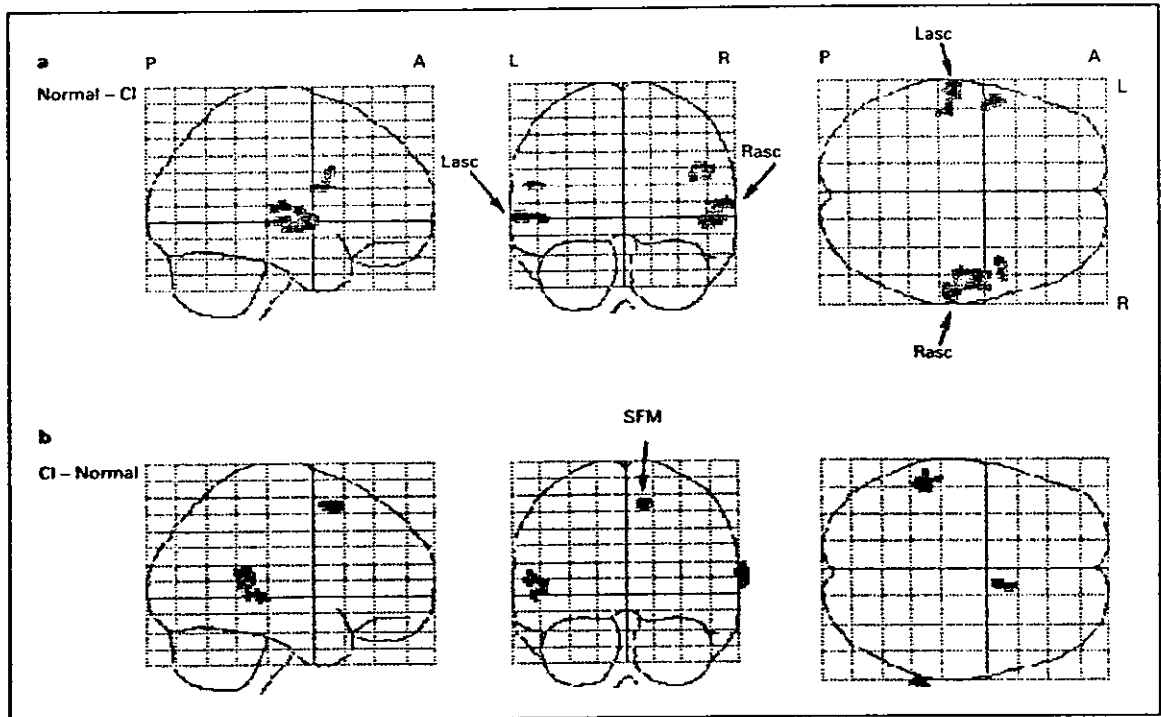
Fig. 4. Regions where activation with word stimuli was stronger in normal subjects than in CI users (a) and vice versa (b). The areas were thresholded at uncorrected p < 0.001.

Brain Activation by Word Stimuli

Word stimuli (table 3) activated bilateral auditory association cortices (left: Lasc, right: Rasc) in normal subjects (fig. 3a).

In the CI users (fig. 3b), in addition to bilateral auditory association cortices (Lasc and Rasc), the right superior frontomedian cortex (SFM) presumably including the supplementary motor area and the neighboring midline anterior cingulate gyri were also activated. In association cortices, the peak Z values in CI users were smaller than in normal subjects, and the statistical parametric map images gave an impression of broader activation (small activated foci scattered in the bilateral association cortices, in contrast to more compact, concentrated activated foci in normal subjects). However, since the activated foci in the temporal lobe largely overlap with word stimulation and the difference is not so easy to determine as with tone bursts, we investigated the differential activation between CI users and normal subjects (fig. 4). Activation in bilateral association cortices near primary areas, corresponding to Brodmann's area 42, was higher in normal subjects. In CI users, activation in the right SFM was

Table 3. Foci of maximal activation by word stimuli in 3 areas of interest are shown in the order of Z values calculated by SPM99

	Area	Z value	Coordinates (x, y, z)
Normal subjects	Lasc	5.52	(–58, –22, 2)
		4.96	(–58, 0, –10)
		3.90	(–68, –22, 6)
	Rasc	5.49	(66, –22, 2)
		5.02	(68, –14, 2)
		4.74	(60, –8, 4)
CI users	Lasc	5.01	(–60, –36, 6)
		4.51	(–54, –24, –6)
		4.19	(–50, –38, 4)
	Rasc	4.43	(62, –16, 10)
		4.32	(68, –40, 12)
		4.17	(72, –20, 0)
	SFM	4.30	(12, 12, 54)
		4.20	(4, –4, 60)

Lasc and Rasc = Left and right auditory association cortices, respectively; SFM = right superior frontomedian cortex. Coordinates (mm) are shown in the standard stereotaxic space of Talairach. Only the areas with peak Z values exceeding 3.3 are shown, i.e. p < 0.0005.

higher than in normal subjects. We also found that activation in CI users is higher at the posterior end of bilateral association cortices (posterior end of Brodmann's area 22).

Discussion

Differences in Cortical Activation between Normal Subjects and CI Users
Tone Burst Stimuli

Cortical activation patterns by the tone burst stimuli showed an outstanding difference between normal subjects and CI users. Whereas a small area in the *contralateral* primary auditory cortex was activated in normal subjects, a much broader area in the *ipsilateral* primary cortex was predominantly activated, with 2 separate peaks, in early CI users. No comparable plasticity in the auditory primary cortex has been reported. The broadness of this ipsilateral primary cortex activation may be due to stimulation of a larger population of cochlear neurons, in contrast to a very limited stimulation in normal subjects, owing to inevitable current leaks of CI devices and/or may signify that such ipsilateral projection is new and therefore preliminary, failing to converge on a small central processing area. The functional auditory projection to the primary cortex was shown to shift from contralateral to bilateral after unilateral deafness, possibly due to disinhibition from the contralateral inputs [Vasama et al., 1995; Scheffner et al., 1998; Bilecen et al., 2000; Ponton et al., 2001], especially with left ear unilateral deafness [Khosla et al., 2003]. Although the contralateral activation remained dominant in such cases, predominant ipsilateral activation was found in the very acute phase of sudden deafness [Suzuki et al., 2002; Po-Hung Li et al., 2003]. In the present study, the ipsilateral projection (right ear to right primary cortex) in early CI users may partly be attributed to lack of inhibition from the contralateral (left) ear. However, since we tested only right-sided CI users, we dare not assert that the left-sided early CI users will show similar ipsilateral activation, since a difference in cortical activation according to the deafened side in patients with unilateral deafness was found [Khosla et al., 2003].

Our present results may not be compared directly to previous studies, since no previous PET studies in CI users employed tone burst or pure-tone stimuli. Electric and magnetic responses over the ipsilateral (right) hemisphere were observed in 1 patient implanted on the right side with tone burst stimuli [Pelizzone et al., 1986].

Giraud et al. [2001] found activation of the contralateral (right) primary cortex with a mixture of speech and noise stimuli in early left-sided CI users. One study with a brief square pulse, which corresponds to a click stimulus in normal-hearing subjects, showed that the activation was contralateral in 2 of the 3 subjects tested and was ipsilateral in 1 subject [Truy et al., 1995], although the length of the usage of devices was not noted. Thus, the results were variable depending on the side of stimulation, type of stimulus and period of rehabilitation. However, PET studies with postlingual CI users including long-term users, whose subjects were similar to ours, have shown that the white noise activated the contralateral primary cortex more strongly than the ipsilateral cortex [Naito et al., 1995; Okazawa et al., 1996]. Assuming that the difference between tone burst stimuli and white noise stimuli could be ignored, we hypothesize that the prominent right-sided ipsilaterality observed in the present study may diminish in the long-term use of CI devices, i.e. in the course of auditory rehabilitation.

In discussing the lateralization of cortical responses, it is important to note the large difference among different types of sound stimulation. With meaningful speech stimuli, bilateral temporal gyri (primary and association cortices) are strongly activated with higher activation on the left side [Scott et al., 2000]. With word stimuli, the pattern of activation is similar but less strong with less marked left-sidedness [Wise et al., 2001]. With pure-tone or tone burst stimuli, a small area of the contralateral temporal gyrus (primary cortex) is activated [Lauter et al., 1985]. Cortical activation with white noise or click stimuli, which has a wide frequency range, shows an intermediate and obscure pattern between speech and pure tone, i.e. bilateral (contralateral > ipsilateral), broad but weaker activation than that caused by speech stimuli [Naito et al., 1995]. Moreover, the significant activation of the primary cortex was not consistently shown (only small to no statistical significance) in previous PET studies with white-noise stimuli [Okazawa et al., 1996; Naito et al., 1997, 2000]. Lateralization differences of cortical activation according to gender have been controversial, although a recent large-scale report denied intersexual differences [Frost et al., 1999]. Hemispheric asymmetry affected by aging was also reported [Bellis et al., 2000]. However, these were shown only with speech stimuli and only the laterality, not the precise location of activated foci, was noted. Such differences with pure-tone stimuli were not reported and seem not plausible with bilaterally normal-hearing ears, considering the predominant contralateral connection of the primary cortex. In fact, exact age/gen-

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der matching has not been considered essential in the PET studies in CI users [Naito et al., 2000]. Therefore, we consider that the lateralization differences between CI users and normal subjects with tone burst stimuli is significant, although our control subjects and CI patients were not exactly age/gender matched.

The activation of the left auditory association cortex by tone bursts, although not very strong, was somewhat unexpected because all CI users distinguished between tone burst and word stimuli. In a previous study, the multitalker babble activated bilateral association cortices in CI users but not in normal subjects [Wong et al., 1999]. However, this result is not directly comparable to ours, either, because the multitalker babble retains much more of the characteristics of speech [Wong et al., 1999].

Word Stimuli

In CI users, lower activation peaks in the bilateral association cortices were observed with word stimuli, along with the activation of the SFM presumably including the supplementary motor area and adjacent midline cingulate gyri, which was absent in normal subjects. The lower peak levels in association cortices can be explained by the lower word recognition scores in these early CI users. In contrast, rehabilitated, long-term CI users showed stronger activation of association cortices than normal subjects [Wong et al., 1999; Naito et al., 2000].

The activation of the supplementary motor area may be attributed to a new recruitment of normally unused areas to process the stimuli. In long-term CI users, such an activation of the supplementary motor area and cingulate gyri was reported in one study [Naito et al., 2000] but not in another [Wong et al., 1999]. As the latter included patients with longer CI use, recruitment of these brain areas may disappear in the long term. Activation of the anterolateral right hemisphere was reported also in patients with unilateral sudden deafness [Vasama and Makela, 1995]. The SPM studies also implied activation of more peripheral areas in the association cortex in early CI users. Concerning the peripheral temporal areas, activation of the left inferolateral temporal cortex was reported in aphasia, which probably contributed to better performance after rehabilitation [Warburton et al., 1999]. The supplementary motor area, which is concerned with internal rehearsal of motion, has been shown to be activated also by word retrieval tasks [Warburton et al., 1996]. Naito et al. [2000] hypothesized, using a task using silent repetition of heard words, a stronger recruitment of an internal playback mechanism embedded in a network arrangement involving the auditory cortex, Broca's area

and the supplementary motor area. However, in our patients, activation of this area did not require silent repetition of word stimuli, and Broca's area was not activated. In a study with poststroke aphasia, activation of the supplementary motor area was observed only with temporal lesions but not with subcortical lesions, with frontal lesions or in normal subjects [Heiss et al., 1999], suggesting the possibility of compensatory activation.

Central Plasticity or Peripheral Coding Difference?

Before concluding that the differences in activation between CI users and normal subjects are results of plasticity after a long disuse of auditory cortices, we have to discuss the effect of the difference in peripheral coding between CI users and normal subjects. Since the device employed in this study could activate at most 20 channels and the cochlear nerves might have undergone certain degeneration, the quality of signals transmitted by the peripheral nerve of CI users is supposed to be lower than that of normal subjects, which could cause different patterns in cortical activation. Previous studies attributed the higher cortical activation in CI users than normal subjects, which was contrary to our results, to extensive recruitment of cortical neurons (coarse coding strategy) due to degraded signals supplied by peripheral neurons [Wong et al., 1999; Naito et al., 2000]. However, with tone burst stimuli, even though the proportion of cochlear neurons stimulated by the CI device may be larger than that in normal subjects, owing to inevitable current leaks, that cannot explain the activation of the ipsilateral (right) cortex (Rprm1 and Rprm2) in CI users. On the other hand, with word stimuli, the limitation in the number of available CI channels (at most 20) may well cause degradation in the quality of input signals from the periphery to the central nervous system. However, we could not find functional activation studies with normal subjects showing newly recruited brain areas with degraded speech stimuli in contrast to normal speech stimuli, which could underscore the importance of peripheral coding effects. Normal subjects have been shown to understand much deteriorated speech with a small number of channels [Shannon et al., 1995] (>80% sentence comprehension with 3 noise bands, corresponding to 3 channels in CI users), indicating relatively small effects of degraded inputs caused by decreasing the number of channels on speech comprehension. A study involving very well-performing CI users, in whom the peripheral coding effect is supposed to be minimized, also showed differential recruitment of speech processing [Giraud et al., 2000]. Activation of the supplementary motor area and areas periph-
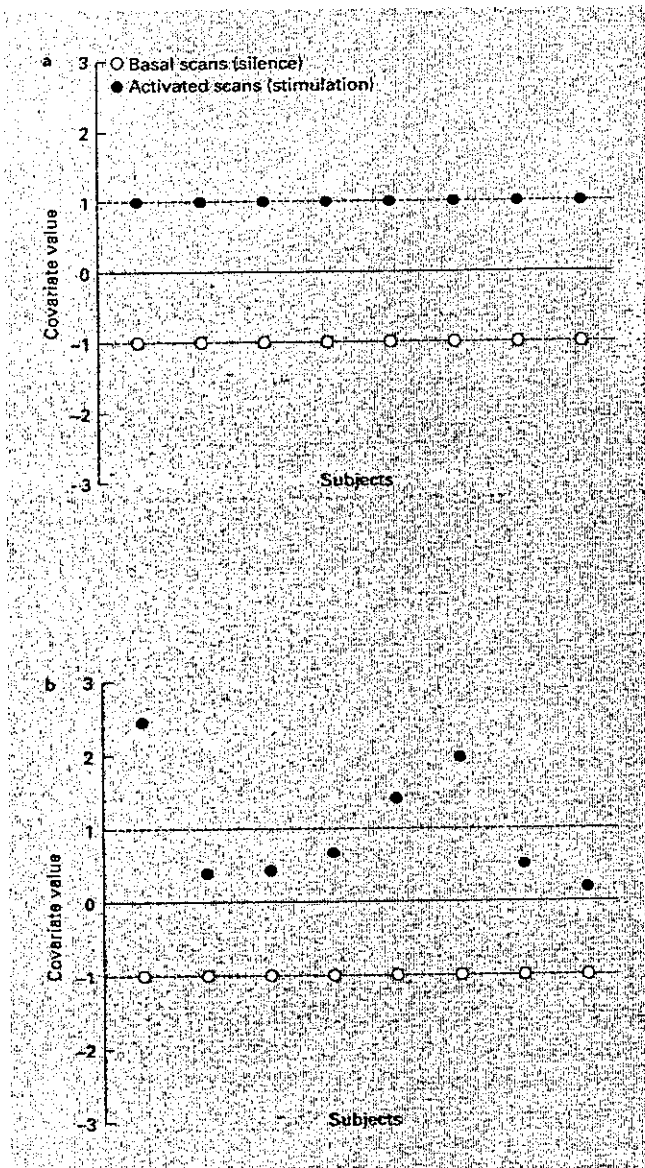
Fig. 5. Conceptual representation of the conditional analysis (a) and the covariate-weighted analysis (b) in SPM. It should be noted that the two methods have no principal differences. To evaluate conditional effects, each nonactivated scan is given the covariate value –1, and the activated scans the value +1 (a). Therefore, the conditional effect is nothing but a particular case of general covariate effects. To investigate covariate-weighted effects, we made a slight modification to the conditional effect evaluation: the activated scans were given covariates weighted by the parameter (word recognition score), centered at +1. This modification is supposed to enhance the brain areas which are more strongly activated in individuals with higher covariate values and less strongly activated in those with lower covariate values, thus approximating covariate-related activation.

eral to the association cortex has been found in aphasic patients, in whom peripheral coding rested intact [Heiss et al., 1999; Warburton et al., 1999]. These proofs, although indirect, seem to support the dominance of central plasticity over peripheral coding effects. In this article, we opt to use the expression 'plasticity' with reservations as described above.

Relationship to Auditory Reconfiguration in Diseases

Interestingly, newly recruited brain areas in early CI users, the supplementary motor area and possibly the areas peripheral to the immediate association cortex, coincided with those in aphasic patients with temporal lobe lesions. This suggests that the plasticity realized at the sudden restart of auditory input after a long disuse of auditory pathways can mimic the alternative recruitment strategy employed in the case of auditory cortex damage. So to speak, at the regain of audition by CI, the brain seems to mobilize all possible cortical areas to decode the auditory inputs. With tone bust stimulation, ipsilateral dominance of the primary auditory cortex was observed, suggesting the possibility of rapid substitution using the ipsilateral primary cortex, in case of malfunction of the contralateral cortex. With word stimuli, recruitment of brain areas which were not activated in normal subjects was observed.

Taking into account the previous studies with CI users [Miyamoto et al., 1999; Wong et al., 1999; Giraud et al., 2000; Naito et al., 2000], the plasticities observed in our early CI users may diminish or disappear in the course of rehabilitation. However, in patients with permanent damages in auditory cortex or pathways, especially in young children, these plasticities have a chance to persist and become effective. For example, interhemispheric transfer of language-processing areas has been reported in children, in disorders affecting the dominant hemisphere, such as tumors [DeVos et al., 1995], epilepsy [Helmstaedter et al., 1994, 1997] and brain damages causing hemiplegia [Bergman et al., 1984]. Right hemisphere restitution in cases with left hemisphere epilepsy was less frequently observed with the onset age of more than 13 years [Helmstaedter et al., 1997]. However, compensatory activations outside the left superior temporal cortex, such as the right hemisphere and the peripheral left temporal regions, were also observed in adult aphasia [Miura et al., 1998; Heiss et al., 1999; Warburton et al., 1999], although the effectiveness in terms of language comprehension might remain insufficient. Thus, we hope that researches on the auditory brain activation in early CI users can contribute to the understanding of the substitutive capacity
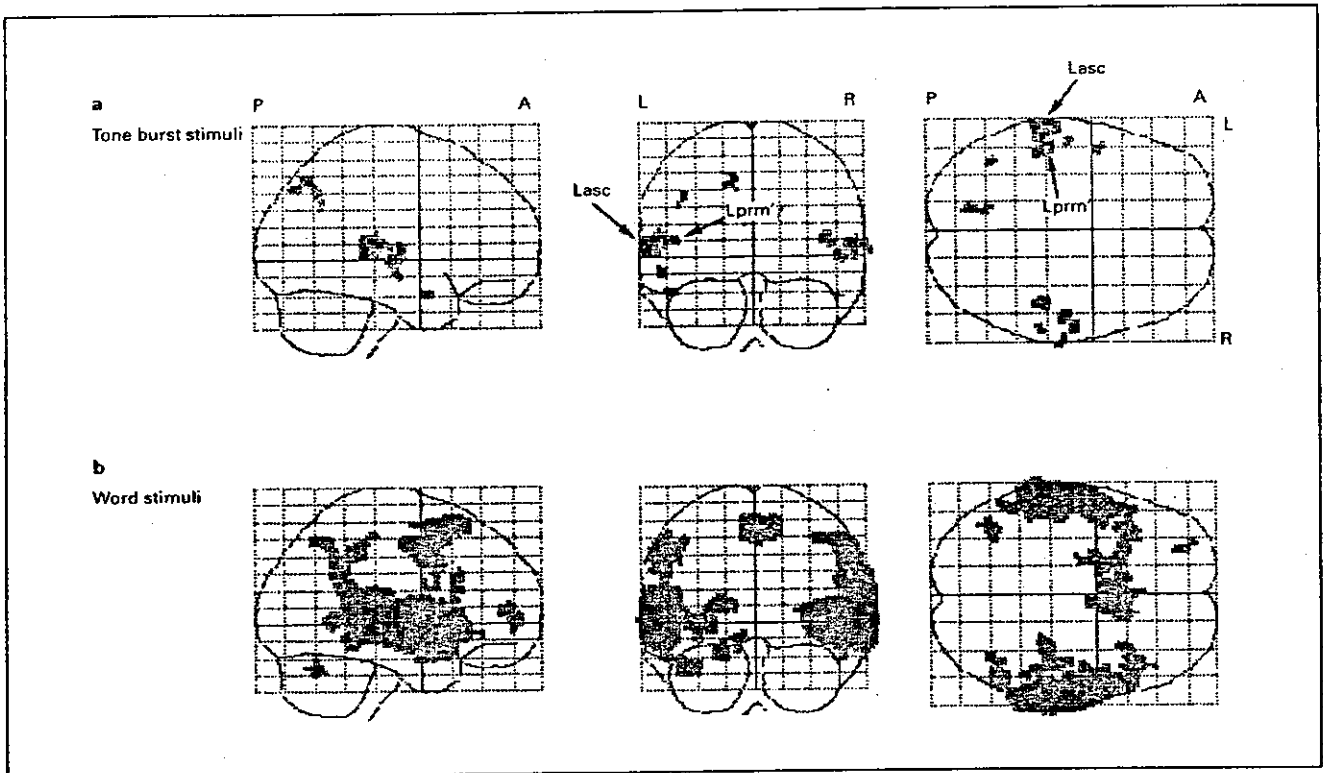
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Fig. 6. a Projection images of statistical parametric maps, showing the covariate-weighted activation (covariate: word recognition score), with tone burst stimuli in CI users. Other imaging parameters are the same as in figure 1. **b** Projection images of statistical parametric maps, showing the covariate-weighted activation (covariate: word recognition score), with word stimuli in CI users. Other imaging parameters are the same as in figure 3.

against disease processes involving auditory central processings.

Implication on the Relationship to Word Comprehension Ability

Early CI users, not yet rehabilitated, tend to show large individual variation in speech comprehension ability. To find out the effect of speech comprehension on the cortical activation, we slightly modified the ordinarily employed *conditional* analysis as follows.

The additional analysis using a covariate concerned only the CI users, and the word recognition score was used as the covariate (*covariate-weighted* analysis). The covariates were divided by their average before use, to adjust the average to + 1.0. Brain activations weighted by a covariate were explored by this analysis. The difference from the usual conditional analysis exists only in that the covariate-weighted analysis allocates different corresponding covariate values (e.g. 0.2, 0.6, 1.9 etc.: positive values

whose average is +1.0) for sound-activated scans and the value −1.0 for all the silent scans whereas conditional analysis allocates the same values for all the sound-activated scans (+1.0) and for all the silent scans (−1.0) [Frackowiak et al., 1997], in forming the general linear model. Therefore, brain areas which are activated proportionally to a covariate are supposed to be more emphasized than in the conditional analysis. Figure 5 demonstrates the basic concept of these SPM analyses. It will be understood that this additional analysis is only a slight modification of the conventional one. For *tone burst stimuli*, the covariate-weighted activation analysis showed a pattern different from the simple conditional activation studies (fig. 1b and 6a). The activation for Lprm' (Z value: 4.64) exceeded those for the right primary cortex (Rprm1: 4.35 and Rprm2: 3.88). For *word stimuli*, the covariate-weighted activation studies with word recognition scores (fig. 6b) revealed activation patterns different from those in the simple conditional activation study (fig. 3b) in two

points: (1) the activated foci in the bilateral association cortices look more compact with higher Z values at peaks (5.84 for the right side, 5.44 for the left), and (2) SFM activation was less significant. In summary, these patterns were more similar to the cortical activation in normal subjects (fig. 1a and 3a), indicating that good word recognizers tended not to employ these plasticities. From another point of view, one can presume that preserved auditory connections are important for good word comprehension in early CI users. In relation to this, the recovery after aphasia caused by temporal lobe lesions was shown to be closely related to the degree of preservation of the left temporal areas and their reintegration into function networks, rather than to compensatory activation of other cortical areas, such as contralateral (right) temporal cortices [Heiss et al., 1999; Warburton et al., 1999].

Acknowledgments

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The Role of the External Auditory Canal in the Development of the Malleal Manubrium in Humans

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Objective: To determine if the external auditory canal (EAC) plays a role in the induction and proper positioning of the malleal manubrium in humans.

Study Design: Retrospective study between 1994 and 2002.

Setting: Academic, tertiary care referral medical center.

Patients: Fifty-five ears of 50 patients with congenital atresia (n=47) or stenosis (n=8) of the EAC, for which meatoplasty was performed at the University hospital between 1994 and 2002.

Main Outcome Measures: The presence of the manubrium was examined during surgery, and the corre-

lation between the presence of the manubrium and the grade of the microtia was evaluated.

Results: The manubrium was identified in all ears with EAC stenosis, whereas it was absent in all ears with EAC atresia. No correlation was observed between manubrium formation and auricular deformity.

Conclusions: Our results demonstrated a close relationship between the formation of the EAC and that of the malleal manubrium in humans. This is consistent with the recent findings in knockout mice. This information is useful for surgical intervention in cases of congenital EAC anomalies.

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C ONGENITAL ANOMALY OF the external auditory canal (EAC) is a relatively rare clinical entity and consists of a series of malformations of the auricle and EAC, the latter varying from slight narrowing to complete absence of the EAC.[1-5] The incidence ranges from 1 in 10000[6] to 1 in 15000[7] births. A combination of reconstruction of the EAC and auricle is usually performed to achieve improvement in hearing and cosmetic appearance. The reconstruction of the anomalous EAC is one of the most challenging procedures in otology because it is often accompanied by middle ear anomalies, such as facial nerve aberration, deformity of the ossicles, defect of the oval window, and lack of mastoid pneumatization.[1-5,8,9] These malformations are considered to result from developmental arrest between 6 and 10 weeks of fetal life.[10,11] Knowledge of the development of middle ear elements is indispensable to safely and successfully complete surgery for congenital EAC anomalies. The patterns of formation of the

middle ear elements and their mutual interaction, however, have not been fully explored in humans.

Preoperative evaluation with high-resolution computed tomography (HRCT) of the temporal bone is essential for surgical planning because it provides important information on the type and severity of anomalies of the EAC and middle ear elements, including the ossicles. The development of the middle ear has been

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evaluated by a grading system based on HRCT of the temporal bone.[1] It has been reported that the severity of EAC and middle ear anomalies correlate with the surgical outcome.[12]

Recent molecular and genetic analyses using knockout mice enhance our understanding of ear development and the pattern of formation of middle ear elements.[13-16] For example, disappearance of the tympanic ring due to retinoic acid treatment and

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Table 1. Classification of 55 Ears With External Ear Canal Anomalies According to Schuknecht's Criteria[3]

Group	Unilateral		Bilateral	Total
	Right	Left		
Stenotic group				
Type A	0	0	0	0
Type B	3	1	4	8
Atretic group				
Type C	11	12	11	34
Type D	2	3	8	13
Total	16	16	23	55

Table 2. Relationship Between the Type of External Ear Canal Anomaly and Grade of Microtia in 55 Ears With Congenital External Ear Canal Anomalies

Group	Grade			Total
	I	II	III	
Stenosis group				
Type A	0	0	0	0
Type B	5	1	2	8
Atresia group				
Type C	9	7	18	34
Type D	1	1	11	13
Total	15	9	31	55

Table 3. Presence of the Manubrium in Relation to the Classification of External Auditory Canal Anomalies

Group	Malleal Manubrium	
	Present	Absent
Stenotic group		
Type A	0	0
Type B	8	0
Atretic group		
Type C	0	34
Type D	0	13
Total	8	47

duplication of the ring in Hoxa-2 null mutant embryos resulted in alterations in EAC formation.[15] In addition, Prx1 and Goosecoid (Gsc) genes are known to be essential for middle ear and EAC development, and target mutation of these genes results in the lack of tympanic ring and EAC and the hypomorphism of the manubrium.[17-19] These findings suggest that the formation of the EAC may depend on the formation of tympanic ring. Moreover, it has been reported that the presence of EAC plays an essential role in the induction and proper positioning of the malleal manubrium,[13,16] which originates from the mesenchyme of the proximal area of the first branchial arch and provides the connection between the tympanic membrane and middle ear through skeletogenesis of the mesenchyme.[13] These results obtained from genetically modified mice suggest a close correlation between the formation of the ma-

nubrium and the EAC. However, such a correlation has not been well documented in humans.

The purpose of the present study was to evaluate the relationship between the presence of the manubrium and the appearance of the EAC in humans by comparing them in ears with atresia or stenosis of the EAC.

METHODS

Between August 1992 and October 2002, reconstruction of the EAC was performed in 66 ears in 58 patients with EAC anomalies who had no known genetic abnormality. Eleven ears, in which the mesotympanum was not explored, were excluded. Thus, 55 ears of 50 patients (42 male and 8 female patients; mean age, 13.7 years [range, 6-34 years]) were included in the study. Eighteen patients had bilateral EAC anomalies, and 32 patients a unilateral anomaly (16 right-sided and 16 left-sided). Five patients underwent bilateral canaloplasties, and 45 patients underwent unilateral canaloplasty. Schuknecht[3] established a classification (types A-D) of the atresia based on HRCT and surgical findings. In that classification, the EAC anomaly is limited in type A to the fibrocartilaginous part; the EAC is stenotic, and cholesteatoma sometimes develops in the EAC. In type B, narrowing and in some cases tortuosities of both the fibrocartilaginous and bony parts of the EAC are found. Type C has a totally atretic EAC with well-developed pneumatization of the tympanic cavity. Type D has poor pneumatization of the temporal bone with severe anomaly of the middle ear structures. We included types A and B in the stenosis group and types C and D in the atretic group.

The degree of microtia was classified into grades I to III according to the classification of Marx.[20] Grade I microtia shows a mild deformity, with the auricle being slightly smaller than normal and each part being clearly distinguished. In grade II microtia, the size of the EAC is one half to two thirds of the normal size and its structure is partially retained. In grade III microtia, the auricle is severely malformed and usually exhibits a peanut shape.

The appearance of the ossicles was evaluated during surgery. We focused on 3 anatomical structures of the malleus. We evaluated the fusion of the malleus head with the incus body, presence of the neck and the lateral process of the malleus, and extension of the vertical process (ie, manubrium) below the lateral process. The surgical findings and the grades of microtia were compared between atresia and stenosis groups.

RESULTS

The number of ears in each of 4 atresia types (A-D) is given in **Table 1**. The stenosis group consisted of 8 ears of type B, whereas the atresia group consisted of 34 ears of type C and 13 ears of type D. The side of involvement was not associated with the type of EAC anomalies. The severity of microtia in relation to the degree of EAC stenosis is given in **Table 2**. Fifteen ears were assigned as grade 1 microtia, 9 ears as grade II, and 31 ears as grade III. Microtia was classified as grade I in 5 of 8 ears of type B, whereas microtia grade III was found in 11 of 13 ears of type D. Type C ears showed all grades of microtia with no prevalence. In general, the formation of the auricle was more severely affected in the atresia group compared with the stenosis group.

The relationship of the presence or absence of the manubrium and atresia or stenosis of the EAC is given in **Table 3**. The presence of manubrium was con-
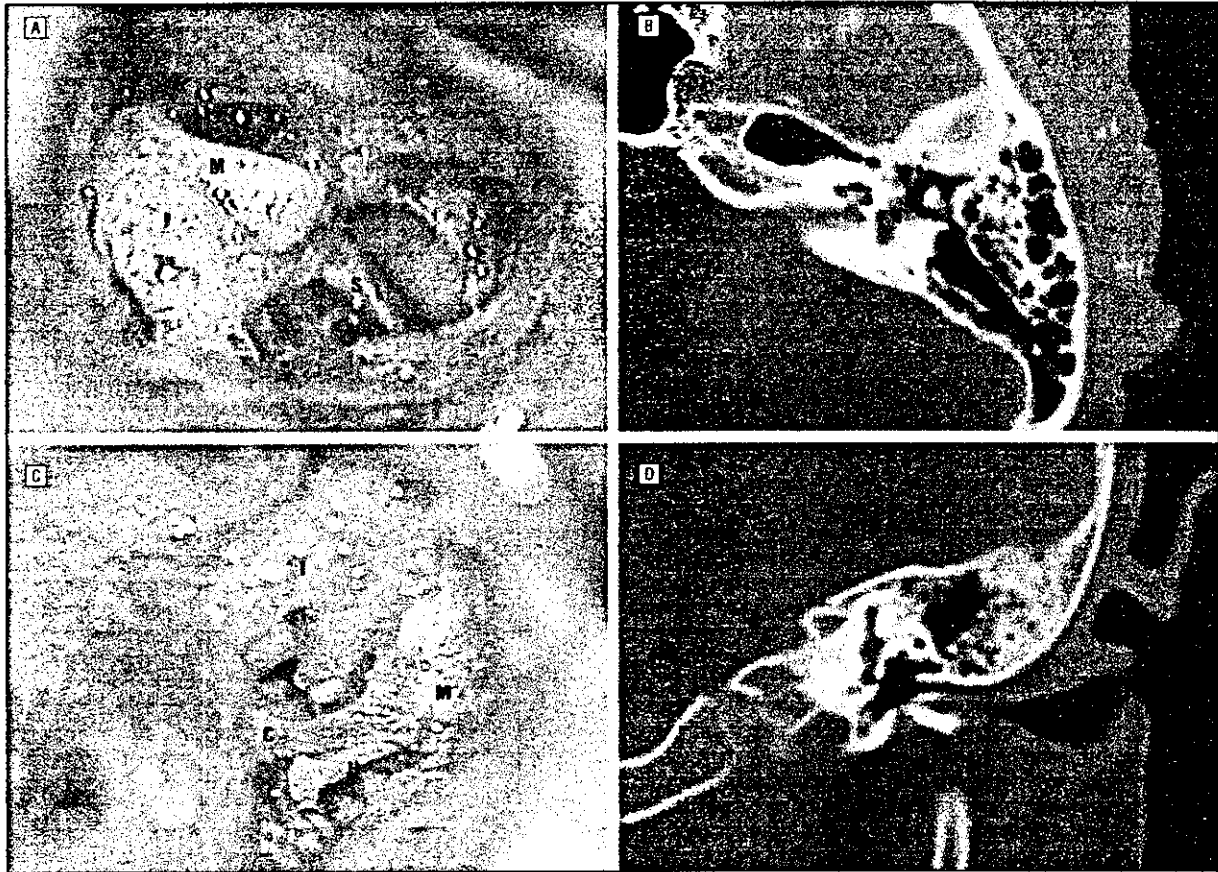
Coronal computed tomographic (CT) images and surgical view of temporal bones in a patient with external auditory canal (EAC) atresia (A and B) and stenosis (C and D). A, Surgical view of the right ear with EAC atresia. B, Axial CT image of the ear shown in A. C, Surgical view of the right ear with EAC stenosis. D, Coronal CT image of the ear shown in C. M indicates malleus; I, incus; H, manubrium; C, chorda tympani; and S, stapes.

firmed in all 8 ears of the stenosis group; however, the manubrium was shorter and angled toward the promontory in 5 of the ears. In the atresia group, the manubrium was absent in all ears. The head of the malleus and the body of the incus were commonly fused in this group. The **Figure** shows the HRCT and surgical findings of representative cases from each group.

COMMENT

Congenital anomalies of the EAC are often associated with absence or deformity of the manubrium or its angulation toward the promontory.[1-3] Furthermore, the head of the malleus and the body of the incus are sometimes fused.[1-5,9] The relationship between the pattern of the ossicles and the EAC condition, however, is not well understood. The present study clearly demonstrated a close relationship between the formation of the EAC and that of the malleal manubrium.

Schuknecht[3] reported that in type C anomaly, the manubrium was "usually" absent, and when present, it was deformed and angled toward promontory. However, in that study, there was no reference to the correlation between the condition of the manubrium and ECA appearance. In addition, the relationship between the grade of the microtia and the presence of the manu-

brium was not clearly delineated. The present study demonstrated that the presence of the manubrium was dependent on the formation of the EAC but not on the severity of the microtia.

The EAC develops from the first branchial groove between the mandibular and hyoid arches of the dorsal and ventral portions.[21,22] In humans, at 4 to 5 weeks of gestation, a solid core of epithelial cells, derived from the ectoderm of the first groove, comes into contact with the endoderm of the first pharyngeal pouch, in the area of the tympanic ring.[21,22] Then, the mesoderm grows between the ectoderm and the endoderm, and the contact is disrupted. By the eighth week of gestation, the primary meatus is formed like a funnel-shaped tube because of the deepening of the first branchial cleft toward the tympanic cavity.[21,22] The primary meatus corresponds to the lateral third of the EAC, which is later surrounded by cartilage that forms from the surrounding mesoderm.[21,22] During the ninth week of gestation, the ectoderm of the first branchial groove thickens and grows medially toward the tympanic cavity.[21,22] During the 21st week of fetal life, resorption of the cord epithelial cells begins to occur, leading to the formation of a canal.[21,22] By the 28th week, the deepest cells of the ectoderm plug remain, forming the superficial layer of the tympanic membrane.[21,22] The medial two thirds of the EAC is derived from the new ectoder-

mal tube and becomes the bony portion of the canal.[21,22] If this canalization process is arrested prematurely, it is possible for a more normally developed tympanic membrane and bony ear canal to exist in association with an atretic or stenotic membranous canal.[21,22]

There is a close relation between the development of the tympanic ring and the formation of the manubrium.[13] The manubrium of the malleus is inserted in the tympanic membrane. This process is essential for transferring vibrations in the membrane to ossicle chain. The anatomical structure of the insertion of the manubrium between 2 epithelia is due to the development of the EAC, tympanic membrane, and the middle ear cavity.[13] In particular, the tympanic ring plays a central role in the anatomical structure.[13] Moreover, the development of the tympanic ring is induced by the formation of the EAC, and the development of the manubrium is dependent on the formation of the EAC. These correlations may explain why the manubrium was absent in the atresia group but present, albeit short and deformed, in the stenosis group.

In conclusion, we have demonstrated in the present study that the formation of the manubrium was closely associated with the appearance of the EAC, suggesting that the EAC also plays an essential role in the induction and proper location of the malleal manubrium in humans.

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5 難聴の遺伝子治療

石本　晋一，山岨　達也

はじめに

　遺伝子治療とは「疾患の治療を目的とした遺伝子または遺伝子を導入した細胞をヒトの体内に投与することである」と定義される。つまり目的とするDNAを医薬品として体内へ投与することで単一遺伝子異常疾患の異常遺伝子を修復したり，欠損遺伝子を補充したりすることで疾患を治癒させるという治療法である。

　遺伝子治療の進歩は原因遺伝子の研究と遺伝子導入技術（遺伝子組み換え技術・ウイルスベクターの開発ほか）の進歩によるところが大きい。WatsonとCrick（1954）によるDNAの構造発現と機能における意味づけは生物学に革命的な影響を与えた。さらにmRNAと遺伝情報のセントラルドグマ（各遺伝子情報はDNAからRNA，そして蛋白へと伝達されること）が明らかにされて以降，遺伝子研究は急激な進歩を遂げ，2003年にはヒトゲノム30億塩基の配列が解明されるまでに至った。遺伝子導入技術としてはウイルスゲノムが永久的に細胞ゲノムに組み込まれることから，既に1966年にはウイルスを遺伝子導入に用いることが予想され，1960年代終わりには遺伝子治療の最初の試みが行われた（S. Rogers）。現実味を帯びたのは1980年初期にレトロウイルスベクターが開発されてからであり，わが国では北海道大学でアデノシンデアミナーゼ欠損症の男児に遺伝子治療を行ったのが1例目である。現在では家族性高脂血症，嚢胞性線維症などの遺伝病，パーキンソン病な

どの神経変性疾患のほか，虚血性心疾患，各種悪性腫瘍など多くの分野で遺伝子治療が臨床試験されている。耳鼻咽喉科領域では，鼻腔，喉頭[1]，中耳[2]，内耳などで基礎実験が行われているが臨床応用には至っていない。近年，内耳を標的器官とした遺伝子治療に関しての論文が国内外から多数報告され，有毛細胞・蝸牛神経障害の感音難聴に対しての遺伝子治療の研究は加速度的に発展している。今回，感音難聴の原因の多くを占める有毛細胞の障害，蝸牛神経細胞の変性に関する遺伝子治療の近年の研究論文を報告するとともに今後の発展，可能性に関して紹介する。

1. 内耳における遺伝子治療の意義と適正

　感音難聴の多くを占める内耳有毛細胞，蝸牛神経核細胞の障害に対しては現在のところ適切な治療法がない。それは哺乳類においては，感覚受容体である蝸牛有毛細胞やラセン神経節細胞は消失した場合，再生されないからである。そこで新たな内耳障害の治療法の一つとして遺伝子治療が期待されている。

　内耳において遺伝子導入の役割は大きく二つある。一つには遺伝性難聴のように，ある特定の遺伝子の欠損あるいは変異が確認でき，将来難聴が生じることが予測される，または難聴が進行する可能性が高い場合に，欠損遺伝子を蝸牛に導入して有毛細胞などの障害が生じることを予防することである。もう一つは加齢変化，

表1. 遺伝子導入法の代表的なベクターとその一般的な特徴

遺伝子導入法	長　所	短　所
アデノウイルスベクター	ベクター作製方法が確立している 高力価のベクターが調合できる 非分裂細胞への遺伝子導入可能	抗原性，細胞毒性がある，作製法が複雑 染色体に組み込まれず繰り返し投与が必要 遺伝子発現が一過性
アデノ随伴ウイルス（AAV）ベクター	病原性，細胞毒性がない 染色体の特定部位への組み込み可能 非分裂細胞への遺伝子導入可能	ベクターの大量生産困難 4.5kb以上の遺伝子は導入できない
レトロウイルスベクター	ベクター作製方法が確立している 染色体に組み込まれる	非分裂細胞に導入できない 細胞遺伝子に挿入変異を起こす可能性あり 増殖性ウイルスに病原性あり
ヘルペスウイルスベクター	感染する宿主細胞のスペクトルが広い DNAに組み込まれず核内に存在 長期にわたりウイルスDNAが存在	病原性・細胞障害性がある
パピローマベクター	遺伝子を安定に高発現させる 感染性がない	遺伝子導入効率が低い ある種の細胞をトランスフォームさせる
カチオニックリポソーム	複数遺伝子の導入可能	染色体に組み込まれず繰り返し投与が必要 導入遺伝子がリソソームで分解 細胞毒性がある
膜誘導型リポソーム	高い導入効率 複数遺伝子の導入可能	作製法が複雑 染色体に組み込まれず繰り返し投与が必要 導入遺伝子がリソソームで分解

騒音曝露，薬剤投与などにより有毛細胞が変性・消失して難聴が生じた場合，有毛細胞，ラセン神経節などを新たに再生させて機能回復することである。

　内耳において遺伝子治療が適当であるとする理由は三つあり，①内腔が液体で占められるためベクターが迅速に分布することが期待できる，②手技的にベクターの投与が比較的容易である，③実験上遺伝子発現の分布や程度を定量できる，ということである。このことから，内耳は遺伝子治療のよい標的組織とみなされている[3)4)]。

　内耳における遺伝子導入方法はこれまでいろいろ試みられている。通常，DNAそのものを標的細胞に取り込ませることは困難であり，ベクター（担体）を用いて標的細胞のDNAに組み込ませることになる。ベクターは大きくウイルス性，非ウイルス性のものに分類される。ウイルス性ベクターとしてはヘルペスウイルスベクター，アデノ随伴ウイルスベクター，アデノウイルスベクター，レトロウイルスベクターなどさまざまなベクターがこれまでin vitro, ex vivo, in vivoで用いられている[3)~11)]。一方，非ウイルス性のベクターの代表的なものはリポソームである[12)13)]。これはDNAを脂質膜でできたリポソームの内部に取り込ませ標的細胞の細胞膜と融合させ，DNAを細胞内に誘導する方法である。各ウイルスベクター，非ウイルスベクターには長所・短所があり，表1に示す。

　また投与法についても，主にモルモット，マウスを用いてin vivoでさまざまな経路からの投与法が試みられている。主な投与方法は鼓室階に開けた小孔または蝸牛窓経由での蝸牛外リンパ腔への投与である。ほかに蝸牛中央階に開

けた小孔[14)15)]または内リンパ嚢[16)]からの蝸牛内リンパ腔投与，さらに外側半規管[17)]から蝸牛へ投与することが試みられている。

2. 難聴モデルの遺伝子治療の進歩

感音難聴に対する遺伝子治療の研究は大きく二つの方向で進んでいる。一つは神経栄養因子を含む増殖因子・防御因子をコードした遺伝子の導入による騒音曝露，薬剤投与に誘導される急性難聴に対する有毛細胞および蝸牛神経核変性の保護効果である。もう一つは分化誘導遺伝子の導入による有毛細胞の再生である。

1) 保護効果(外リンパ腔投与による保護効果)
*in vivo*で内耳内に投与されたものには，インターロイキンレセプター (IL-1 receptor) に対する antagonist の cDNA[18)]や，神経賦活因子 (GDNF，NT3，BDNF) が挙げられる。これらの神経賦活因子などを内耳に投与したことで聴器毒性，音響外傷による有毛細胞のアポトーシス，ラセン神経節細胞の変性が予防できたと報告されている。これらの遺伝子をアデノウイルスベクターに組み込んで投与した場合の作用機序は線維芽細胞をはじめとした蝸牛構成の細胞に遺伝子が導入されたことにより細胞，組織内で蛋白が発現されて効果を示すためといわれている。

Suzuki ら[19)]はゲンタマイシンによる蝸牛・前庭障害に対して GDNF を組み込んだアデノウイルスベクター (Ad.*GDNF*) の治療効果について検討した。ゲンタマイシンを中耳腔に投与して前庭蝸牛障害を作製した場合，鼓室階への人工外リンパ液投与群では内外有毛細胞の障害が顕著に認められたのに対して Ad.*GDNF*投与群では有毛細胞の障害がわずかに認められたに過ぎなかった。Yagi ら[7)]はエタクリン酸とカナマイシンでモルモットを群にして，その後7日目に Ad.*GDNF*と人工外リンパ液を投与した後，3週間目に断頭してラセン神経節細胞を評価したところ，Ad.*GDNF*投与群ではラセン神経節

細胞が顕著に残存した。また Hakuba ら[20)]はモルモットで内耳虚血モデルを作製して Ad.*GDNF*を鼓室階に投与して7日目の内有毛細胞の障害を観察したところ，非治療側と比較して有意に内有毛細胞の障害を防ぐことができたと報告している。

最近では薬物投与・音響曝露では有毛細胞の障害がフリーラジカルによるため，エダラボンをはじめとするフリーラジカルスカベンジャーで有毛細胞の障害が抑制できることが明らかになってきた[21)]。Kawamoto ら[22)]は活性酸素の発生を抑制する作用のある catalase, CuZn superoxide dismutase (SOD1)，Mn superoxide dismutase (SOD2) を組み込んだアデノウイルスベクターをエタクリン酸とカナマイシンで内耳障害を起こしたモルモットの鼓室階に浸透圧ポンプを用いて投与し，顕著に内外有毛細胞の障害が抑制されたと報告している。Oshima ら[12)]はカナマイシンで内耳有毛細胞を障害したモルモットの蝸牛鼓室階に hepatocyte growth factor (HGF) を組み込んだリポソームを投与したところ，有意に内外有毛細胞の障害および蝸牛神経核のアポトーシスを抑制したと報告している。

2) 有毛細胞の再生(内リンパ腔投与)
近年の遺伝子研究の成果により胎生時期の耳胞から内耳有毛細胞の分化の過程が明らかになり，有毛細胞に分化・誘導するために必要なさまざまな遺伝子が判明してきた。特に*Math1*遺伝子は有毛細胞分化を誘導し，*Hes1*, *Hes5*は有毛細胞分化を抑制する遺伝子であることが*in vitro*さらにはノックアウトマウスで報告された[23)~25)]。このことは蝸牛支持細胞が有毛細胞に分化する能力を有している前駆細胞である可能性を示唆している。Kawamoto ら[14)]は有毛細胞分化誘導遺伝子である*Math1*遺伝子を組み込んだアデノウイルスベクター (Ad.*Math1*)をモルモットの蝸牛中央階に投与してコルチ器の支持細胞に感染させ2カ月後に外有毛細胞の外側に幼若な有毛細胞が再生したと報告した(図1)。

図1. *Math1*遺伝子導入60日後の再生した有毛細胞（bar：2μm）

図2. Myosin ⅦA陽性の再生した有毛細胞に蝸牛
神経の樹状突起が伸展

ヘンゼン細胞領域，bar：25μm

これは哺乳類において初めて*in vivo*で有毛細胞の再生に成功した報告である。さらに驚くことに蝸牛神経のニューロフィラメントが再生された有毛細胞へ伸展し，再生された細胞は神経細胞として働く可能性が高いことを示唆していた（図2）。またIzumikawaら[26]はエタクリン酸とカナマイシンで内耳有毛細胞を障害したモルモットの蝸牛中央階にAd.*Math1*を投与して有毛細胞の再生に成功し，さらにABRで非治療側と比較して有意に聴力を回復させたと報告し

た。さらに電子顕微鏡写真での観察では，本来の有毛細胞と異なる形態をした有毛細胞が再生し，これはHensen cellが分化し伸長したことを示した。Minodaら[27]はサイクリン依存性キナーゼのインヒビターであるskip2を組み込んだアデノウイルスベクター（Ad.*Skip2*）とAd.*Math1*を組み合わせて投与した場合，再生した有毛細胞の数が増加したと報告している。

3. 今後の難聴に対する遺伝子治療の進展と課題

Kawamotoら[14]の有毛細胞再生の成功により内耳の遺伝子治療は大きく進歩した。そして，Izumikawaら[26]の研究はKawamotoら[14]の研究を継承し，さらに再生した有毛細胞が感覚細胞としての機能を十分に有していることを示した。今後の内耳遺伝子治療研究の目標の一つはいかに多くの有毛細胞を効率よく適切な位置に再生させるかであり，これを解決して初めて臨床応用が現実味を帯びるといえる。このほかにも遺伝子治療を臨床応用するに際して多くの課題が残されている。その一つはアデノウイルスの有する抗原に対してのヒトの免疫応答である。ヒトでは幼児期にアデノウイルスに感染して抗体を有しているため，初回投与を行っても抗原抗体反応が生じる可能性が強い。さらに複数回投与した場合は非常に強い免疫応答が生じ，有毛細胞の障害が顕著に認められる[28]。今後は免疫応答を最小限に抑えるウイルスベク

ターの開発，感染効率の高いリボソームの改良
など技術的な開発が必要になるものと思われ
る。ウイルスベクターをヒトの内耳に投与した
際の安全性など倫理的なハードルも大きいが，
近年の遺伝子治療の急速な発展により内耳遺伝
子治療がヒトに応用される日も近いと思われる。

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Overexpression of ErbB-2 Protein in Human Middle Ear Cholesteatomas

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Objective: The purpose of this study is to verify the hypothesis that ErbB-2 protein is overexpressed in human middle ear cholesteatomas and to elucidate the relationship between overexpression of ErbB-2 protein, cell proliferation, and apoptosis. *Study Design:* Prospective review of 20 patients between 2001 and 2003 with middle ear cholesteatoma. *Methods:* Middle ear cholesteatoma matrix and retroauricular skin were immunostained with anti-ErbB-2, Ki-67, and single-stranded DNA (ssDNA) antibody. The distribution of immunoreactivity to these antibodies and labeling indices were compared between cholesteatoma and retroauricular skin. *Results:* In matrix of middle ear cholesteatoma, ErbB-2 and ssDNA were expressed in the keratinocytes of all layers and Ki-67 was expressed in the keratinocytes of the basal, lower spinous, and occasionally granular layer. In retroauricular skin, ErbB-2 and Ki-67 were expressed in the keratinocytes of the basal and occasionally lower spinous layer and ssDNA was expressed in the keratinocytes of all layers. Labeling indices against anti-ErbB-2, Ki-67, and ssDNA antibody were significantly greater in cholesteatoma as compared with retroauricular skin. *Conclusions:* In cases of cholesteatoma, ErbB-2 protein was overexpressed and cell proliferation and apoptosis of keratinocytes were accelerated. ErbB-2 protein could modulate terminal differentiation and apoptosis in the keratinocytes of all layers in cholesteatoma matrix and cell proliferation in the keratinocytes of the basal and lower spinous layer in normal skin. *Key Words:* Middle ear cholesteatoma, retroauricular skin, ErbB-2, Ki-67, single-stranded DNA.

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INTRODUCTION

The product of neu oncogene was referred to as HER-2 or ErbB-2 because of the homology to the human epidermal growth factor receptor (HER) gene and c-erbB proto-oncogene. The ErbB family includes the epidermal growth factor receptor (EGFR), HER-2, HER-3, HER-4 (also designated as ErbB-1, ErbB-2, ErbB-3, ErbB-4), and Xmrk.

ErbB1, ErbB3, and ErbB4 bind multiple ligands of epidermal growth factor (EGF) family.[1,2] Ligand binding to the extracellular domain of ErbB receptors was suggested to cause receptor homo- and heterodimerization. Intracellular signal diversification results from different utilization of downstream pathways by different ErbB receptor combinations.[3] Although no direct ligand for ErbB-2 has been identified, it has been shown that ErbB-2 is activated by heterodimerization and/or multimerization with other ErbB receptors.[4,5] Deregulated ErbB signaling has been implicated in various malignancies.[6–8] Expression of ErbB-2 protein has been also demonstrated in chronic inflammatory disorders such as lichen planus and normal skin keratinocytes.[8–11]

In general, activation of the ErbB family members leads to a cellular proliferative response. In certain instances, however, activation of an ErbB receptor can induce differentiation, cell cycle arrest, and even apoptosis.[11] An increasing rate of cell proliferation and apoptotic cell death has been demonstrated to play a crucial role in the pathogenesis of human middle ear cholesteatoma.[12] In the present study, we hypothesized that the keratinocytes of human middle ear cholesteatoma overexpress ErbB-2 protein, and therefore, to elucidate the relationships among overexpression of ErbB-2, cell proliferation, and apoptosis, we conducted immunohistochemical staining using anti-ErbB-2, Ki-67, and ssDNA antibody.

MATERIALS AND METHODS

Tissues

Twenty cholesteatoma matrices and 20 retroauricular skin samples were obtained during tympanoplasty from 20 patients (13 males and 7 females, ranging from 13 to 77 years old; average, 48 years old) with acquired pars flaccida type cholesteatoma. Informed consent for using cholesteatoma matrix and retroauricular skin was obtained from all patients, but informed consent for using ear canal skin was not sought, because enlargement of the raw surface of ear canal by harvesting of an ear canal skin specimen could influence the healing process after tympanoplasty. Six cholesteatoma matrices and three retroauricular skins

that did not contain any cell components were excluded. Overall, 14 cholesteatoma matrices and 17 retroauricular skin samples were enrolled in this study.

Immunohistochemical Staining

The formalin-fixed and paraffin-embedded specimens were sliced into 4 μm sections and mounted on silane-coated slides. Slides were deparaffinized in xylene and dehydrated through graded alcohol. Sections were soaked in 10 mmol/L EDTA and autoclaved for 30 minutes for antigen retrieval for anti-ErbB-2 and Ki-67 antibody. Antigen retrieval for anti-ssDNA antibody was performed by incubating the slides with 5 N HCl for 20 minutes at room temperature. Endogenous peroxidase was blocked with 3% hydrogen peroxidase in methanol for 20 minutes. Nonspecific antibody binding was blocked with the blocking buffer. The slides were incubated overnight in a moist chamber at 4°C with primary rabbit polyclonal anti-ErbB-2 antibody, mouse monoclonal anti-Ki-67 antibody (clone: MIB-1), and rabbit polyclonal anti-ssDNA antibody (Dako Japan, Kyoto, Japan) diluted 1:100, 1:100, and 1:500, respectively, in the blocking buffer. After three rinse in PBS, the slides were incubated with Histofine MAX-PO (MULTI) (Nichirei, Tokyo, Japan) for 30 minutes at room temperature. Immunoreaction was visualized by applying 3 to 3'-diaminobenzidine as a chromogen. After another rinse, the slides were counterstained with Mayer hematoxylin, dehydrated, cleared, and cover-slipped. Negative controls were established by replacing primary antibody with the blocking buffer. Labeling indices of three antibodies were estimated by the percentage of immunostained positive cells scored under a light microscope at a 200-fold magnification. A minimum of 200 cells were counted in two different areas of each section. Labeling indices of three antibodies were compared between cholesteatoma and skin groups using Mann-Whitney test.

RESULTS

Immunohistochemical Staining with Anti-ErbB-2 Antibody

In all cholesteatoma matrices, ErbB-2 protein was expressed in the keratinocytes of all layers on the plasma membrane (Fig. 1A.). ErbB-2 protein was detected in the keratinocytes of the basal and occasionally lower spinous layer in retroauricular skin in the cytoplasm in a vesicular pattern (Fig. 2A.). In the negative controls, no staining was seen in either cholesteatoma or retroauricular skin. Labeling indices of anti-ErbB-2 antibody in cholesteatoma matrix and retroauricular skin were 61.2 ± 11.3% (mean ± SD) and 25.3 ± 7.5%, respectively (Fig. 3.). There was a significant difference (P < .01) in labeling indices between cholesteatoma and retroauricular skin.

Immunohistochemical Staining with Anti-Ki-67 Antibody

In cholesteatoma matix, Ki-67 antigen was detected in the nuclei of the keratinocytes of the basal, lower spinous, and occasionally granular layer (Fig. 1B). In retroauricular skin, Ki-67 antigen was detected in the nuclei of the keratinocytes of the basal and occasionally spinous layer (Fig. 2B). In the negative controls, no staining was seen in either cholesteatoma or retroauricular skin. Labeling indices of anti-Ki-67 antibody in cholesteatoma matrix and retroauricular skin were 32.8 ± 9.2% and 9.4 ± 2.3%, respectively (Fig. 3). There was a significant difference (P < .01) in the labeling indices between cholesteatoma and retroauricular skin.

Immunohistochemical Staining with Anti-ssDNA Antibody

In all cholesteatoma matrices, ssDNA was detected in the nuclei of the keratinocytes of all layers (Fig. 1C.). ssDNA was also detected in all layers in retroauricular skin, although the staining intensity was lower compared to cholesteatoma matrix (Fig. 2C.). In the negative controls, no staining was seen on either cholesteatoma or retroauricular skin. Labeling indices of anti-ssDNA antibody in cholesteatoma matrix and retroauricular skin were 64.7 ± 11.6% and 45.0 ± 13.3%, respectively (Fig. 3). There was a significant difference (P < .01) in labeling indices between cholesteatoma and retroauricular skin.

DISCUSSION

Cholesteatomas are derived from the skin of the tympanic membrane or adjacent ear canal skin, which may not be directly comparable to retroauricular skin. For the ethical reason described previously, we thought it the second best to substitute retroauricular skin for ear canal skin.

In this study, we showed the immunoreactivity of anti-ErbB-2 antibody in the keratinocytes of the basal and occasionally lower spinous layer in retroauricular skin. The staining pattern showed an intracellular vesicular pattern. For the first time, we showed the immunoreactivity of ErbB-2 of keratinocytes of all layers in cholesteatoma. ErbB-2 protein was expressed on cell surfaces in all layers. In general, activation of the ErbB family members is considered to lead to a cellular proliferative response. In certain instances, however, activation of an ErbB receptor can induce differentiation, cell cycle arrest, and even apoptosis.[11] Lewis et al.[11] reported that inhibition of ErbB2 led to a suppression, although statistically insignificant, of UV-B-induced HaCaT keratinocyte apoptosis. This result also supports the relevance between the expression of ErbB-2 and apoptosis. Kansra et al.[13] showed that ErbB-2 protein localized intracytoplasmically in the keratinocytes in the basal and lower spinous layers of skin moved to the cell surface as the cells approached the upper differentiated layers. Although retroauricular skin did not express a cell surface pattern in the upper differentiated layers, the ErbB-2 was expressed on cell surfaces in all layers in cholesteatoma matrix, suggesting the acceleration of cell differentiation—that is, apoptosis. The lack of the expression in a cell surface pattern might have been ascribed to methodology, including the antigen retrieval procedure and the fixation time by 10% buffered formalin.

Ki-67 is a non-histone protein expressed during the non-G0 phases of the cell cycle. A monoclonal antibody, MIB-1, which was raised to Ki-67, has been shown to have a good correlation with other markers of cell proliferation in human breast cancers. In the current study, we showed the immunoreactivity of anti-Ki-67 antibody in the keratinocytes of the basal and occasionally lower spinous layer in retroauricular skin and in the keratinocytes of the basal, lower spinous, and occasionally granular layer in cholesteatoma matrix. The distributions of immunoreactivity in both cholesteatoma and retroauricular skin were substantially the same. The labeling index of Ki-67 of cholesteatoma was significantly greater than that of normal skin, suggesting that the keratinocytes of cholesteatoma are less differenti-