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HISTONE DEACETYLASE INHIBITION ENHANCES ADENOVIRAL VECTOR TRANSDUCTION IN INNER EAR TISSUE

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Abstract—Adenovirus vectors (AdVs) are efficient tools for gene therapy in many tissues. Several studies have demonstrated successful transgene transduction with AdVs in the inner ear of rodents [Kawamoto K, Ishimoto SI, Minoda R, Brough DE, Raphael Y (2003) J Neurosci 23:4395–4400]. However, toxicity of AdVs [Morral N, O'Neal WK, Rice K, Leland MM, Piedra PA, Aguilar-Cordova E, Carey KD, Beaudet AL, Langston C (2002) Hum Gene Ther 13:143–154.] or lack of tropism to important cell types such as hair cells [Shou J, Zheng JL, Gao WQ (2003) Mol Cell Neurosci 23:169–179] appears to limit their experimental and potential clinical utility. Histone deacetylase inhibitors (HDIs) are known to enhance AdV-mediated transgene expression in various organs [Dion LD, Goldsmith KT, Tang DC, Engler JA, Yoshida M, Garver RI Jr (1997) Virology 231:201–209], but their effects in the inner ear have not been documented. We investigated the ability of one HDI, trichostatin A (TSA), to enhance AdV-mediated transgene expression in inner ear tissue. We cultured neonatal rat macular and cochlear explants, and transduced them with an AdV encoding green fluorescent protein (Ad-GFP) under the control of a constitutive promoter for 24 h. In the absence of TSA, GFP expression was limited, and very few hair cells were transduced. TSA did not enhance transduction when applied at the onset of Ad-GFP transduction. However, administration of TSA during or just after Ad-GFP application increased GFP expression in supporting cells approximately fourfold. Moreover, vestibular hair cell transduction was enhanced approximately sixfold, and that of inner hair cells by more than 17-fold. These results suggest that TSA increases AdV-mediated transgene expression in the inner ear, including the successful transduction of hair cells. HDIs, some of which are currently under clinical trials (Sandor et al., 2002), could be useful tools in overcoming current limitations of gene therapy in the inner ear using Ad-GFP. Published by Elsevier Ltd on behalf of IBRO.

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Abbreviations: AdV, adenovirus vector; Ad-GFP, adenovirus vector encoding green fluorescent protein; Ad-LacZ, adenovirus vector encoding •-galactosidase; CAR, coxsackie and adenovirus receptor; DMEM, Dulbecco's Modified Eagle's Medium; FBS, fetal bovine serum; GFP, green fluorescent protein; HATs, histone acetyltransferases; HCs, hair cells; HDACs, histone deacetylases; HDIs, histone deacetylase inhibitors; PAGE, polyacrylamide gel electrophoresis; PFA, paraformaldehyde; PI, propidium iodide; PVDF, polyvinylidene difluoride; RAR, retinoic acid receptor; RXR, retinoid X receptor; T-PER, tissue protein extraction reagent; TSA, trichostatin A.

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The major cause of sensorineural hearing loss and vestibular disorders is the loss of hair cells (HCs) in the inner ear (Nadol, 1993). This can occur for many reasons, including aging, genetic disorders and exposure to noise or toxins. Moreover, although HC regeneration occurs in birds and fish, the loss of HCs in mammals is currently irreversible. It is thus important to develop therapies that can prevent HC loss or induce HC regeneration in patients.

Potential treatments for inner ear disorders include gene therapy. It has been demonstrated experimentally that gene transfer into the inner ear can induce the expression of protective substances (Kawamoto et al., 2003), correct genetic disorders (Maeda et al., 2007), and induce HC regeneration (Izumikawa et al., 2005). Gene transfer into the inner ear also has the advantage that this site is relatively isolated from other tissue and the spread of gene transfer vectors to other organs is likely to be limited.

Adenovirus vectors (AdVs) have been widely explored for gene therapy because of their ability to mediate transgene expression in many cell types. However, adenoviral toxicity restricts their use for clinical medicine, and they do not efficiently transduce all types of cells. This is particularly true of HCs, which are one of the most important targets for gene therapy of the inner ear. Augmentation of gene expression efficacy from AdVs may be helpful for inner ear gene therapy because it would potentially decrease the quantity of vector required for therapy and thus decrease the potential for toxicity. It may also increase the numbers and types of inner ear cells that can effectively be transduced.

Acetylation of core histones is an important regulator of gene expression in eukaryotic cells, since it reduces the density of nucleosomes and enhances exposure of DNA to the transcriptional machinery of the cell. Histones may be involved in efficient transduction with vector DNA, as well. Viruses produce histone-like proteins and recruit them to their DNA, which can increase transcriptional access (Grove and Saavedra, 2002). A histone deacetylase inhibitor (HDI): trichostatin A (TSA) has been shown to enhance AdV-mediated transgene expression in a number of cell types (Dion et al., 1997), and this property may be helpful in overcoming some limitations of gene therapy using AdVs. However HDIs have not been evaluated for their potential to enhance AdV-mediated gene expression in the inner ear. The present study was designed to assess the effects of TSA on transgene expres-

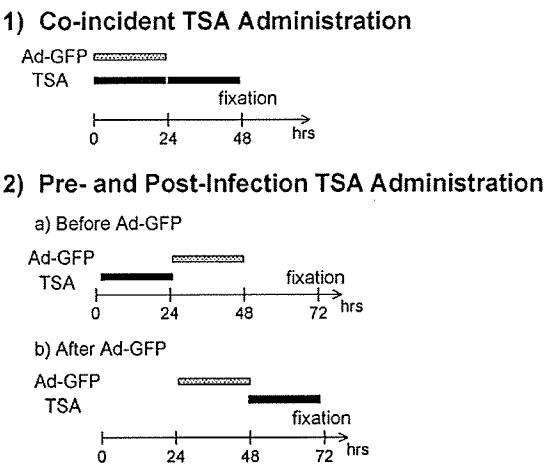

Fig. 1. Experimental protocols used in the present study. TSA was applied during, before or after exposure of the tissue to Ad-GFP.

sion mediated by AdVs in inner ear cells, and to determine whether TSA altered core histone acetylation.

EXPERIMENTAL PROCEDURES

Adenoviral vectors (AdVs)

Replication-incompetent AdVs (E1/E3 deleted, CMV promoter, serotype 5 expressing green fluorescent protein (Ad-GFP) or • -galactosidase (Ad-LacZ)) were used.

Cell culture and treatment

As a positive control for the effects of TSA, the Rat-1 fibroblast cell line was infected with Ad-GFP at 30 PFU/cell. After infection, the cells were cultured in the presence or absence of 500 nM TSA for 48 h. The effect of TSA on GFP expression was evaluated by fluorescence microscopy.

Tissue culture of vestibular maculae and organ of corti

Utricular and saccular maculae and organs of Corti were dissected from Wistar rat pups at postnatal days 3–5 (P3–5). The vestibular explants were then transferred into a 24-well plate and cultured with the HC layer uppermost. The explants were maintained in culture medium composed of Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) and 30 U/ml penicillin, to which HEPES buffer had been added to a concentration of 25 mM.

Culture wells contained 500 • l of medium and were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. The experimental protocol adopted for this study was approved by the Animal Subjects Committee of the San Diego VA Medical Center.

Administration of TSA and Ad-GFP or Ad-LacZ

The explants were incubated in the serum-free medium containing 1• 10^7 PFU/ml of Ad-GFP or Ad-LacZ for 24 h. The medium was then replaced with fresh DMEM containing 10% FBS, and TSA (500 nM final concentration) was added to the medium at designated times (Fig. 1).

Measurement of transduction efficiency

Forty-eight hours after transduction with Ad-GFP, the explants were fixed with 4% paraformaldehyde (PFA) for 30 min. Explants were first evaluated as whole-mounts under fluorescence and confocal microscopy. They were then embedded in OCT compound and sectioned at 10 • m on a cryostat. The sections were nuclear-stained with DAPI and immunostained for myosin 7A to distinguish HCs from supporting cells. The percentages of GFP-positive cells were normalized to the number of DAPI-positive cell nuclei. Expression of Ad-LacZ was examined by whole mount X-gal staining followed by sectioning. Explants were fixed with 4% PFA and immersed in X-gal solution. They were then embedded in OCT and sectioned at 10 • m.

Western blotting for detection of histone acetylation

TSA-treated and normal control vestibular maculae were evaluated for acetylation of histones by Western blotting. Twenty vestibular organs from P4 rats were lysed in 400 • l T-PER (Tissue Protein Extraction Reagent). The samples were then spun down at 1000 rpm for 2 min and the supernatant discarded. The tissue was then homogenized. NuPAGE LSD sample buffer and NuPAGE Reducing Agent (Invitrogen, Carlsbad, CA, USA) were mixed with the samples and they were heated to 70 °C for 3 min. The samples were then kept on ice for a few minutes. Thirty microlitres of tissue lysates were separated by SDS-polyacrylamide gel electrophoresis (PAGE) on 7.5% gels and electrotransferred to polyvinylidene difluoride (PVDF) membranes (Immobilon-P; Millipore, Bedford, MA, USA). The membranes were blocked with 5% nonfat dried milk in TBS–Tween [50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 0.1% Tween 20] for 60 min at room temperature. The blots were incubated with a primary antibody (mouse monoclonal anti-acetyl-lysine antibody AKL5C1, (sc-32268; Santa Cruz Biotechnology, Santa Cruz, CA USA) that detects N-espilon-acetylated lysine residues) in blocking buffer overnight at 4 °C and then incubated with horseradish peroxidase-

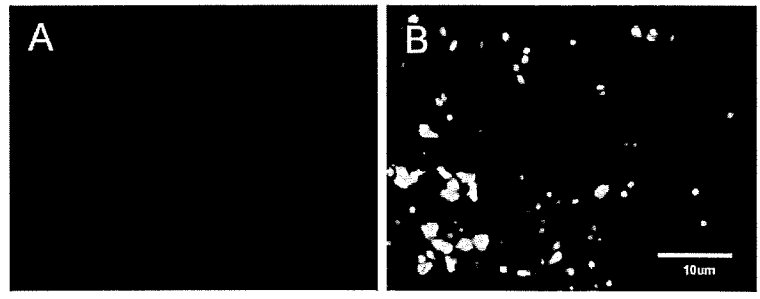

Fig. 2. Effect of TSA on Ad-GFP transduction of rat fibroblasts. (A) Ad-GFP alone: Only a few GFP-positive cells are observed. (B) Ad-GFP• TSA: Many more GFP-positive cells are present and transduction levels are increased, as has been reported previously [4].

labeled anti-mouse IgG antibody (sc-2005; Santa Cruz) as a secondary antibody, followed by chemiluminescent detection of labeling at the molecular weights corresponding to core histones H3 and H4. To verify protein loading, the PVDF membranes were immediately stripped by placing the membrane in stripping buffer (0.5 M NaCl and 0.5 M acetic acid) for 30 min at room temperature. The membrane was washed once for 10 min in TBS–Tween, reblocked, and blotted with an antibody to •-actin as an internal control for protein quantity.

Acetylation of histones in tissue

We also evaluated histone acetylation in TSA-treated explants by immunohistochemistry using an antibody against acetylated histone. Vestibular macula explants were embedded in OCT compound and sectioned at 10 • m on a cryostat. The sections were then reacted with rabbit monoclonal anti-acetylated histone H3 (Lys23) antibody (#9674; Cell Signaling Technology, Beverly, MA, USA) and FITC-labeled anti-rabbit IgG (#0936; DAKO, Glostrup, Denmark) as a secondary antibody, followed by fluorescence microscopy.

Effects of TSA on the expression of an endogenous gene using a transgenic reporter mouse

In order to examine the effects of acetylation on an endogenous gene encoding the same protein encoded by Ad-GFP, we used transgenic mice in which GFP is expressed in HCs under the control of a pou4f3 promoter construct (Erkman et al., 1996). Vestibular maculae and organ of Corti from P3–P5 mice were cultured with or without TSA for 2 days. The intensity of HC GFP fluorescence was then evaluated by image analysis.

PI evaluation of cell viability

In order to assess the toxicity of Ad-GFP itself and of Ad-GFP plus TSA, we evaluated the viability of cells using propidium iodide (PI) exclusion. Explants were treated with Ad-GFP, with or without TSA as above. They were then incubated with PI (P-3566, 1:2000; Molecular Probes, Invitrogen) in PBS solution for 15 min at room temperature. The explants were washed with HBSS three times and fixed with 4% PFA for 30 min, followed by evaluation under light and fluorescence microscopy. Cells exhibiting nuclear staining with PI were scored as nonviable.

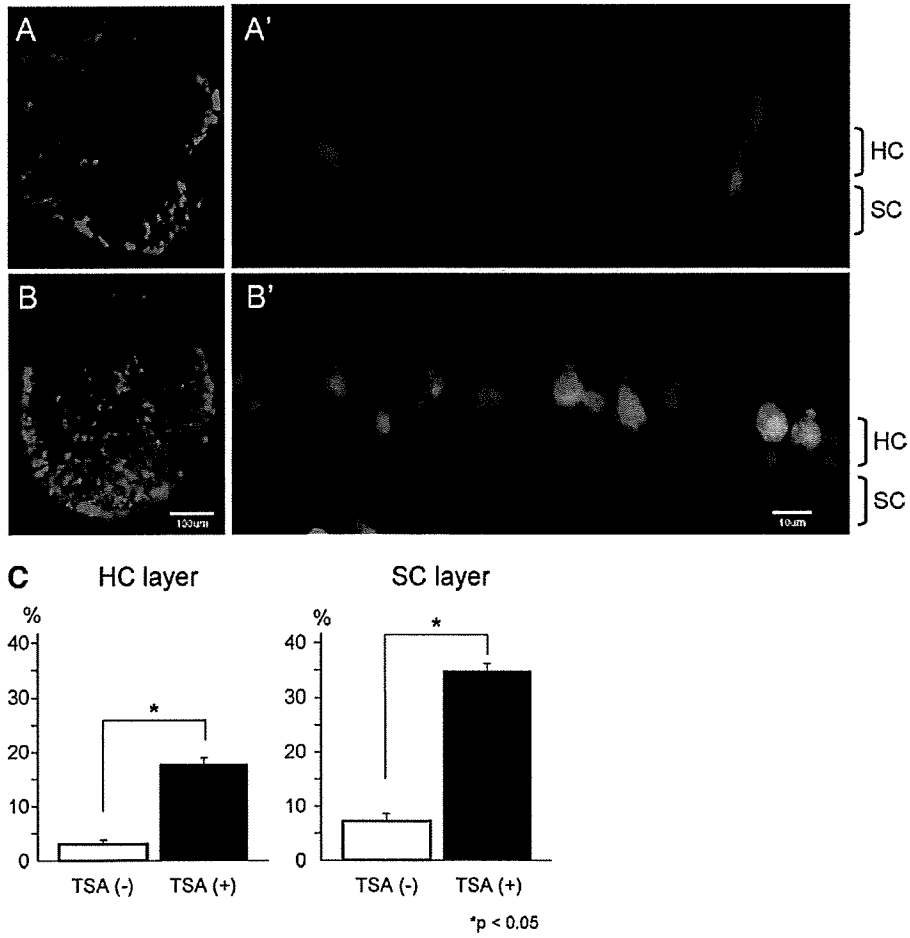

Fig. 3. Effect of simultaneous TSA administration on Ad-GFP transduction of neonatal vestibular maculae, surface preparations (A, B), sections (A', B') and cell counts (C). Ad-GFP alone: Cells are transduced primarily in the peripheral region (A). Sections reveal that only two HCs and one supporting cell are transduced (A'). Ad-GFP with TSA: GFP-positive cells are present throughout the epithelium (B). Sections reveal that there are many GFP-positive HCs and supporting cells (B'). (Green: GFP; Red: Myosin 7A; Blue: DAPI). (C) Graph illustrating Ad-GFP transduction efficiency by cell type in vestibular maculae: TSA enhanced GFP expression in both the hair cell (HC) layer and the supporting cell (SC) layers. However, the enhancement of HC transduction was somewhat greater.

Statistical analysis

Quantitative differences were evaluated using Student's t-test. Significance was evaluated at a level of P• 0.05. Data are presented as means and standard errors, along with the number of explants used in each condition.

RESULTS

Effect of TSA in cell lines

Rat1-fibroblasts, which were obtained from American Type Culture Collection, were infected with Ad-GFP and cultured in the presence or absence of TSA. As shown in Fig. 2A, B, TSA significantly augmented expression of GFP, even though there was no significant difference in the density of the cultured cells themselves.

Effect of TSA in inner ear tissues

The effect of TSA on Ad-GFP expression in inner ear tissues is illustrated in Figs. 3–8. In the absence of TSA, GFP-positive cells were primarily localized in peripheral regions of vestibular maculae (Fig. 3A). However, in explants treated simultaneously with TSA, the number of GFP positive cells was substantially increased and GFP positive cells were observed not only in the periphery but also in the central region of the macula (Fig. 3B). Sections revealed that both supporting cells and HCs were transduced with Ad-GFP in the absence of TSA (Fig. 3A'), although HCs were much less commonly transduced in agreement with previous studies (Dazert et al., 2001; Jero et al., 2002; Yagi et al., 1999). TSA significantly increased Ad-GFP expression in both supporting cells and HCs (Fig. 4B'), with the result that substantial numbers of HCs were transduced. In the HC layer, transduction efficiency rate increased from 3.09• 0.84% to 17.6• 1.35% (mean• SE); that is, by sixfold (P• 0.05, n• 7) (Fig. 3C). In the supporting cell layer, it increased from 7.18• 1.35% to 33.18• 1.66% (mean• SE); that is by 4.2 fold (P• 0.05, n• 7) (Fig. 3C). Thus the enhancement in HC transduction was somewhat greater than that seen in supporting cells. TSA also enhanced Ad-LacZ transduction (Fig. 4).

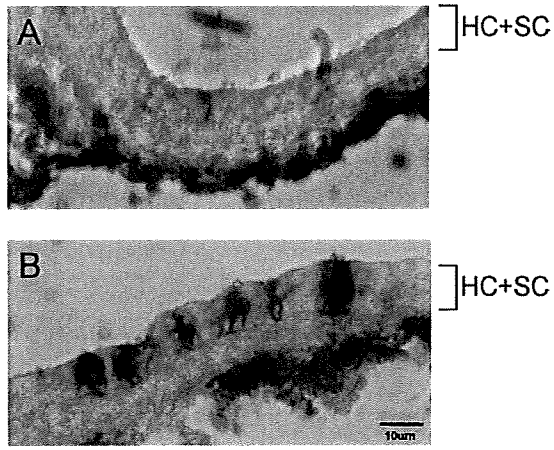

Fig. 4. Typical TSA effect on transduction with Ad-LacZ in vestibular maculae. (A) Ad-LacZ alone: Only stromal cells are strongly transduced. (B) Ad-LacZ plus TSA: Both HCs and supporting cells (SC) are strongly transduced. Because the number of samples used was small (n• 3), quantification was not performed.

In organ of Corti explants, we used the same treatment paradigm as in the vestibular epithelium. TSA was added to the media at the same time as Ad-GFP. TSA also enhanced Ad-GFP transduction in both supporting cells and HCs (Fig. 5). However, inner HCs were consistently transduced at higher levels than were outer HCs with TSA (Fig. 5B' and B''). In inner HCs, TSA enhanced transduction efficiency from 3.33• 1.86% to 58.33• 2.94% (mean• SE); that is, by more than 17 fold (P• 0.001, n• 6). For outer HCs, it increased transduction from 3.33• 0.97 to 5.83• 0.64; that is by less than twofold (P• 0.2, n• 6).

Prior treatment with TSA has no effect on Ad-mediated transgene expression

When TSA was administered 24 h after Ad-GFP application, histone deacetylase (HDAC) inhibition enhanced Ad-GFP expression to a degree comparable to that seen with simultaneous treatment when compared to Ad-GFP alone (Fig. 6B). Thus in vestibular sensory epithelium, transduction efficiency in the HC layer increased from 3.92• 0.05% to 19.47• 5.98% (mean• SE); that is, fivefold (P• 0.05, n• 11). In the supporting cell layer efficiency increased from 12.4• 0.48 to 32.75• 6.33 (mean• SE); that is, 2.5 fold (P• 0.05, n• 11). However, when TSA was added to the media 24 h prior to Ad-GFP but then discontinued upon Ad-GFP treatment, there was no effect of TSA on transduction efficiency (Fig. 6A). These results suggest that the effect of TSA on adenoviral transgene expression is not mediated by an increase in uptake of Ad-GFP, but rather is mediated by the augmentation of transgene expression.

Association of core histone hyperacetylation with the enhancement of Ad gene expression

Histones are acetylated at their lysine residues. Their acetylation was detected by Western blotting of macular explants using an anti-acetyl-lysine antibody. TSA induced subtantially more acetylation of histones H3 and H4 in inner ear cells (Fig. 7). The amounts of •-actin protein observed in the blots were similar. We also evaluated histone acetylation by immunohistochemisty, using an anti-acetylhistone antibody (Fig. 8). In explants without TSA, few cells displayed visible immunolabeling (Fig. 8A). However, in explants exposed to TSA, the nuclei of almost all cells were labeled (Fig. 8B), indicating extensive hyperacetylation.

Influence of TSA on expression of an endogenous gene

In order to assess whether TSA might influence the expression of an endogenous gene, we used explants from transgenic mice in which an integrated gene construct directs the expression of GFP to HCs. No significant differences in HC GFP expression were observed with TSA

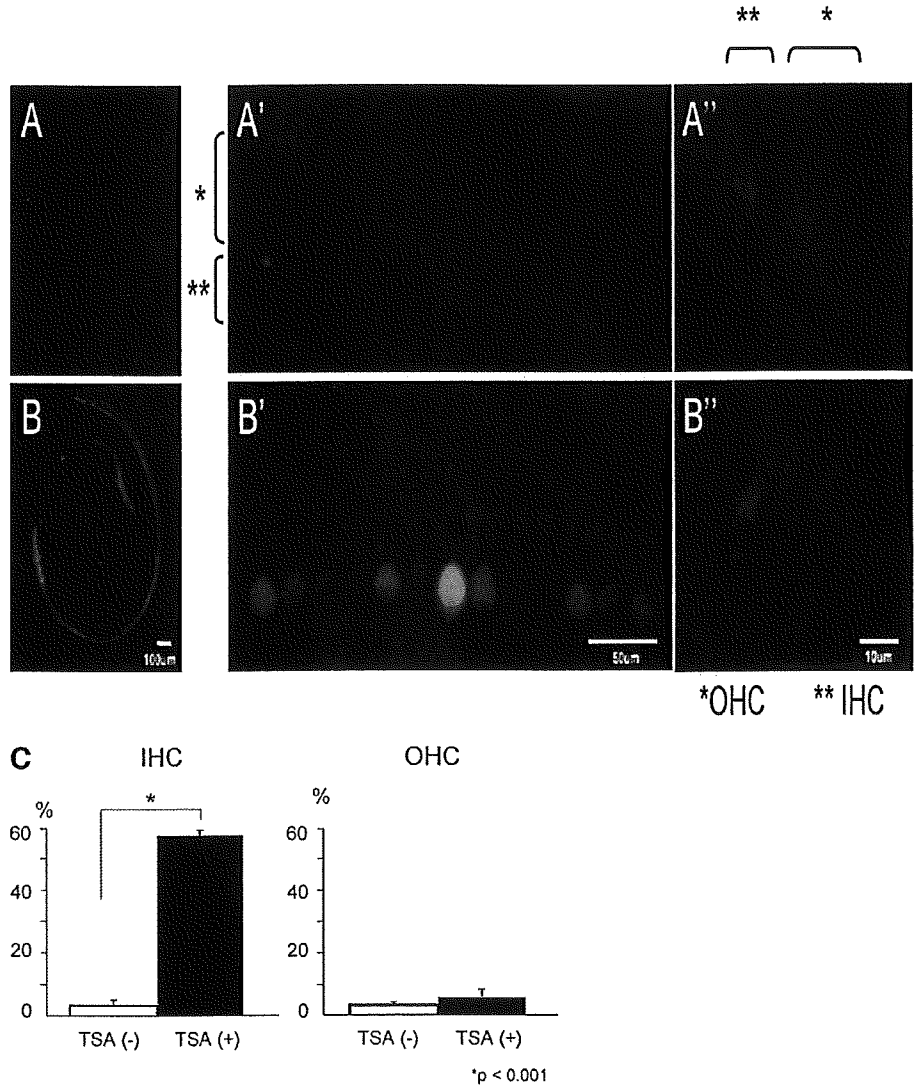

Fig. 5. Effect of TSA on AdV transduction of cochlear explants, surface preparations (A–A', B–B') and sections (A", B") and cell counts (C). Ad-GFP alone: Cochlear cells are transduced, but expression is weak and few hair cells are transduced (A, A', A"). Ad-GFP plus TSA: Many more cells are transduced, and expression levels are higher. Inner HCs are preferentially transduced (B, B', B") (Green: Ad-GFP; Red: Myosin 7A; Blue: DAPI). (C) Graph illustrating Ad-GFP transduction efficiency by cell type in the organ of Corti: TSA enhanced GFP expression in HCs and supporting cells. However, the enhancement of inner HC transduction was much greater.

(Fig. 9A, 9B), suggesting that TSA does not influence the regulatory DNA of the integrated transgene, nor does it influence the fluorescence of GFP directly.

The effect of TSA on adenoviral toxicity

We determined the viability of sensory epithelial cells using a PI exclusion assay. Exposure to AdV increased PI staining in explants, suggesting toxicity to inner ear cells (Fig. 10A) although it was not severe with the amount of Ad-GFP used for the present study. The addition of TSA had no significant effect on PI labeling of explants (Fig. 10B) (P• 0.3, n• 7), indicating that decreased toxicity is not responsible for the observed effects of TSA on transduction.

DISCUSSION

AdVs are powerful tools for gene therapy. Several studies have demonstrated successful gene transduction of cells in the inner ear using AdVs, indicating their potential utility at this site. Luebke et al. (2001) reported effective transduction and no apparent ototoxicity using AdVs. Staecker et al. (2007) observed functional HC regeneration following Math1 gene transfer using AdVs. However inconsistent results have been reported, especially in terms of the tropism of AdVs for HCs; for example, Holt et al. (1999) reported successful Ad-GFP transduction in HCs but in contrast, Shou et al. (2003) found that Ad-GFP preferentially transduced supporting cells, but rarely if ever HCs.

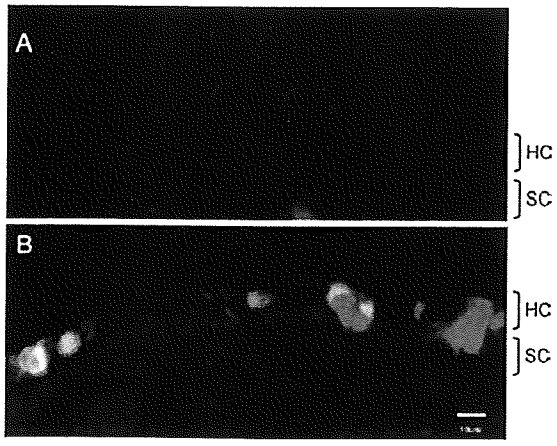

Fig. 6. Effect of time of TSA administration on AdV transduction in vestibular maculae. (A) TSA administration before Ad-GFP: Only a few cells are transduced in the epithelial region. (B) TSA administration after Ad-GFP: Many more GFP-positive cells are present (Green: Ad-GFP; Red: Myosin 7A; Blue: DAPI).

Our present study is consistent with the latter finding. Very few HCs were transduced in the absence of TSA, while moderate numbers of supporting cells showed reporter gene expression. AdV transduction efficiency can also be dependent upon age (Umegaki et al., 2003), and it is possible that the effects of TSA might also change with the developmental stage. In preliminary experiments for this study, we found that in vitro Ad-GFP transduction of inner ear sensory epithelia was more efficient at P1 that at P3–P5 (data not shown). It will be of interest to evaluate the effects of TSA on AdV transduction at both earlier and later stages of development than used for the present study.

Treatment with TSA significantly enhanced Ad-GFP gene expression in supporting cells. In addition, to our surprise, TSA induced transgene expression in many HCs as well. Also, in the cochlea, TSA enhanced gene expression to a much greater extent in inner HCs than in outer HCs. Little is known about differences in the TSA sensitivity of cells, but it appears that HCs are much more sensitive to TSA than supporting cells and that inner HCs are more sensitive than outer HCs. The differential sensitivity does not appear to be related to differences in histone aceytlation between the various cell types, since we observed similar enhancement of histone acetylation in supporting cells and HCs (Fig. 8), and since Chen et al. (2009) recently observed that treatment of neonatal mouse organ of Corti with TSA induced robust acetylation of histones in outer HCs as well as inner HCs.

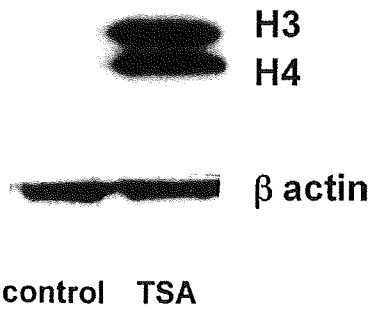

Fig. 7. Acetylation of histones in vestibular sensory epithelia was analyzed by Western blotting using anti-acetyllysine antibody. H3 and H4 indicate the position of histone H3 and H4, respectively. These data suggest that, in the tissue treated with TSA, histones are highly acetylated.

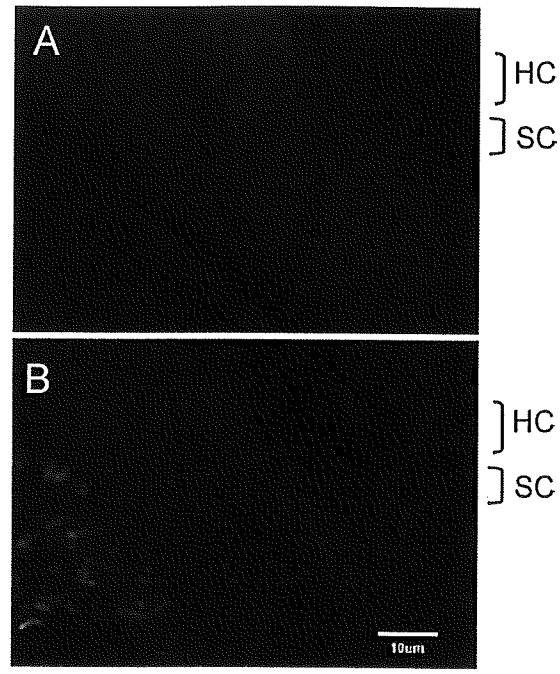

Fig. 8. The acetylation of histones in tissues was analyzed by staining with anti-acetylated Histone 3. (A) an untreated explant: There are a few acetylated nuclei in the epithelial layer. (B) TSA-treated explant: almost all cells show acetylated histones in their nuclei.

The mechanism by which HDAC inhibitors influence Ad-GFP transduction is not clear. Some authors suggest that transduction efficacy is increased via the up-regulation of the coxsackie and adenovirus receptor (CAR) on target cells (Kitazono et al., 2001a). However in our experiment, treatment with TSA prior to Ad-GFP transduction had no effect on AdV-mediated gene expression, suggesting that the augmentative effect of TSA is not likely to involve an increase in Ad-GFP uptake, which could be mediated by CAR.

Alternatively, as noted above, acetylation of histones is known to play an important role in the regulation of gene transcription in eukaryotic cells. Acetylation and deacetylation are catalyzed by specific enzyme families, histone acetyltransferases (HATs) and deacetylases (HDACs) and hyperacetylation of core histones in the nucleus is associated with enhanced gene expression. Much less is known about the role of histones in Ad-GFP gene transcription. The present study demonstrated that TSA caused both hyperacetylation of core histones and enhanced AdV-mediated transgene expression in inner ear cells. However,

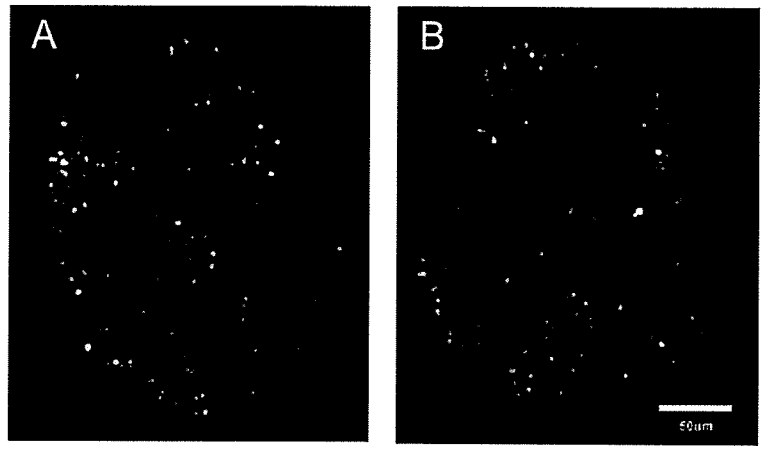

Fig. 9. Lack of effect of TSA on endogenous gene expression in the vestibular maculae of transgenic mice expressing GFP in HCs. (A) Untreated macula. (B) TSA-treated macula. There is no difference in gene expression between the two conditions.

the adenoviral genome is not integrated into the host genome and may not use histones of host origin, although adenovirus has its own histone-like proteins (Lischwe and Sung, 1977; Sung et al., 1977), which are known to be acetylated (Fedor and Daniell, 1980). While a direct causal relationship between enhanced gene expression and hyperacetylation of Ad-GFP histone-like proteins was not demonstrated, our study suggests that acetylation may be important for the regulation of Ad-GFP insert transcription. This could occur in a manner analogous to the effects of core histone acetylation on host cell gene expression. However, as noted above cells with similar levels of histone acetylation showed different levels of transduction enhancement. Moreover, it should be noted that there are many other cellular and nuclear proteins whose acetylation can be enhanced by TSA, including a number whose acetylation state influences gene transcription. This includes both specific and general transcription factors (Das and Kundu, 2005).

It is also not clear why HCs should be more sensitive to TSA than other inner ear cells. However, Gaetano et al. (2000) reported that TSA ehancement of AdV-mediated gene expression is particularly strong in cells bearing retinoic acid receptor (RAR) and retinoid X receptor (RXR) complexes. The CMV promoter used in Ad-GFPs contains repeated RAR elements, and these authors concluded that RA may co-operate with TSA on the CMV promoter, with TSA enhancing access to RAR elements by RA. RARs and RXRs are known to be present in the developing sensory epithelia of the inner ear (Romand et al., 1998). Moreover, HCs have been found to be particularly sensitive to the effects of RA (Kelley et al., 1993; Raz and Kelley, 1999).

While AdV-transduced expression of GFP, as well as of LacZ, was amplified by TSA treatment, endogenous GFP expression in the HCs of a transgenic mouse was not affected. This is perhaps not surprising, since GFP is expressed at high levels in the HCs of these mice, and thus the regulatory and transcriptional DNA of active genes is presumably highly acetylated (Verdone et al., 2006; Heintzman et al., 2007). While TSA has been found to regulate some endogenous genes (Dombrowsky and Uhlig, 2007), the effects of HDAC inhibition vary depending

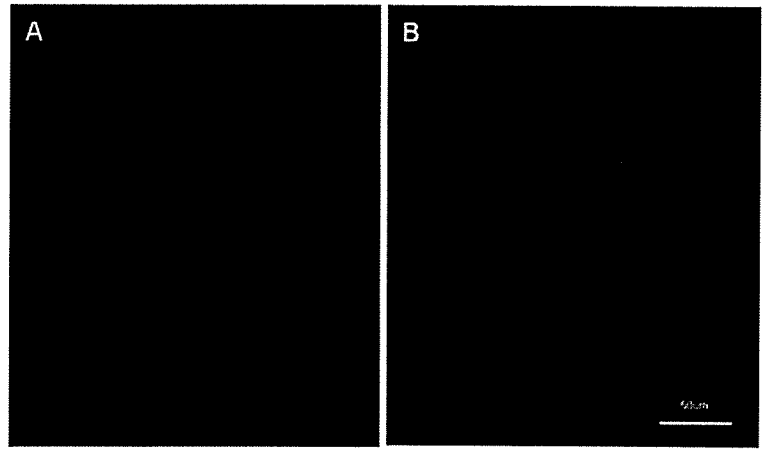

Fig. 10. Effects of TSA on cell viability in the vestibular macula. (A) Ad-GFP alone: While some cells are labeled with PI, the number is small. (B) Ad-GFP plus TSA-treated explant: the number of PI-positive cells was not significantly different from (P• 0.3, n• 7).

upon the gene. In fact, Van Lint et al. reported that the expression of only approximately 2% of cellular genes is affected by histone hyperacetylation due to HDIs (Van Lint et al., 1996). This may be because epigenetic downregulation of eukaryotic genes is more likely to be caused by histone or DNA methylation than by hypoacetylation (Zhang and Reinberg, 2001).

Whatever the mechanism by which TSA enhances Ad-GFP transduction, this effect is not restricted to Ad-GFPs. Similar effects of TSA have been reported in other viral vectors and even nonviral transfection methods such as lipofectamine [our data not shown; Tobias et al., 2000; Kitazono et al., 2001b; Yamano et al., 2000].

CONCLUSION

The present study demonstrates the potential utility of HDAC inhibitors for overcoming limitations of AdV-mediated gene therapy targeting HCs of the inner ear.

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論　　説

耳鼻咽喉科手術トレーニング

伊　藤　壽　一

Surgical Training in Otorhinolaryngology

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It is very difficult to master surgical skills in Otorhinolaryngology because of the anatomical complexixy of the field. There are several ways to gain surgical skills before being involved in surgery on humans, especially for young surgeons.

This paper introduces the concept of surgical dissection in the field of nasal surgery using a human cadaver and points out certain problems with using human cadavers. Future surgical training systems such as computer-simulated surgical training system are also described.

Key words : surgical training, nasal surgery, cadaver, computer-simulated surgical training system

はじめに

外科手術手技の習得には分野を問わず困難と時間を伴うものである．耳鼻咽喉科の分野でも事情は同じで，手技によって異なるが数年から10年程かけて一つの手技を習得することになる．このことは以前に本誌にて「耳科トレーニング」について述べた[1]．耳科手術は耳鼻咽喉科・頭頸部外科学領域の手術の中で習得に非常に時間がかかり，また困難なものである．従来，耳科手術の習得には熟練した術者の助手を務めたり手術ビデオを見たりして徐々にその技術を向上させるという方法が取られてきた．耳科手術など，顕微鏡を使用する手術と他の手術との決定的な違いは，手術は術者一人に委ねられるということであり，モニターを使っても上級者が同じ術野で直接指導するのが困難な点にある．顕微鏡下での手術と同様，内視鏡下の手術も手術が術者一人に委ねられるという点では同様に指導・習熟に困難をきたす．耳鼻咽喉科領域では主に鼻副鼻腔手術に対し内視鏡を用いる．内視鏡下鼻副鼻腔手術（Endoscopic Sinus Surgery：以下 ESS と略す）は耳科手術に比べ比較的経験の浅い医師が手術を始めることが多いが，また手術に伴う事故の多いことも事実である．これはトレーニングもそこそこに実際の手術を行ってしまうことが多かったことにもよると思われる．内視鏡下の手術でも何らかの手術トレーニングを行ってから実際の手術を始めることが必須であるが，顕微鏡下の耳科手術同様これまでこのようなトレーニングを行っている施設はわが国では非常に限られた施設のみであった．本稿では京都大学耳鼻咽喉科・頭頸部外科学のヒト cadaver を用いた ESS 手術実習プログラムを紹介するとともに，耳科手術トレーニングもあわせ，これからの耳鼻咽喉科手術トレーニングシステムのありかたについて言及したい．

鼻・副鼻腔手術実習マニュアル

鼻・副鼻腔手術実習を行うためには側頭骨手術実習[2]の場合と同様，優れた実習マニュアルが不可欠である．欧米では各施設で優れた鼻・副鼻腔手術マニュアルを作

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表1　鼻・副鼻腔手術講習会マニュアル

1) 準備
　実習室の機器の点検
　手術器具の点検
　Cadaverの状態の確認
2) CT読影と基本構造の観察
　3DCTでの読影
　1. 鼻前頭洞管とAgger Nasi Cell鈎状突起の位置関係
　2. 中鼻甲介水平部，上鼻甲介の同定
　3. 前篩骨動脈，後篩骨動脈の位置関係
　4. 視神経管，翼口蓋管の位置
　5. 基本構造の確認
　①下鼻甲介
　②鈎状突起（天蓋，眼窩内側壁の付着部，上顎自然口の探索）
　③中鼻甲介（篩骨洞ブラ）
　④上鼻甲介
　⑤蝶形骨洞自然口
3) 鼻中隔矯正術
4) 前部篩骨洞開放
5) 後部篩骨洞，蝶形骨洞開放
6) 後鼻神経切断術
7) 下垂体，頸動脈
8) 斜台部
9) 前頭洞の解放，Modified Lothrop
10) Medial Maxillectomy
11) 前頭蓋底開放

成しそれによって実習を行っている．実習書は図を多用し，よく工夫されているが，側頭骨実習マニュアルに比べその数はそれ程多くはない．頭蓋内部の記載がやや不十分なものや必ずしも実際の手術を想定していないものなども多い．われわれは独自の鼻・副鼻腔手術実習マニュアルを作成しそれに沿って実習を行っている[3]．表1にわれわれの作成した実習書を紹介する．

鼻・副鼻腔手術実習の問題点

鼻副鼻腔手術実習の必要性はいうまでもないが，実習室の整備には当然費用がかかる．われわれは側頭骨実習の際と同様の実習室を使用しているが，使用する内視鏡機器を整備するには側頭骨実習に比べさらに費用を要する．また，機器の多様化のため実習室のみで収容できる人数に限りがある．

しかし，もっとも大きな問題点はヒトcadaverの入手の問題である．ヒト側頭骨（cadaver）に比べ鼻・副鼻腔手術実習で使用するcadaverはwhole head cadaverであり，またその使用目的のため，一般の学生実習で用いられるようなホルマリン固定ではなく，凍結のものを用いることが多い．このためcadaverの入手だけでなく保存にもかなりの設備とスペースが必要となる．Cadaverの入手に関しては限られた数であれば各大学の解剖学教室，病理学教室の協力のもとに，ホルマリン固定のcadaverを使用する方法があり，実際このような方法で実習を行う場合もある．しかし，それでは実際必要とする数に対して大きく不足する．京都大学では海外から輸入したヒトwhole head cadaverを使用する場合が多いが，かなり費用がかかる（1個のwhole head cadaverで1000ドル位必要な場合もある）．さらに輸入元の米国でも多くの施設でヒトcadaverを使用するため，絶対数が不足する傾向にある．

一方，このような実習に対する倫理的，法的問題は解決されていない．学生の解剖学実習で使用する屍体は以前は身元不明者，また有志からの献体であったが，いずれにせよ学生実習のためのものであり，法的に定められた実習室で行うことが義務付けられている．屍体をその一部であれ，解剖学実習室以外で，手術実習のため行うことに対する法的正当性は認められていないのが現状である．またわれわれの行っているような輸入cadaverを使用することに対しては，その是非に関しての規定もあいまいな状態である．

耳鼻咽喉科以外の外科系領域でも手術トレーニングの必要性が論議されている．脳神経外科領域では耳鼻咽喉科の側頭骨実習と同様にcadaverを使用した実習を行っている施設もある．また一般外科でも昨今の医療事故の問題からもcadaverを使用しての実習の必要性が論議されている．いずれの分野においてもcadaverを使用する限り，耳鼻咽喉科領域での実習と同様の問題，とくにcadaverの入手問題と法的問題は解決されていない．最近では外科系全体がまとまってこれらの問題につき討議し，解決への道を模索しようとする動きがある．いずれにせよこのような手術手技トレーニングが必須であることは議論の余地がないので法的整備も早急にする必要がある．

今後の方向性

側頭骨実習の際にも述べたが，現在行われているようなcadaverを用いての実習は，費用の問題，cadaver入手が困難な点，倫理的な問題もあろうが，手術のスキルアッ

プ，医療事故などを避けるためにも必須であり，少数の施設だけでなくできるだけ多くの施設で行うべきであろうと思われる．

一方 cadaver を使用せずに手術手技トレーニングを行う機器なども開発されている．側頭骨実習の場合は3次元 CT を元にして，合成樹脂などで実際の側頭骨と解剖学的にもまた骨削開用ドリルでの削開感触もかなり類似したものが作成でき，それによって実習を行うことが可能となっている．本トレーニング素材は非常に有用であるが，血管，神経などの軟部組織を構築できないという欠点も有する．また最近では手術術野をコンピューターで再現し，バーチャル手術トレーニングを行うソフト，機器も開発されている．このようなソフトを使用すると解剖学的知識の習得には有用であるが，この機器の欠点は，実際の骨削開，軟部組織の操作などの感触を得ることができない．近い将来にはこのような機器を利用した手術トレーニングが cadaver を使用したトレーニングに代わっていくものと思われるが，現時点ではまだ cadaver を使用したものには遠く及ばない．

前回も述べたが，手術トレーニングに対する学会認定も必要となってくると思われる．

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3）中川隆之：鼻・副鼻腔手術講習会マニュアル．

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ORIGINAL ARTICLE

Subjective visual vertical in patients with ear surgery

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Abstract

Conclusion. Dysequilibrium is one of the most important side effects of ear surgery. The subjective visual vertical can be used as a good indicator for the evaluation of otolithic function in patients with ear surgery. *Objective.* To investigate the influence of various types of ear surgery on the otolithic organs. *Methods.* Seventy-one patients underwent ear surgery. Subjective visual vertical (SVV) test was performed before and after ear surgery. We investigated the directional changes of SVV before and after the ear surgery. *Results.* The postoperative SVV of two patients who underwent translabyrinthine removal of vestibular schwannoma shifted toward the operated side, but following other surgical procedures the SVV tended to shift toward the healthy side.

Keywords: *Utriculus, subjective visual horizontal, SVH, vestibular, tympanoplasty, otoliths*

Introduction

It is known that the perception of vertical not only depends on visual information but is also affected by the head position relative to the direction of gravity. Measurement of the subjective visual vertical (SVV) is used clinically as a method to assess the degree of otolithic dysfunction, primary vestibular nerves, and central graviceptive pathways [1]. Significant tilts of the SVV have been described in patients with peripheral vestibular disorders and patients who have undergone labyrinthectomy, as well as in patients with brainstem and cerebellar lesions [2–6]. Patients with various unilateral vestibular lesions, including vestibular neurectomy and labyrinthectomy, consistently tilt the SVV towards the side of the lesion [2–6].

Tribukait and Bergenius [7] reported that patients with stapedotomy showed significant deviations of the subjective visual horizontal (SVH) toward the healthy ear and it contrasts with the consistent deviation of the SVV toward the affected ear in acute vestibular lesions such as vestibular neurectomy and labyrinthectomy [2–6]. The goals of this study were to measure the SVV and to define the influence of the otolithic functions before and after the various types of ear surgery.

Material and methods

We investigated SVV in 71 patients with unilateral ear disease. They underwent various types of ear surgery in our hospital from 2006 to 2008. There were 38 women and 33 men; their mean age was 44 years, ranging from 5 to 81 years. The operated side was the right ear in 32 patients and the left in 39. All surgery was carried out under general anesthesia. Four surgeons performed ear operations on a total of 71 patients. Surgical procedures were as follows: tympanoplasty in 48 patients, cochlear implants in 11, stapes surgery in 4, exploratory tympanotomy in 3, translabyrinthine removal of vestibular schwannoma in 2, canal plugging in 1, partial removal of temporal bone in 1, and removal of external ear osteoma in 1 (Table I). Types of tympanoplasty were type I, 11 cases; type III, 26 cases; type IV, 8 cases; and first-stage operation, 3 cases. A surgical bur was used for all surgical procedures.

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Table I. Patients and surgical procedures.

Surgical procedure	*n*	Disease/condition	*n*
Tympanoplasty	48	Cholesteatoma	20
		Chronic otitis media	22
		Malformation of auditory ossicles	5
		Transaction of auditory ossicles	1
Cochlear implant	11	Deafness	11
Exploratory tympanotomy	4	Perilymphatic fistula	3
		Temporal bone fracture	1
Stapes surgery	3	Otosclerosis	2
		Foreign body in middle ear	1
Translabyrinthine removal of vestibular schwannoma	2	Acoustic tumor	2
Canal plugging	1	Benign paroxysmal positional vertigo	1
Partial removal of temporal bone	1	Middle ear tumor	1
Removal of external ear osteoma	1	External ear osteoma	1

The SVV was measured by means of a small rotatable luminous line in the upright body position in a completely darkened room. The patient was seated in front of the SVV device. After the luminous line was tilted automatically, the subject was asked to rotate the bar to the position they felt vertical using a hand controller. The SVV measurement was performed 10 times for each subject and its mean value was regarded as the measured value. In this study, we designated tilting of the SVV toward the operated side as negative, and tilting to the healthy side as positive. SVV was tested preoperatively and postoperatively to investigate any direction changes. In most patients, SVV was measured on postoperative day 1, but in a few patients who required bed rest it was tested a few days after the surgery.

Postoperative positional nystagmus was recorded with an infrared CCD camera. We measured SVV in 21 healthy volunteers, in whom the mean ± SD of SVV was 0.05 ± 0.73. Based on this result, the upper limit of the normal range was set as ±2.0° and tilts greater than −2.0° to +2.0° were determined to be pathologic [7].

We classified the patients into three groups by the deviation value of SVV as deviation toward the healthy side, deviation toward the operated side, and no deviation (SVV <2.0°). We designated the patient with preoperative pathologic SVV and postoperative normal SVV as deviation toward the healthy side and the patients with both preoperative and postoperative pathologic SVV as no deviation.

Results

Tympanoplasty (48 patients) (Figure 1)

Postoperative SVV tilted to the healthy side in 18 patients (37.5%), to the operated side in 3 (6.2%), and there was no deviation in 27 (56.3%) (Table II). There was significant change of SVV between the preoperative and postoperative values (p = 0.0003, Wilcoxon test). Postoperative positional nystagmus was observed in nine patients (18.8%); toward the operated side in seven and toward the healthy side in two (Table III). There were no obvious differences in the results of the SVV tilt according to the types of tympanoplasty.

Cochlear implant (11 patients) (Figure 2)

Postoperative SVV tilted to the healthy side in four patients (36.4%), to the operated side in one patient (9.1%), and there was no deviation in six patients (54.5%) (Table II). There was no significant difference in the SVV values. Postoperative nystagmus toward the operated side was observed in four patients and to the healthy side in one (Table III).

Exploratory tympanotomy (four patients) (Figure 3)

Postoperative SVV tilted to the healthy side in one patient (25.0%) and there was no deviation in three patients (75.0%) (Table II). Postoperative positional nystagmus toward the operated side was observed in two patients (Table III).

Stapes surgery (three patients) (Figure 3)

Postoperative SVV tilted to the healthy side in all patients. The postoperative SVV was significantly different from preoperative SVV (p = 0.0495, Mann-Whitney U test). Postoperative nystagmus toward the operated side was observed in two patients (Table III).

Translabyrinthine removal of vestibular schwannoma (two patients) (Figure 3)

Postoperatively SVV tilted to the lesion side and postoperative positional nystagmus toward the healthy side was observed in two patients (Tables II and III).

Other ear surgeries (Figure 3)

The postoperative SVV tilted to the healthy side in patients with canal plugging for benign paroxysmal

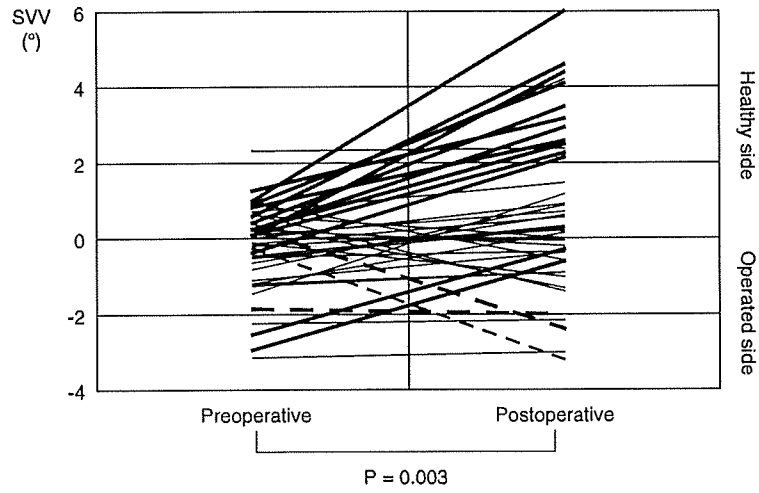

Figure 1. Preoperative and postoperative SVV changes following tympanoplasty in 48 patients. The solid thick line indicates the SVV shift to the healthy side, the solid thin line indicates no deviation, and the dotted line indicates the SVV shift to the operated side. Positive values in this figure represent the deviation toward the healthy side.

positional vertigo (BPPV). There were no pathologic SVV deviations in patients with removal of external ear osteoma and removal of the temporal bone. In the patient with BPPV, the positional nystagmus was directed toward the operated side in the 5 days postoperatively, but the direction of the nystagmus changed to the healthy side later. On the other hand, the postoperative SVV remained tilted to the healthy side. In the patients with removal of the temporal bone, the nystagmus was toward the healthy side. Preoperative and postoperative SVV results are summarized in Table II.

Discussion

The SVV test in upright body position is a simple and quick otoneurological test that provides information on the tonic afferent balance between the otolithic organs [8]. The SVV test result might indicate an otolithic disorder, in the same way that spontaneous nystagmus reflects the semicircular canal afferent balance [9]. Ear surgery has an influence not only on the otolith organs but also on the semicircular canals. In the patients with translabyrithine removal of vestibular schwannoma who had a spontaneous nystagmus, we speculate that semicircular canal and otolithic influences had affected the severe deviation of postoperative SVV. However, most patients in this study did not have spontaneous nystagmus, but had positional nystagmus or pathologic SVV shift. In these patients, we cannot completely rule out the influence of the semicircular canals, but we speculate that the otolithic condition mainly affected the postoperative

Table II. Preoperative and postoperative SVV ranges and postoperative direction of SVV tilting.

Surgical procedure	No. of patients	Range of preoperative SVV deviation (mean value)	Range of postoperative SVV deviation (mean value)	Postoperative SVV direction change		
				To the operated side	To the healthy side	No change
Tympanoplasty	48	–3.14° to 2.32° (–0.24°)	–3.22° to 6.01° (0.86°)	3 (6.2%)	18 (37.5%)	27 (56.3%)
Cochlear implant	11	–2.22° to 1.52° (0.25°)	–8.72° to 5.30° (0.03°)	1 (9.1%)	4 (36.4%)	6 (54.5%)
Exploratory tympanotomy	4	–3.58° to 3.11° (–1.21°)	–2.39° to 2.21° (0.06°)	0	1 (25%)	3 (75.0%)
Stapes surgery	3	0.10° to 1.33° (0.84°)	5.96° to 6.93° (6.32°)	0	3 (100%)	0
Translabyrinthine removal of vestibular schwannoma	2	–0.05° to 0.69°	–13.7° to –8.76°	2 (100%)	0	0
Canal plugging	1	2.38°	7.72°	0	1	0
Partial removal of temporal bone	1	–0.17°	–1.03°	0	0	1
Removal of external ear osteoma	1	0.27°	1.99°	0	0	1

Table III. Occurrence of postoperative nystagmus and pathologic SVV shift (greater than −2° to 2°).

Surgical procedure	Rate of positional nystagmus	Nystagmus		Deviation of SVV	
		Direction	*n*	Side	*n*
Tympanoplasty	9/48 (18.8%)	Operated side	7	Healthy side	3
				Operated side	2
				No deviation	2
		Healthy side	2	Healthy side	1
				No deviation	1
		No nystagmus	38		
Cochlear implant	5/11 (45.5%)	Operated side	4	Healthy side	2
				No deviation	2
		Healthy side	1	Operated side	1
		No nystagmus	6	Healthy side	2
				No deviation	4
Exploratory tympanotomy	2/4 (50%)	Healthy side	2	No deviation	2
		No nystagmus	2	Healthy side	1
				No deviation	1
Stapes surgery	2/3 (66.6%)	Operated side	2	Healthy side	2
		No nystagmus	1	Healthy side	1
Translabyrinthine removal of vestibular schwannoma	2/2 (100%)	Healthy side	2	Operated side	2
Canal plugging	1/1 (100%)	Operated side	1	Healthy side	1
Partial removal of temporal bone	1/1 (100%)	Healthy side	1	No deviation	1

deviations of SVV when absence of spontaneous nystagmus is considered.

It has been reported that vibration is an excitatory stimulus for semicircular canal and otolith afferents, and the net effect of an oscillating mechanical stimulus delivered to the hair bundle of a vestibular receptor cell is excitatory [10]. Vibration to the surface of the bony labyrinth using a conventional surgical drill dislodges the otoconia from the utricle [11] and it is suggested that the use of the surgical drill affects the otolithic organs. It was reported that the utricular nerve has monosynaptic and disynaptic connection with the abducens motoneurons and also has polysynaptic connections with the inferior oblique and trochlear motoneurons [12]. In the otolithic organ, the utricular nerve potentially affects SVV more than the saccule [13,14]. Polysynaptic connections between the utricular nerve and the inferior oblique and trochlear motoneurons seemed to play a role in eye rotation during head tilt [12], and hence we speculate that these nerves also contribute to the SVV deviations. In this study, we investigated the influence of ear surgery on otolithic function by measuring the SVV in the upright position. In previous studies, the significant tilting of the SVV to the lesion side had been described in patients with peripheral vestibular disorders including labyrinthectomy [1–6]. Postoperatively, SVV in an upright position tilted toward the operated ear in patients with vestibular neurectomy or cochleo-vestibular neurectomies [2–6]. These abnormalities in vertical perception are due to changes in the afferent gravioceptive pathways in the vestibular nerve. In this study, the SVV also tilted toward the operated side after translabyrithine removal of vestibular schwannoma. However, in other ear surgeries, the SVV tended to tilt toward the healthy side.

Tribukait and Bergenius [7] reported that in the acute stage after stapedotomy, subjective visual horizontal (SVH) in an upright position significantly tilted toward the healthy side, and these results indicate an increase in the resting activity of the utricular afferent nerve. In this study, SVV of all patients with stapes surgery tilted to the healthy side and postoperative nystagmus toward the operated side was observed in two patients. Stapes surgery opening the vestibule may have caused slight local labyrinthitis and irritation of the adjacent sensory structures, especially of the otolithic organs, leading to increase in the resting activity of the utricular afferent nerve.

Dizziness after cochlear implant surgery is a potential complication. Opening the labyrinth and inserting

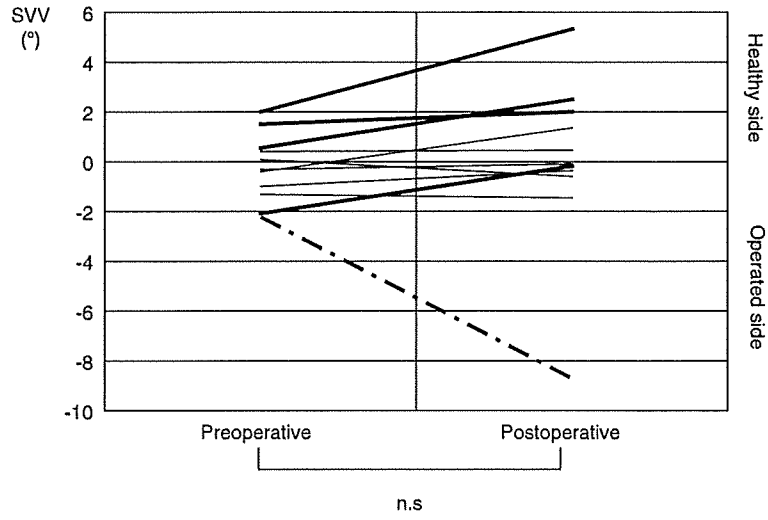

Figure 2. Preoperative and postoperative SVV changes following cochlear implant in 11 patients. The solid thick line indicates the SVV shift to the healthy side, the solid thin line indicates no deviation, and the dotted line indicates the SVV shift to the operated side. Positive values in this figure represent the deviation toward the healthy side.

the electrode after cochleostomy cause acute transient labyrinthitis. Temporal bone studies have shown that electrode insertion into the scala vestibule damages the osseous spiral lamina, basilar membrane, and vestibular receptors [15,16]. However, cochlear implant surgery does not always induce dizziness. In this study, the SVV tilted to the healthy side in four patients (36.4%), but it tilted to the operated side in one patient and postoperative positional nystagmus was observed in five patients. The mean values of postoperative SVV were smaller than those for stapes surgery and canal plugging. We assume that the cause of the small value change is due to the inner ear functions of the cochlear implant patients impaired preoperatively. The SVV of the other patient shifted markedly to the operated side and had a nystagmus toward the healthy side. The occurrence of severe inner ear damage was suspected in this case and the severity of vestibular function was speculated with the degree of SVV deviation.

In patients with a refractory posterior canal type BPPV, plugging of the posterior canal was performed using a transmastoid approach. Postoperatively the persistent positional nystagmus disappeared. The

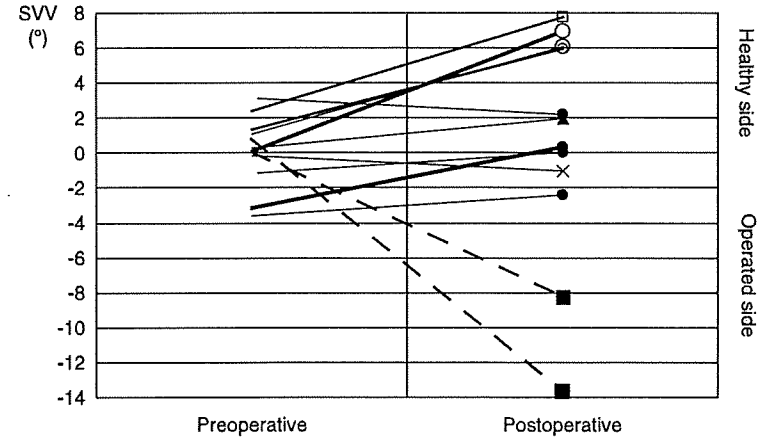

Figure 3. Preoperative and postoperative SVV changes with other surgeries in 12 patients. There was significant change of SVV between the preoperative and postoperative SVV in patients with stapes surgery. The solid thick line indicates the SVV shift to the healthy side, the solid thin line indicates no deviation, and the dotted line indicates the SVV shift to the operated side. Positive values in this figure represent the deviation toward the healthy side. ■, translabyrithine removal of vestibular schwannoma; ○, stapes surgery; ▲, removal of external ear osteoma; □, canal plugging; ●, exploratory tympanotomy; x, partial removal of temporal bone.

patient suffered a mild hearing loss temporarily but it soon improved. SVV tilted to the healthy side for 2 months after operation. Temporary hearing impairment was reported in previous studies [17,18] and it seemed to be due to an effect to the inner ear caused by the disrupted membranous labyrinth. Canal plugging slightly affects cochlear function, but also increases the resting activity of the utricular afferent. These results indicate that inner ear fenestration, such as stapes surgery, cochlear implant, and canal plugging activate the utricular afferents. However, other ear surgeries apart from inner ear fenestration also tend to move the SVV to the healthy side.

After tympanoplasty, the SVV of most patients shifted to the healthy side, as inner ear fenestration. In these cases the postoperative shift may be due to manipulation of the auditory ossicles or the ambient structures, or the effects of the surgical drill, or both. Not only tympanoplasty type III or IV requiring manipulation of stapes but also type I tympanoplasty cause the pathologic SVV. In patients undergoing tympanoplasty, postoperative SVV tilted to the operated side in three patients (6.2%), and their SVV ranged from –2.02° to –3.22°, two of them had postoperative nystagmus and had dizziness. The nystagmus direction was toward the operated side and no patient had any hearing impairment after surgery. It is suspected that the SVV results indicated the damage of the utriculus in these patients, but the damage did not include the cochlear function.

The patients with pathologic SVV did not always have postoperative nystagmus and the directions of the SVV tilt were not consistent with the direction of the nystagmus, and some of the patients with no nystagmus showed pathologic SVV deviation. These results indicate that the origin of the postoperative positional nystagmus and the postoperative SVV tilt are different.

In the patient with partial removal of temporal bone for external canal carcinoma, there was only a slight SVV deviation after the operation, probably because the vestibular organ was preserved. In this study, deviated postoperative SVV was normalized afterwards in almost all patients, including those who underwent translabyrithine removal of vestibular schwannoma. Ushio et al. [19] reported on long-lasting deviation of the SVH in patients with definite unilateral vestibular deafferentation and suggested the resection of the vestibular ganglion cells to be a contributory factor.

Disequilibrium is one of the most important complications following ear surgery. Postoperative nystagmus is an important indicator of disequilibrium. The SVV test in an upright body position can contribute to assessment of the otolithic dysfunction in a short time without complex equipment. Measurement of postoperative SVV seems to be an important clinical parameter to evaluate vestibular function such as observation of postoperative nystagmus.

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