The causative relationship between the presence of immune response and HC loss is not completely understood. Scala tympani application of adenovirus vectors into the guinea pig cochlea does not lead to transduction of HCs [Raphael et al., 1996; Stöver et al., 1999; Suzuki et al., 2000]. It is therefore likely that HC degeneration occurs as a secondary response to the presence of the virus in the inner ear. The hypothesis that HC loss is due to the immune response is strongly supported by our data that immune suppression with DEX reduces threshold shift and HC loss following the second inoculation of the adenovirus vector. It was suggested that the inflammatory response to CMV might be the primary cause for the hearing loss. Similarly, hearing loss due to CMV-induced labyrinthitis was also reduced by immune suppression [Darmstadt et al., 1990]. In addition, the primary and secondary inner ear immune response has been investigated using keyhole limpet hemocyanin [Harris, 1983; Woolf and Harris, 1986; Tomiyama and Harris, 1989; Ma et al., 2000]. A secondary immunization with keyhole limpet hemocyanin has been shown to cause severe hearing loss and histological changes [Woolf and Harris, 1986; Ma et al., 2000]. Taken together, our data demonstrate that the immune response elicited by the second application of a replication-deficient adenovirus vector is more damaging to the inner ear than the direct impact of the virus itself.

The influence of immunosuppressive agents on the outcomes of adenovirus gene therapy has been studied in other systems. Agents such as FK506, cyclosporin A and deoxyspergualin reduce inflammation and permit prolonged transgene expression. The mechanism of action of these agents is thought to involve prevention of neutralizing antibody formation [Vilquin et al., 1995; Smith et al., 1996; Yang et al., 1996a, b; Kaplan and Smith, 1997; Bouvet et al., 1998; Cichon and Strauss, 1998; Kuriyama et al., 1999]. Administration of monoclonal antibodies such as anti-CD40 ligand antibody and CTLA4Ig around the time of the first vector administration has also been shown to prevent the formation of neutralizing antibody against the adenovirus and permit successful readministration of the virus [Lei et al., 1996; Yang et al., 1996a, b; Scaria et al., 1997; Jooss et al., 1998].

The lower efficiency of transgene expression we observed following the second administration of the reporter gene vector without DEX treatment (group I), as compared with our routine findings after 1 inoculation, and with the pattern and extent of transgene expression in group II may also be related to the inflammatory response. Several reports have documented a decrease in transgene expression following readministration [Smith et al., 1993; Kozarsky et al., 1994; Yei et al., 1994]. The decrease in efficiency may be explained by the presence of neutralizing antibodies. Such antibodies have been detected in the sera of animals after a single administration [Kuriyama et al., 1999]. It is presently unclear whether the reduction in transgene expression following the second inoculation is due to the lower efficiency of transfection or impaired/reduced gene expression in transduced cells. We speculate that a large part of the vector is destroyed by the neutralizing antibody prior to being internalized in the target cells, as previously suggested [Kuriyama et al., 1999]. Kuriyama et al. have investigated reapplication of the adenovirus vector via the intraportal (systemic) route, which differs from the ear-specific administration in our study. Nevertheless, their data show that intraportal readministration of adenoviruses failed to induce detectable levels of transgene expression and that immunosuppressive therapy could improve transgene expression in their animals.

Glucocorticoids are widely used as immunosuppressive agents for the therapeutic management of various inner ear diseases such as Ménière's disease, sudden deafness and autoimmune-related hearing loss [Kanzaki, 1994]. The therapeutic impact of glucocorticoids in the inner ear is probably related to some of their known activities, including: (a) secretion of cytokines and prostaglandin; (b) expression of surface receptors for complement and immunoglobulins; (c) phagocytosis, and (d) antibacterial and antifungal activities [Kitajima et al., 1996]. Glucocorticoid function is mediated primarily by their action on monocytes/macrophages and T cells [Munck et al., 1984; Boumpas et al., 1991; Russo-Marie, 1992; Stam et al., 1993]. Glucocorticoids inhibit the secretion of IL-2, which is needed for T cell proliferation [Gillis et al., 1979; Boumpas et al., 1991]. We speculate that the mechanism of the reduction of the HC damage due to repeated adenovirus inoculation was via decreased inflammation and suppressed immune response of both the humoral and cellular types.

While the use of immunosuppression enhances the outcome of a second application of a replication-deficient adenovirus vector, it does pose other risks that should be considered as therapies are designed. Most notably, immune suppression exacerbates the outcome of infection with wild-type (replication-competent) viruses, as shown for CMV [Mayo et al., 1978] and adenovirus [Ardehali et al., 2001]. Therefore, future use of immunosuppression for enhancing gene therapy protocols will need to be carefully balanced between enhancing the therapeutic success of the replication-deficient vector and exposing the body to potentially lethal infections by wild-type viruses.

The data we present suggest that the use of viral vectors may be enhanced by the reduction of side effects and that immune suppression may help reduce the side effects associated with the readministration of the vector. Therefore, the use of DEX or other immunosuppressive agents may be beneficial in gene transfer experiments and in the development of clinically applicable gene therapy protocols.

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Cochlear damage caused by continuous and intermittent noise exposure

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Abstract

We compared the extent of permanent threshold shifts (PTS) and cochlear hair cell damage caused by continuous noise exposure with those caused by intermittent noise exposure. Twenty male pigmented guinea pigs that had been exposed to a one-octave band of noise at 4 kHz for 5 h were placed in four groups: exposure to 115 dB SPL continuous noise (group 1, $n=5$), 115 dB SPL intermittent noise (group 2, $n=5$), 125 dB SPL continuous noise (group 3, $n=5$), and 125 dB SPL intermittent noise (group 4, $n=5$). PTS at 2, 4, 8, and 16 kHz were assessed by means of auditory brainstem responses measured before noise exposure and 10 days after. The guinea pigs were killed 15 days after noise exposure, and the number of hair cells missing counted in surface preparations of the organs of Corti stained with rhodamine phalloidin. Groups 1 and 3 had significantly greater PTS ($P<0.05$) at all frequencies than intermittent groups 2 and 4. Group 3 had the greatest PTS at all the frequencies. Intermittent 125 dB noise total energy was greater than that of continuous 115 dB noise, but the latter elicited more PTS than the former. The extent of hair cell damage was comparable to the physiological findings. This indicates that continuous noise causes greater damage to the cochlea than intermittent noise of the same intensity and that, at the intensities tested, damage to the cochlea is not proportional to the total noise energy.
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Key words: Cochlea; Hair cell; Noise-induced hearing loss

1. Introduction

Most studies of noise-induced hearing loss in humans and animals involved continuous noise exposure. Sound stimulus in daily life, however, is generally intermittent, and employees in most industrial plants are exposed intermittently to noises of fluctuating intensities. In assessing the hazard associated with intense sound exposure, intermittent stimulation clearly deserves special attention.

Intermittent noise is reported to cause smaller temporary threshold shifts (TTS) and less hair cell damage compared to continuous noise of the same intensity, both in laboratory animals (Eldredge et al., 1959; Schmidek et al., 1975; Kryter et al., 1966; Saunders et al., 1977; Ward, 1970; Clark and Bohne, 1992; Fredelius and Wersall, 1992) and humans (Sataloff et al., 1969, 1983; Schmidek and Carpenter, 1974; Patuzzi, 1998). Although those studies compared the extent of TTS for various interrupted and even intermittent exposures, few attempts have been made to determine the effects of intermittence on noise-induced permanent threshold shifts (PTS) (e.g. Saunders et al., 1977).

Because most industrial exposures are not continuous but intermittent or fluctuating (Goodfriend, 1971), it is important to determine the effects of the interruption of exposure on threshold shifts and hair cell loss. Campo and Lataye (1992) and Clark and Bohne (1992) speculated that the small amount of hair cell damage caused by intermittent noise exposure could be related to the recovery periods between noise phases. Several studies have been published on threshold shifts produced after repeated exposure for periods longer than a few hours (Eldredge et al., 1959; Ward, 1970; Saunders et al., 1977) or a few days (Johnson et al., 1976; Clark et al., 1987), but few have addressed the issue of threshold shifts produced after repeated exposure with short breaks of a few seconds. It is necessary to determine

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whether a short interval in pulsating exposure is sufficient to initiate the recovery process.

The equal-energy hypothesis, initially proposed by Eldred et al. (1955), assumes that hearing damage is a function of the total acoustic energy received. It states that the hearing organ reacts in a uniform manner to sounds of various intensities and durations provided that the total sound energy over the measurement period remains constant. Animal studies, in which the applicability of this principle was investigated, have questioned its generality and validity. It seems to be applicable at low intensities, but perhaps not at high ones. For a single uninterrupted exposure of approximately equal energy to chinchillas, Ward et al. (1981) found that the total-energy principle holds until the SPL involved exceeds the critical level of 112 dB. The chinchilla is considerably more sensitive to noise damage than the guinea pig, by 15–25 dB (Drescher and Eldredge, 1974), apart from the consideration of differential conduction of sound to the cochlea (Mitchell, 1976). A 115 dB SPL exposure in guinea pigs therefore is similar to a 99 dB SPL noise exposure in chinchillas (Drescher and Eldredge, 1974) and would be below the critical level. These findings indicate that the critical intensity for the guinea pig is above 125 dB SPL.

To investigate the effect of intermittence on PTS and the applicability of the equal-energy principle, we compared PTS and hair cell loss by exposing guinea pigs to continuous or intermittent noise at two levels, 115 and 125 dB SPL. We speculated that the extent of damage that occurred across these exposure parameters would be consistent with the equal-energy principle, regardless of the type of exposure.

2. Materials and methods

2.1. Animals

Twenty male pigmented guinea pigs (250–350 g) with normal Preyer's reflex were tested. Animals were assigned to one of four groups: group 1 ($n = 5$, 5 ears) was exposed to continuous 115 dB SPL noise, group 2 ($n = 5$, 5 ears) to intermittent 115 dB SPL noise, and groups 3 and 4 ($n = 5$, 5 ears each), respectively, to continuous and intermittent 125 dB SPL noise. This study was reviewed by the Committee for Ethics in Animal Experiments of the University of Tokyo and was carried out under Japanese law and the guidelines for Animal Experiments at the University of Tokyo.

2.2. Experimental protocol

To avoid the potential protective effects of conditioning, guinea pigs were kept in a quiet room after arrival. On day 3, normal hearing was confirmed by auditory brainstem response (ABR) threshold measurements at 2, 4, 8, and 16 kHz. Two days later (day 5), the animals underwent a 5 h noise exposure (4 kHz octave band noise). Ten days after that exposure, ABR thresholds were again obtained. Five days later (15 days post-exposure) the animals were killed under deep anesthesia and their temporal bones prepared for histological analysis of organ of Corti damage.

2.3. Noise exposure

All exposures were carried out in a single-walled, sound-deadened chamber, with two wire-mesh cages centered in the sound field. One animal per cage was exposed and the guinea pigs were allowed to move freely during exposure. The sound chamber was fitted with speakers driven by a noise generator and power amplifier. A 0.5 inch Bruel and Kjaer condenser microphone and Fast Fourier Transform analyzer were used to calibrate and measure sound levels at multiple locations within the sound chamber to ensure stimulus uniformity of ±1 dB within the exposure area.

All the subjects were exposed for 5 h to an octave band of noise (OBN) centered at 4 kHz. Noise was presented continuously or intermittently. Intermittent noise was presented at two pulses per second on 200 ms plateau, with a rise–fall time of 25 ms and 250 ms interval. Duration of a brief tone is the time interval between the half-maximum amplitude points on the rising and falling portions of the brief tone envelope, therefore, the duty cycle of the intermittent tone used is 45% (Fig. 1).

The ratio of the power of the intermittent tone used versus the continuous tone is calculated as follows:

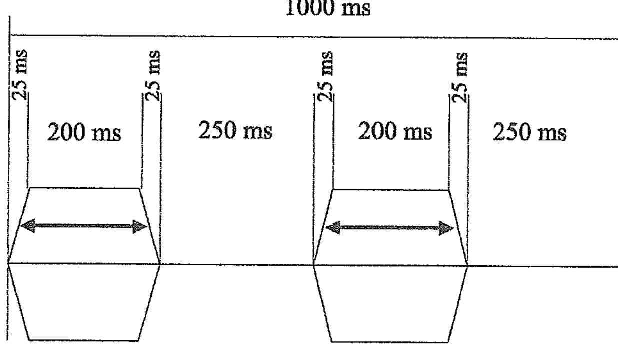

Fig. 1. Parameters of the intermittent noise used. The noise was presented at two pulses per second on a 45% duty cycle, with a rise–fall time of 25 ms. The duration of a brief tone is the time interval between the half-maximum amplitude points on the rising and falling portions of the brief tone envelope (arrow). Therefore, the duty cycle of the intermittent tone used is 225/500 = 45%.

$$
\begin{aligned}
&1/t_1+t_2+t_3+t_4\left(\int_0^{t_1}[t/t_1]^2\mathrm{d}t+t^2+\int_0^{t_3}[1-t/t_3]^2\mathrm{d}t\right)\\
&=1/t_1+t_2+t_3+t_4(1/t_1^2\,[t^3/3]_0^{t_1}+t_2+\\
&\quad[t]_0^{t_3}-2/t_3[t^2/2]_0^{t_3}+1/t_3^2\,[t^3/3]_0^{t_3})\\
&=1/t_1+t_2+t_3+t_4(t_1/3+t_2+t_3/3)\\
&=0.4333
\end{aligned}
$$

(t_1 = rise time, t_2 = plateau time, t_3 = fall time, and t_4 = interval time).

The ratio of the power between 125 dB SPL and 115 dB SPL is 10. Therefore, the ratio of the power of noises used is 125 dB cont.:125 dB int.:115 dB cont.: 115 dB int. = 10:4.333:1:0.4333.

2.4. ABR measurement

Hearing thresholds in the left ears of all the animals were evaluated by ABR 2 days before noise exposure and 10 days after it. Threshold shifts were determined by subtracting the hearing threshold after noise exposure at each frequency from values obtained before exposure. The guinea pigs were anesthetized with a mixture of xylazine (10 mg/kg, i.m.) and ketamine (40 mg/kg, i.m.). Differential needle electrodes were placed subcutaneously below the test ear (reference) and at the vertex (active). A ground electrode was positioned below the contralateral ear. The sound stimulus consisted of a 15 ms tone burst, with a rise–fall time of 1 ms at frequencies of 2, 4, 8, and 16 kHz. The intensity was varied in 5 dB steps. Hearing thresholds were defined by visual interpolation between the lowest intensity producing a definite, repeatable response and an intensity 5 dB less, at which no ABR response was elicited. Animals with baseline thresholds greater than one standard deviation beyond the normative lab baseline were excluded from the study.

2.5. Histological examination

2.5.1. Preparation technique

The guinea pigs were killed 15 days after noise exposure (5 days after the last ABR measurement) by decapitation under deep anesthesia with a mixture of xylazine and ketamine. Temporal bones were removed immediately, the round and oval windows exposed and the perilymphatic spaces perfused with 2% paraformaldehyde in 0.1 M phosphate buffer at pH 7.4 for 1 h, then washed in 0.1 M phosphate buffer. The bony shell of the cochlea was removed, and the specimens were soaked in 0.3% Triton X-100 for 5 min to make the hair cell membranes permeable, and then whole mounts of the organ of Corti were stained for actin for 60 min with rhodamine phalloidin, to outline hair cells and their stereocilia for a quantitive assessment (Raphael and Altschuler, 1991). After staining, tectorial membrane was removed and the osseous spiral lamina partially dissected away, and individual turns of the organ of Corti mounted on glass slides.

2.5.2. Analysis of the cochlea

Slides were observed under a fluorescence microscope at a magnification of 40×. From the area next to the apex (0.45 mm) to the base (18 mm), cells in each of four rows were counted and each cell was evaluated as present or missing in 0.45 mm reticule. Cells with an identifiable cell body and cuticular plate were considered remaining cells. Distinctive scar formation produced by convergence of adjacent phalangeal processes was regarded as a sign of a missing hair cell (Engström et al., 1966; Bohne, 1976; Hawkins, 1976), although formation of a scar may not preclude the presence of a hair cell buried beneath the scar (Sobkowicz et al., 1996). The average total number of outer hair cells (OHC) and inner hair cells (IHC) counted in the reticules with scattered hair cell loss was considered the bin width. When hair cells were concentrated in a reticule, missing hair cells were counted based on the previous OHC and IHC counts. The percentages of missing hair cells were determined by counting the number of missing cells and dividing this figure by the total number of cells (present and missing) for each animal at each 0.45 mm length of tissue, and the average for each reticule plotted along the cochlea, producing an average cytocochleogram.

2.6. Statistical analysis

Sigma stat statistical software was used. Threshold shifts after noise exposure at each frequency, as well as the percentages of missing OHC and IHC, were compared among groups 1, 2, 3, and 4 by a one-way analysis of variance (ANOVA). When significant differences were found, they were compared with each other by means of the Student–Newman–Keuls test. $P<0.05$ was considered statistically significant.

3. Results

3.1. Hearing loss induced by continuous and intermittent noise exposure

Hearing thresholds before noise exposure were essentially equivalent in all the ears. There were no significant differences at any frequency across groups (Fig. 2). The exact values for the pre- and post-noise exposure hearing threshold are also shown in Table 1. Fig. 3 shows the threshold shifts after noise exposure. Animals

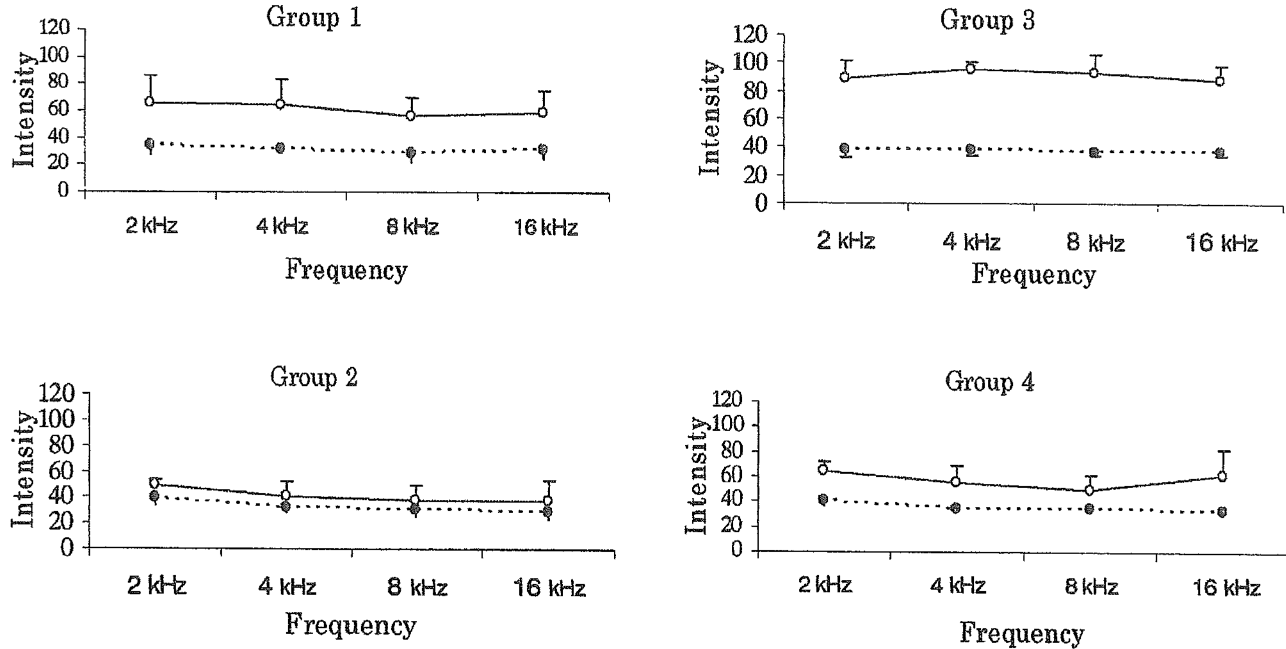

Fig. 2. Hearing threshold (mean ± S.D.) tested pre-noise (dotted line) and post-noise (solid line) exposure for each group.

that received 5 h of continuous 115 dB SPL noise exposure (group 1) had approximately 32 dB SPL threshold elevations at 2 and 4 kHz, and 28 dB SPL at 8 and 16 kHz. Those animals exposed to 5 h intermittent 115 dB SPL noise (group 2) had the smallest threshold shifts: 10 dB at 2 and 4 kHz, 6 dB at 8 kHz, and 9 dB at 16 kHz. Average threshold shifts in those exposed to 125 dB SPL continuous noise (group 3) were the highest among the groups: approximately 51 dB, 57 dB, 55 dB and 50 dB at 2, 4, 8, and 16 kHz, respectively. Animals exposed to 125 dB SPL intermittent noise (group 4) had threshold shifts of approximately 25, 22, 16, and 28 dB at 2, 4, 8, and 16 kHz, respectively. Animals in group 1 had significantly ($P < 0.05$) greater threshold shifts at all the frequencies as compared to those in group 2. Similarly, when compared to animals in group 3, threshold shifts in group 4 were significantly ($P < 0.05$) smaller at all the frequencies. At the two intensities tested, continuous noise exposure caused greater threshold shifts than intermittent noise.

The total energy of the intermittent 125 dB SPL noise was 4.333 times greater than that of the continuous 115 dB SPL noise. When compared to group 4, however, group 1 guinea pigs had significantly greater threshold shifts ($P < 0.05$) at 4 and 8 kHz.

3.2. *Histological findings*

Fig. 4 shows the average percentages of missing OHC and IHC throughout the cochlea for all groups. In all four groups, row 1 OHC showed the most damage, whereas IHC were virtually undamaged. Intermittent 115 dB SPL noise (group 2) caused the least OHC damage, whereas the greatest OHC loss occurred in

Table 1
Pre- and post-noise hearing threshold (mean ± S.D.) across frequencies, shown in Fig. 2

	2 kHz	4 kHz	8 kHz	16 kHz
Pre-noise				
Continuous 115 dB ($n = 5$)	34.0 ± 6.5	32.0 ± 2.7	28.0 ± 6.7	31.0 ± 6.5
Intermittent 115 dB ($n = 5$)	39.0 ± 5.5	32.0 ± 4.5	31.0 ± 6.5	29.0 ± 6.5
Continuous 125 dB ($n = 5$)	38.0 ± 5.7	38.0 ± 4.5	37.0 ± 2.7	37.0 ± 2.7
Intermittent 125 dB ($n = 5$)	40.0 ± 3.5	34.0 ± 2.2	34.0 ± 2.2	33.0 ± 2.7
Post-noise				
Continuous 115 dB ($n = 5$)	66.0 ± 19.5	61.0 ± 19.2	52.0 ± 13.5	51.0 ± 16.3
Intermittent 115 dB ($n = 5$)	45.8 ± 4.9	44.2 ± 10.7	43.3 ± 11.2	42.5 ± 15.0
Continuous 125 dB ($n = 5$)	89.2 ± 12.4	95.0 ± 5.5	83.3 ± 13.7	79.1 ± 10.7
Intermittent 125 dB ($n = 5$)	67.0 ± 7.6	56.0 ± 12.9	51.0 ± 11.9	61.0 ± 22.2

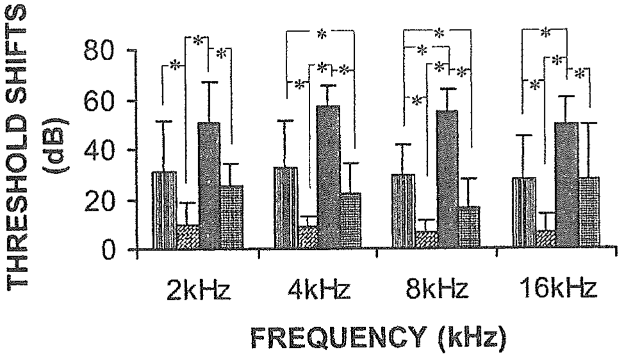

Fig. 3. ABR threshold shifts (mean ± S.D.) for each frequency 10 days after noise exposure. Vertically striped bars: group 1 (continuous 115 dB SPL, $n = 5$); diagonal-brick bars: group 2 (intermittent 115 dB SPL, $n = 5$); dark gray bars: group 3 (continuous 125 dB SPL, $n = 5$); square-filled bars: group 4 (intermittent 125 dB SPL, $n = 5$). Guinea pigs in group 1 have significantly ($P < 0.05$) greater threshold shifts at all frequencies as compared with those in group 2. Similarly, as compared with group 3, at all frequencies threshold shifts are significantly ($P < 0.05$) smaller in group 4. Asterisks indicate significant differences ($P < 0.05$).

animals exposed to continuous 125 dB SPL noise (group 3). These findings are consistent with the magnitude of the threshold shifts.

OHC loss in rows 1, 2, and 3 differed significantly among the groups, but there were no significant differences in IHC loss. Group 1 had significantly ($P < 0.05$) greater row 1 OHC loss as compared to group 2. Moreover, OHC in rows 2 and 3 were more severely damaged in group 1 than in group 2 ($P < 0.05$). Similarly, group 3 had significantly ($P < 0.05$) greater loss of row 1 OHC as compared with group 4. OHC in rows 2 and 3 of group 3 also were more severely damaged than those in group 4 ($P < 0.05$). A comparison of the extent of hair cell loss in groups 1 and 4 showed that group 1 had significantly ($P < 0.05$) greater loss of row 1 OHC than did group 4. There were no significant differences in OHC loss between rows 2 and 3.

At the highest intensity studied, the area of total OHC damage is positioned appropriately for the spectral characteristics of the noise exposure used. The greatest loss of OHC occurred in the lower second

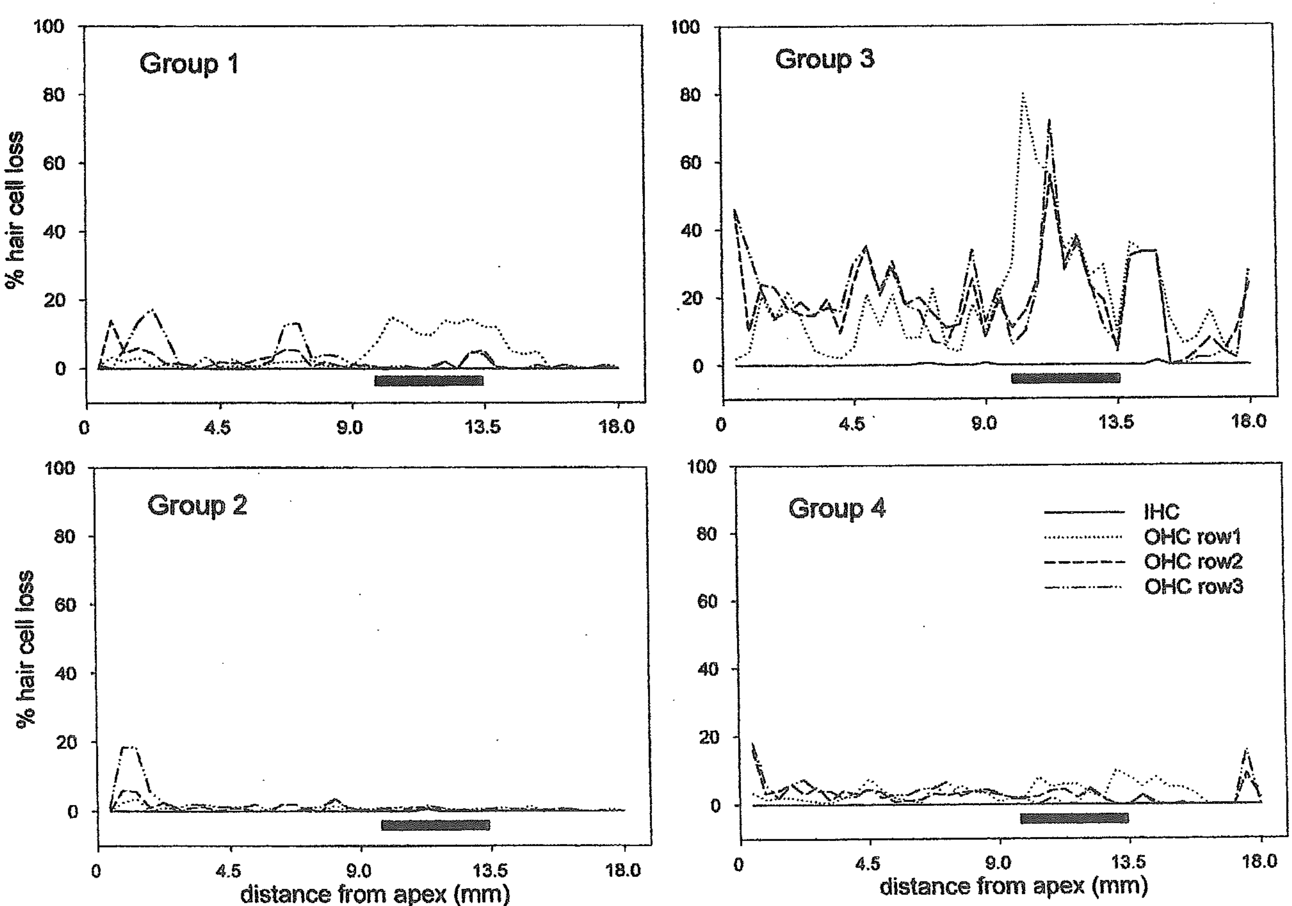

Fig. 4. Average cytocochleogram for each group. The main lesion caused by 4 kHz OBN is located 11.4 mm or more from the apex, at which point row 1 OHC are most damaged. Group 3 shows the most severe hair cell damage, then groups 1, 4, and 2 in that order. Bar indicates 9.69–13.5 mm from the apex.

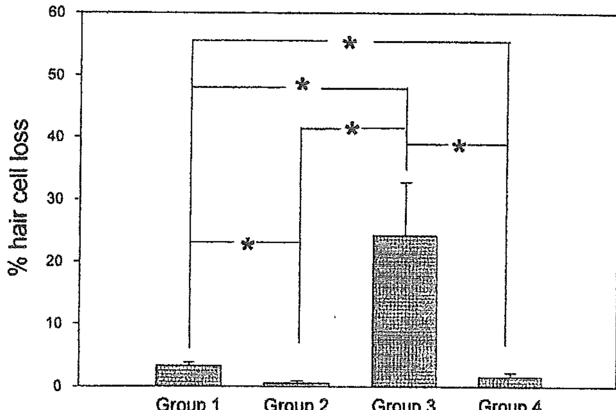

Fig. 5. Means and S.D.s of row 1 OHC loss 11.4 mm or more from the apex. Asterisks indicate significant differences ($P < 0.05$). The percentage of missing cells is larger in group 1 than in group 2, and in group 3 than in group 4.

and basal turns, 11.4 mm or more from the apex. Because this region was expected to be the most affected by the noise exposure used, and the row 1 of hair cells was the most damaged, we compared the extent of row 1 OHC loss in this area across the groups (Fig. 5). The percentage of missing row 1 OHC was 4.2 ± 1.2 in group 1, which was significantly ($P < 0.05$) higher compared to group 2 (0.3 ± 0.5). Similarly, the percentage of row 1 OHC in group 3 was 24.3 ± 7.9, which was significantly higher ($P < 0.05$) compared to group 4 (2.1 ± 1.8).

4. Discussion

We found intermittent noise exposure produced significantly smaller PTS and less hair cell loss than in guinea pigs exposed to continuous noise. Although the total energy of the intermittent 125 dB SPL noise was approximately four times that of the continuous 115 dB SPL noise, the former produced smaller PTS and less hair cell loss. These findings indicate that the introduction of even very brief silent intervals during noise exposure may reduce cochlear damage caused by such exposure and that damage to the cochlea is not proportional to the total noise energy at the intensities tested.

Human and animal studies have shown that interruption of noise exposure by regular rest or quiet periods provides the ear with some protection from the development of TTS (e.g. Ward, 1970; Kryter et al., 1966; Schmidek et al., 1975; Patuzzi, 1998) or PTS and that there is less hair cell loss than under continuous noise exposure of equal energy (e.g. Campo and Lataye, 1992; Clark et al., 1987; Ward, 1991; Clark and Bohne, 1992; Fredelius and Wersall, 1992). In the Clark and Bohne (1992) study, chinchillas were exposed to an octave-band noise centered at 0.5 KHz, 95 dB SPL, for 6 h/day for 36 days or for 15 min/h for 144 days. They evaluated behavioral measures of hearing loss and histopathological cochlear changes produced by exposure to low-frequency noise on interrupted schedules. The two schedules had similar duty cycles but different periods. Results indicated that these interrupted exposures produced smaller TTS and PTS, and less cochlear damage than continuous exposure of equal energy. Ward (1991) divided a continuous exposure into 45 periods given once a day Monday through Friday, for 9 weeks to chinchillas (interrupted exposure) and showed there was a slight reduction in damage as compared with uninterrupted exposure. Division of each of the 45 daily exposures into short noise bursts presented at regular intervals (interrupted–intermittent exposure) decreased damage even more. Fredelius and Wersall (1992) exposed guinea pigs to 108, 114, and 120 dB SPL for 6 h with and without a 1 h break after the first 3 h. Using scanning electron microscopy, they found that the introduction of a rest period during exposure to high sound energy produced a significant decrease in hair cell damage. Erlandsson et al. (1980) exposed guinea pigs to intense pure tones or impulse noise with variation in the intensity or duration of exposure. They found that above a certain critical level of intensity the damage started to increase more rapidly. In their study, animals exposed to impulse noise for 6 h, at 102 dB had slightly higher mean damage than those exposed to a corresponding continuous pure tone. TTS and PTS in the chinchilla were studied by Blakeslee et al. (1978) and Henderson et al. (1979) after exposure to continuous noise for 10 days or interruption by nighttime rest. In those investigations, TTS were almost the same in the groups, but far greater damage was found in cochleas of the uninterrupted exposure group.

As stated, there are discrepancies in the findings of previous studies, which may be due in part to the noise parameters used. Intermittent noise regimens used previously were: (1) several hours per day for several days, (2) several 1 h exposures with a 1 h break between exposures, and (3) impulse noise at various rates. In our study, guinea pigs were exposed for 5 h to an OBN centered at 4 kHz presented continuously or intermittently. Intermittent noise was presented at two pulses per second on a 45% duty cycle with a rise–fall time of 25 ms, which differed from previous studies. On the other hand, our lab's previous studies show that cochlear damage in the guinea pigs is almost established 10 days after noise exposure when they are exposed to 115 dB and 125 dB 4 kHz octave-band noise for 5 h (Yamasoba et al., 1999, 2001; Shoji et al., 2000). Brief interruption of noise exposure clearly resulted in signifi-

cantly smaller PTS and less hair cell damage than found for continuous noise exposure.

Previous studies showed that the degree of protection from noise damage afforded by periodic interruption of exposure depends both on the noise and rest parameters. Dolan et al. (1976) noted that when the noise level exceeded 123 dB, chinchillas incurred marked damage during the initial exposure period. Eldredge et al. (1959) found that when there was a very short rest period (<1 s) between successive exposures, no recovery occurred in guinea pigs; neither the loss of cochlear potentials nor sensory cells was decreased by such a short interruption. These reports suggest that if the rest period between exposures is too short and/or the intensity of exposure too strong, intermittency will not result in a decrease in cochlear damage. In our study, however, a very short (250 ms) interruption was sufficient to initiate recovery. The mechanism(s) by which a brief interruption in continuous noise induces the recovery process is unknown. Because the maximum sound pressure levels of the intermittent and continuous noises were equivalent, brief interruption seems chiefly to affect noise-induced metabolic stress. This interruption may have decreased the noise-induced reduction of blood flow, formation of reactive oxygen species, and/or the $[Ca^{2+}]_I$ changes induced by high noise levels. Because no data are yet available, these possibilities have still to be clarified.

In the guinea pig cochlea, the effect of 4 kHz OBN should be evident in the region of 9.69–13.5 mm from the apex, consistent with the guinea pig cochlear frequency map (Tsuji and Liberman, 1997). In this study, histological analysis was performed 5 days after the last ABR measurement. There might have been some subtle histological changes during this period, but the greatest loss of OHC still occurred in the lower second and basal turns, 11.4 mm or more from the apex. Using Greenwood's equation (Greenwood, 1990), we calculated that the 4 and 8 kHz frequency range should have been most affected in the region of maximal structural damage of our study. In this experiment, ears exposed to continuous 125 dB SPL showed the highest threshold shift at 4 and 8 kHz, as expected. Ears of animals in group 1 had significantly greater threshold shifts compared to those in group 4 at 4 and 8 kHz. These physiological findings are consistent with histological findings. On the other hand, when comparing other groups we can also find significant threshold shifts at 2 and 16 kHz, which is out of the region of damage. It shows the insensitivity of ABR to depict the hair cell loss observed in the present study.

Our observations support the speculation that there are significant noise-induced threshold shifts in the absence of significant hair cell loss. Substantial threshold shifts occurred in group 4, but almost no hair cell loss. Many of these threshold shifts presumably reflect damage to stereocilia on sensory cells, ranging from floppy, disarrayed, broken tip links and broken roots to collapsed, fused and elongated stereocilia (Liberman and Kiang, 1978; Liberman and Beil, 1979; Robertson et al., 1980; Slepecky and Chamberlain, 1982; Engstrom et al., 1983; Saunders and Flock, 1986; Tilney et al., 1982) or swelling of afferent nerve terminals on IHC (Pujol and Puel, 1999).

The equal-energy hypothesis, initially proposed by Eldred et al. (1955) assumes that hearing damage is a function of the total acoustic energy received. It postulates that noise damage depends on the total exposure energy, that equal total energies produce equal damage (Ward et al., 1981) and that the hearing organ reacts in a uniform manner to sounds of various intensities and durations, provided that the total sound energy remains constant. Animal studies that examined the applicability of this principle to various indices of hearing damage have challenged its generality and validity. Goulios and Robertson (1983) assessed cochlear damage in guinea pigs immediately following exposure to continuous pure tones of varying intensity and duration. Results were not wholly consistent with the assumptions of the equal-energy principle. Yamamoto et al. (1985) and Patuzzi (1998) found significant differences in humans in the TTS caused by sounds with different ontimes. These findings indicate that the equal-energy assumption may not hold for the prediction of TTS from intermittent noise. Fredelius et al. (1987) exposed guinea pigs to continuous 3.85 kHz pure tones of various durations and intensities, then allowed a 4-week recovery period before morphological changes in the cochlea were assessed. They found that cochlear damage increased with the increase in the sound energy given the animals. Erlandsson et al. (1980) made a histological analysis of damage to the inner ears of guinea pigs that had been exposed to continuous and impulse noises of different intensities and durations and reported that the total-energy hypothesis was oversimplified and inadequate for explaining noise-induced inner-ear damage. In our study, the total energy of the intermittent 125 dB noise was greater than that of the continuous 115 dB SPL, but the latter caused significantly greater PTS and hair cell loss. We therefore conclude that the total-energy principle is not applicable to noise-induced cochlear damage in the intensity range used in our study.

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Adenoviral vector gene delivery via the round window membrane in guinea pigs

[REGENERATION AND TRANSPLANTATION]

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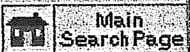

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Abstract

We have found that damage from a local anesthetic solution containing phenol permitted [beta]-galactosidase ([beta]-gal) gene delivery to the guinea pig inner ear via the round window membrane (RWM). RWM damage was evident as degeneration of the outer epithelium. After adenovirus lacZ vector was applied to the damaged RWM, immunohistochemistry showed strong [beta]-gal expression in the RWM, mesothelial cells, organ of Corti, spiral limbus, spiral ligament and spiral ganglion. In the vestibular labyrinth, expression was seen in the sensory and supporting cells, transitional cells, and the dark-cell area. Thus, adenovirus can transfect a variety of inner ear cells in the guinea pig through a damaged RWM.

INTRODUCTION

Virally mediated gene transfer can efficiently bring about expression of transgenes in cells and tissues [1]. As adenovirus has shown a number of advantages in the transfer and expression of therapeutic genes [2], adenovirus-mediated gene transfer has been studied for potential clinical use in treating inner ear disturbances [3–6]. Recently, adenovirus has been demonstrated to transfect hair cells and supporting cells in cultured guinea pig cochlear and vestibular organs [7,8]. So far, the effectiveness of gene transfer in the inner ear of guinea pigs has been assessed after inoculation via the round window membrane (RWM) or through a cochleostomy [4,5,9,10]. Although cochleostomy with creation of a small fenestration has been used to deliver viral gene vectors to the inner ear in rodent experiments, clinical use of this procedure carries risks of traumatic perilymphatic fistula and mechanical damage to inner ear hair cells [10,11]. A less invasive technique for virally mediated gene transfer into the inner ear is needed for therapeutic applications. Recently, gelfoam absorbed with adenoviral vector has proven successful in mediating transgene expression through the RWM into fibroblasts lining the scala tympani and the spiral ganglion cells in the rodent cochlea [12], but transformation by adenovirus transported through the RWM has not yet been demonstrated in hair cells or supporting cells.

The RWM, which may present an important host defense, consists of three layers: an outer epithelium, a middle core of connective tissue, and an inner epithelium [13]. Tight junctions are present near the outer epithelial surface, and desmosomes are distributed extensively throughout the lateral interdigitation [13]. The outer epithelial layer, then, has the structure of a defensive barrier [13]. This outer epithelium of the RWM also is the main barrier to macromolecular transport from the middle ear to the inner ear [14]. We reasoned that injury to the outer epithelium could permit adenoviral transfection of a variety of inner ear tissues through the RWM. We tested this hypothesis in the current study, in which the RWM was damaged by local anesthesia.

MATERIALS AND METHODS

Animals:

We used 18 healthy albino guinea pigs of the Hartley strain (250–350 g) with a normal Preyer's reflex. All experiments were performed in accordance with the guidelines of the University of Tokyo Committee for the use and Care of animals, which were drawn up in accordance with Japanese laws on breeding and treatment for experimental animals.

Adenoviral vectors:

We used a recombinant adenoviral vector with a [beta]-galactosidase reporter gene (bacterial lacZ reporter gene) to transfect a variety of inner ear tissues through the guinea pig RWM. Stock vector solution (~10^9 adenoviral particles/ml in sterile normal Ringer's solution) was used without dilution. Viral suspensions were kept at -80° C until use.

Surgical procedure and virus administration:

The 18 guinea pigs were anesthetized with xylazine (10 mg/kg, i.m.) and ketamine (40 mg/kg, i.m.). An incision was made in the left postauricular region. The cribriform area of the left temporal bone was exposed, and a small hole was made carefully in the tympanic bulla to provide direct visualization of the RWM. In six animals, 20 μl artificial perilymph was placed with a dropper upon the RWM under visualization with a surgical microscope. In 12 animals, 20 μl local anesthetic solution consisting of phenol, 4% xylocaine, peppermint, and dehydrated alcohol was placed upon the RWM with a dropper. Fifteen minutes later, each solution was absorbed and removed using small pieces of sponzel, taking care not to rupture the RWM. In four of the animals treated with local anesthetic solution, 20 μl artificial perilymph also was applied to the RWM (LA + AP group). Twenty minutes later, the four LA + AP animals were killed by decapitation. In the six animals treated with artificial perilymph (AP + Ad.lacZ group) as well as the remaining eight animals treated with local anesthetic solution, 20 μl adenoviral vector suspension (Ad.lacZ) was placed upon the RWM with a dropper (LA + Ad.lacZ group). Twenty minutes after administration of the adenoviral vector, the bony defect in the tympanic bulla was sealed with fascia and the skin closed with sutures. After surgery, lincomycin hydrochloride (10 mg/kg) diluted with 5 ml 0.9% saline was injected s.c. as a prophylactic antibiotic. All guinea pigs in the AP + Ad.lacZ and LA + Ad.lacZ groups were anesthetized and killed by decapitation 3 days after surgery.

Tissue preparation:

After decapitation, the left temporal bone was removed from each animal (the right ears were not studied). In the LA+AP group, the bony labyrinth was briefly rinsed with phosphate-buffered saline (PBS), and fixed with Karnovsky solution for 3 h at 4° C. In the AP + Ad.lacZ and LA + Ad.lacZ groups, the bony labyrinth was briefly rinsed with PBS and then fixed with 4% paraformaldehyde overnight at 4° C. After complete fixation, specimens were rinsed briefly with PBS and immersed in 10% EDTA to be decalcified for 2 weeks. In the LA + AP group, the bony labyrinth were rinsed with PBS and postfixed in 1% osmium tetroxide at 4° C for 1 h. Each cochlea was divided into two parts between the second and third turns. The cochlea was then dehydrated in a graded alcohol series, immersed in propylene oxide, and embedded in epoxy resin. The RWM was sectioned at a thickness of 10 μm with a microtome and then examined with a light microscope. In the AP + Ad.lacZ and LA + Ad.lacZ groups, the specimens were placed in 0.9% saline, dehydrated through a graded alcohol series, and then equilibrated in xylene. Specimens were embedded in paraffin and sectioned in the radial plane at 4–7 μm with a microtome.

Antibody staining:

The paraffin-embedded cochlear sections were dewaxed with xylene, and rehydrated in a graded alcohol series, and then rinsed with PBS. Sections were immersed in 0.3% Triton X for 10 min and washed with PBS. Endogenous peroxidase was blocked with 0.3% hydrogen peroxide for 30 min, and then non-specific binding was blocked with 10% normal goat serum for another 30 min. Next sections were incubated overnight with mouse monoclonal anti-

[beta]-gal (clone Gal-13, Sigma, St. Louis, MO) diluted 1:1000 in PBS. After washing in PBS, sections were incubated with peroxidase-conjugated secondary antibody (Histofine Simple Stain Max-PO; MULT, Nichirei, Tokyo, Japan) for 30 min at room temperature. The bound label was developed with diaminobenzadine. Immunostained cells were compared under a light microscope with negative control sections from the cochlea and vestibular labyrinth.

RESULTS

Immediately after removal of the bony labyrinth, RWM perforation was ruled out in each animal by inspection under a surgical microscope. In the four animals in the LA + AP group, the RWM section showed damage to the outer epithelium (Fig. 1a). The organ of Corti was morphologically intact throughout all turns in the AP + Ad. lacZ group and in the second to apical turns in the animals in the LA + Ad.lacZ group. In six of eight animals in the latter group, occasional inner and outer hair cell loss (70–80%) was seen in the lower half of the basal turn. Expression of [beta]-gal was assessed by immunohistochemistry in all animals throughout the length of the cochlea and in the vestibular labyrinth. In the AP + Ad.lacZ group, no [beta]-gal expression was observed in the RWM, and no [beta]-gal expression was seen anywhere in the cochlea or vestibular labyrinth. In all animals in the LA +Ad.lacZ group, strong expression of [beta]-gal was observed in the RWM and the mesothelial cells in the scala tympani (Fig. 1b). Sections showed inflammation and fibrosis at the scala tympani inside the RWM in the LA + Ad.lacZ group (Fig. 1b). In the second to apical turns of the cochlea, [beta]-gal expression was detected in the inner and outer hair cells (Fig. 2a) and supporting cells (Fig. 2a). Similar expression of [beta]-gal was also observed in the organ of Corti, which showed hair cell loss at the basal turn. Expression of [beta]-gal was detected in the spiral limbus (Fig. 2b), spiral ligament (Fig. 2c), spiral prominence (Fig. 2d) and spiral ganglion (Fig. 2e). However little expression of [beta]-gal was seen in the stria vascularis (Fig. 2c). In the vestibular labyrinth, [beta]-gal expression was seen in the sensory and supporting cells (Fig. 3a), transitional cells, and epithelial cells in the dark-cell area in the ampullar crista (Fig. 3b), and in the sensory and supporting cells of the saccule (Fig. 3c).

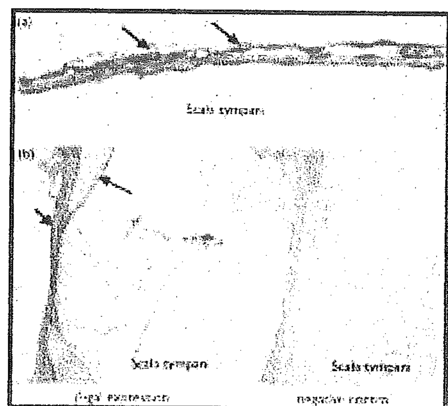

Fig. 1. Findings in the round window membrane (RWM). (a) Damaged outer epithelium (arrows) in the RWM observed in the local anesthetic solution (LA) + artificial perilymph (AP) group. (b) Strong expression of beta-galactosidase ([beta]-gal) in the RWM (short arrow) and in mesothelial cells lining the perilymphatic space (long arrow) in the LA + adenoviral vector (Ad.lacZ) group. Inflammation and fibrous occur at the scala tympani inside the RWM.

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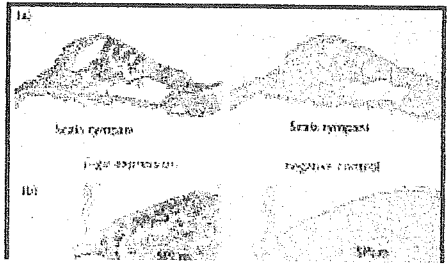
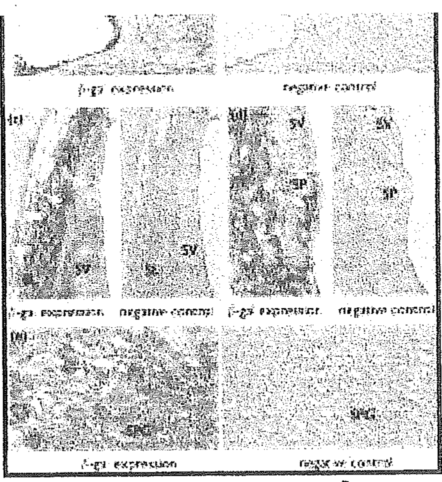


Fig. 2. Expression of [beta]-gal in the cochlea (second turn) in the local anesthetic solution (LA) + adenoviral vector (Ad.lacZ) group. Expression of [beta]-gal in the organ of Corti (a), spiral limbus (b), spiral ligament (c), stria vascularis (c), spiral prominence (d) and spiral ganglion cells (e). (a,b) Strong [beta]-gal expression in the hair cells (short arrows), and supporting cells (long arrow), and in spiral limbus (short arrows). (c,d) Expression of [beta]-gal (short arrows) in the stria vascularis is weaker



than in either the spiral ligament or the spiral prominence. (e) Expression of [beta]-gal in several spiral ganglion cells (short arrows). SPLm, spiral limbus; SL, spiral ligament; SV, stria vascularis; SP, spiral prominence; SPG, spiral ganglion.

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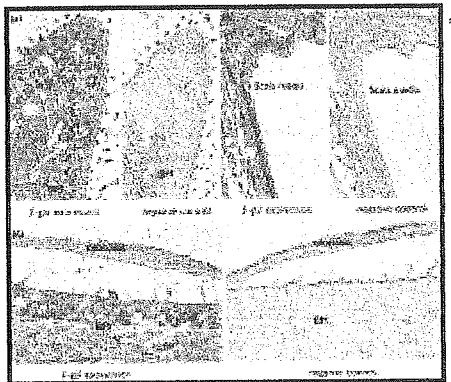

Fig. 3. Expression of [beta]-gal in sensory and non-sensory epithelium in the vestibular labyrinth in the local anesthetic solution (LA) + adenoviral vector (Ad.lacZ) group. (a) Expression of [beta]-gal in the sensory cells (short arrows) and supporting cells (long arrows) in the ampullar crista. (b) Expression of [beta]-gal in the transitional cells (long arrow) and epithelial cells in the dark-cell area (short arrow) of the ampullar crista. (c) Expression of [beta]-gal in the sensory cells (short arrows) and supporting cells (long arrows) of the saccular macula in the LA + Ad.lacZ group. BM, basement membrane.

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DISCUSSION

In the current study, we determined the distribution of cells transfected by adenovirus applied to guinea pig RWM. In all animals in the LA + Ad.lacZ group, transfected cells were detected among the mesothelial cells lining the perilymphatic space, as well as in the organ of Corti, spiral limbus, spiral ligament, spiral ganglion and vestibular sensory epithelium. Thus, our delivery system was able to reliably transfect a variety of inner ear tissues.

In the AP + Ad.lacZ group, no [beta]-gal expression was seen in the RWM, cochlea or vestibular labyrinth. This suggests that the intact guinea pig RWM is a barrier impenetrable to adenovirus. In the LA + Ad.lacZ guinea pigs, adenovirus was able to transfect a variety of inner ear cells through a damaged RWM. The local anesthetic solution, which includes phenol as well as xylocaine, damages the squamous epithelium of the tympanic membrane to permit xylocaine to reach nerve endings in the tympanic membrane and exert an anesthetic effect [15]. In the LA + AP group, the outer epithelium of the RWM was similarly damaged by phenol in the solution, suggesting an increase of the permeability of the RWM. In the LA + Ad.lacZ guinea pigs, the RWM damaged by phenol in the solution may increase its permeability, and thereby may allow the adenovirus vector to transfect to a variety of inner ear cells.

Acute or chronic otitis media may cause labyrinthitis, resulting in a sensorineural hearing loss. Otitis media might also alter RWM permeability for macromolecules [16-18]. During the early stage of purulent otitis media, pore-forming toxins produced by inflammation have been implicated in enhanced permeability to macromolecules such as radioiodinated albumin [17]. At a late stage of experimentally induced serous otitis media, with degeneration of the

outer epithelium of the RWM, ferritin particles may be transported to the scala tympani from the middle ear through the membrane [18]. Adenovirally mediated transfer of genes might take place in the inner ear through the RWM either during an early stage of acute otitis media or a late stage of serous otitis media.

Several investigators have demonstrated the effectiveness of adenovirally mediated gene transfer in guinea pigs by inoculation via the RWM as well as via a cochleostomy [4,5,9,10]. When adenoviral vector was introduced into the perilymphatic space by a single injection, no transfection by adenovirus was detected in the hair cells or supporting cells in the cochlea or vestibular labyrinth [4,5,9]. On the other hand, adenovirus transfected the hair cells and supporting cells after direct infusion of the viral vector using a chronically implanted osmotic pump [10]. These results suggest that gradual prolonged administration is more effective than single injections for adenoviral transfection of the hair cells and supporting cells in the inner ear. In the current study, transport of adenovirus into the scala tympani through the damaged RWM may be relatively slow, facilitating *in vivo* transfection of these cells.

In an *in vitro* study, transgene expression was demonstrated to be similar degrees in the stria vascularis and the spiral ligament [8]. In our current experiment, [beta]-gal expression was weaker in the stria vascularis than in the spiral ligament. Differences in transgene expression between these *in vivo* and *in vitro* studies may involve the sites where adenovirus was administered, (both the perilymphatic and endolymphatic spaces *in vitro*, the scala tympani *in vivo*). Adenovirus transported to the scala tympani from the RWM may reach the stria vascularis mainly via supporting cells and spiral ligament along the basilar membrane. When passing through the perilymph–endolymph barrier between the spiral ligament and the stria vascularis, large particles such as adenoviruses may be impeded, resulting in weaker [beta]-gal transgene expression in the stria vascularis than in the spiral ligament.

In the current study, no perforation of the RWM was detected in animals treated with local anesthetic solution, suggesting that mechanical damage to the cochlea was limited. However, paraffin sections occasionally showed marked morphologic damage to the organ of Corti limited to the basal turn of the cochlea, especially the lower half of the basal turn. Phenol application to the RWM may cause both functional impairment and morphologic damage in the organ of Corti [19]. The hair cell degeneration seen at the lower half of the basal turn in the current study may have involved ototoxic effects of phenol passing through the RWM.

The primary acute pathological finding associated with toxicity of aminoglycosides such as kanamycin or neomycin is cochlear hair cell loss while supporting cells are preserved [20]. In the current study, strong [beta]-gal expression was detected in the supporting cells in all cochlear turns and the vestibular labyrinth, and even supporting cells located where hair cells were missing at the lower half of the basal cochlear turn. This observation suggests that adenovirus delivered via the RWM can transfect the supporting cells even where hair cells are absent. One important goal of gene therapy is to induce hair cell regeneration by promoting plasticity of the supporting cells. The Math 1 gene has been considered to promote appearance of hair cells in developing sensory epithelia [21]. Over-expression of Math 1 has been demonstrated to induce formation of extra hair cells in cochlear explant cultures from postnatal rats [22]. If adenovirus vectors with a Math 1 reporter gene were administered via a damaged RWM early in aminoglycoside-induced ototoxicity, overexpression of the gene in transfected supporting cells might facilitate their differentiation and growth as cochlear hair cells.

CONCLUSION

We found that damaging the outer RWM epithelium with a phenol-containing topical anesthetic solution was a relatively non-invasive way to transfect guinea pig inner ear cells with a [beta]-gal reporter gene carried by an adenoviral vector.

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Key words: Adenovirus; Gene transfer; Inner ear; Phenol; Round window membrane

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