ORIGINAL ARTICLE

Morphologic and Physiologic Changes of Cochlea after Administration of Plasminogen Activator Inhibitor Type 1 in Guinea Pig

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Objective: To find out the effect of PAI-1 through the physiologic and morphologic changes in the cochlea of guinea pigs.

Materials and Methods: Using auditory brainstem responses (ABR), the hearing threshold was measured during the four weeks after placement of saline and PAI-1. Endocochlear potential (EP) was measured at the 2nd turn of cochlea. At the end of the four weeks, the cochleae were harvested and prepared for light microscopy and electron microscopy.

Results: The ABR threshold was elevated mildly to moderately at 30 minutes for the majority of groups, but recovery began within 1-3 days ($p<0.05$). EP was decreased significantly in the PAI-1 groups compared to the control group ($p<0.05$). In groups with permanent hearing loss, the compressed outer hair cells in the organ of Corti and thinned stria vascularis were observed in the LM study. In the SEM study, the stereocilia of outer hair cells appeared to be deleted or bent. Also, the intermediate cells of stria vascularis were missing in the TEM study.

Conclusion: In our study, it appears that the changes of pathophysiology in cochlea can be explained by disturbed microcirculation by PAI-1.

Submitted : 12 August 2011 *Accepted : 13 December 2011*

Introduction

The microcirculation of cochlea is one of the most important factors in the homeostasis of inner ear. It is believed that microcirculatory changes may be the cause of many inner ear diseases. Recent studies have tried to establish the mechanism and role of hypercoagulation and decreased fibrinolytic activity in various microvascular diseases [1-3].

Plasminogen activator inhibitor type1(PAI-1) is a single-chain polypeptide of an approximately 50-kDa that belongs to the serine protease inhibitors superfamily. As a major regulator factor in the plasminogen/ plasmin system, which is important for extracellular protein degradation and fibrinolysis, PAI-1 is a powerful material that diminishes the fibrinolytic activity. In particular, PAI-1 competitively conjugates with both tissue plasminogen activator (tPA), whose major role in the vascular system is thrombolysis, and urokinase plaminogen activator (uPA), which is mainly involved in the invasion of tumor cells. It is normally secreted from endothelial cells, vascular smooth muscle cells, hepatocytes, platelets and adipocytes, with the majority of the circulating PAI-1 secreted from the adipose tissue.

Decreased fibrinolytic activity is an important cause of various diseases related to thrombus since plasma PAI-1 levels are elevated in thrombosis, myocardiac infarction, obese and obstructive sleep apnea

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[4,5]. According to the studies on PAI-1 in type II diabetes mellitus, the plasma concentration of PAI-1 level was elevated in contrast to control groups and some studies also showed that the levels of PAI-1 can be used as an useful indicator for the development and progression of diabetic retinopathy [6,7]. Further the PAI-1 level is regarded as a risk factor in coronary artery diseases along with fibrinogen, t-PA, and von Willebrand factor [8]. But there is no report about the role of PAI-1 in the inner ear pathology.

The etiology and mechanism of diseases potentially caused by the disturbance of inner ear circulation, such as Bell's palsy, vestibular neuronitis and Meniere's disease, are not yet fully understood in the domain of otolaryngology. However, most of these diseases can spontaneously recover in the course of time, so we are considering the disturbed circulation of the cochlea as an etiologic factor. In this study, we investigated the physiologic and morphologic changes of cochlea following direct administration of PAI-1 through the round window in guinea pigs to determine some effects of PAI-1 on the microcirculation of cochlea.

Materials and Methods

Animals and anesthesia

Healthy guinea pigs (250-300 g) with normal Preyer's reflex and normal auditory evoked brainstem responses (ABR) were used in this study. Animals were anaesthetized with an intraperitoneal injection of ketamine hydrochloride (30mg/kg) and xylazine (2mg/kg). Guinea pigs were randomly divided into control group (n=12, 24 ears) and study groups of 10, 20, 40 ㎍/㎖ concentrations of PAI-1 (each n=15, 30 ears) for the evaluation of changes in the auditory threshold and morphology. Each concentration was adopted from the results of pilot studies (5 - 160㎍/㎖) that investigated the optimal concentrations for causing hearing changes. For each different concentration group, three guinea pigs (6 ears) were used to observe the changes in endocochlear potentials, and four guinea pigs (8 ears) were used for LM, SEM, TEM.

The experimental solution, PAI-1, was purchased from Merck Ltd, Seoul, Korea and the normal saline was used for control groups. This study was approved by the Committee for Ethics in Animal Experiments of the Hallym University Medical Center.

The mastoid cavity was opened to expose the round window: normal saline was applied to the control group and concentrations of PAI-1 were also applied to the experimental groups. Pieces of gelfoams stained with 200 ㎕ of each solution were placed on the round window.

ABR recording

Guinea pigs underwent hearing threshold testing by ABR for each ear using Traveler Express (Biologic System Co., Mundelein, IN, USA). Hearing threshold was determined by the disappearance of the Wave I while decreasing the volume in 5 dB steps using click sound stimuli. The hearing threshold was measured using ABR before and after the application of appropriate solutions.

Endocochlear potential (EP) measurement

For the measurment of endocochlear potential, the cochlea helix and round window of guinea pigs with normal hearing were defined and a tungsten micro-needle (2㎛) was inserted into the endolymphatic space at 2nd turn of guinea pig's cochlea. The micro-needle was advanced with a micromanipulator (M3301R+M-3, WPI, Sarasota, FL, USA) through a fenestra opened in the 2nd cochlear turn into the scala media, and reference electrode was placed on the exposed neck muscle. EP value was measured using comizoa CP-201 hardware/ software (Comizoa Co., Ltd, Daejeon, Korea).

Light microscopy (LM)

Cochleae (basal turn) were obtained from both temporal bones of control and study groups(n = 8 ears/ group) for light microscopic study after 4 weeks. Following perfusion fixation by 4% formalin (0.1% glutaraldehyde in sodium phosphate buffer), temporal bones were kept in the same fixative for 3 days. Next, they were decalcified by 10% Ethylenediaminetetraacetic acid (EDTA) for 5 days, washed thoroughly, and embedded in paraffin. The paraffin block was fixed on a wood block and cut into 5 ㎛ in accordance with the cochlear axis. Cut section was deparaffinized in xylene solution, dehydrated in graded ethanol (70-100%) and then stained in hematoxylin and eosin (HE).

Scanning electron microscopic study (SEM)

Two percent glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) was gently irrigated through the oval and the

ruptured round windows, and the bones were kept in the same fixative overnight. The membranous structure was dissected out under a dissecting microscope. Postfixed dissected specimens were dehydrated in graded ethanol, acetone and isoamylacetate. After freeze drying at the critical point, specimens were fixed on stubs that were pasted with 13 mm platinum gold coated in a sputter coater and observed under a SEM (Hitachi S-2500, Tokyo, Japan) (n = 8 ears/group).

Transmission electron microscopic study (TEM)

The bony capsule was opened, membranous tissue was obtained carefully and immediately immersed in a fixing bath containing 2% glutaraldehyde in 0.1 M phosphate buffer for 2 hours. After rinsing, cochleae were dehydrated in a graded series of ethanol and embedded in Epon/Araldite resin. Ultra-thin sections (70 nm) were prepared using a ultramicrotome(Leica Ultracut, Austria) and mounted on copper grids. Sections were stained with uranyl acetate for 20 min, followed by lead citrate for 10 min. Observations were performed under a TEM (Zeiss-109, Germany) (n = 8 ears/group).

Statistical analysis

Statistical comparisons were made between groups according to the difference in hearing changes with the passage of time, followed by different concentrations of PAI-1. Two-wayANOVA was used with $p<0.05$ as the level of significance.

Results

Change of hearing threshold

In all study groups, changes of hearing threshold were measured after the administration of PAI-1 on round window when the same amount of normal saline was administered in the control group. The hearing threshold for most of the study groups went up after 30 min, reached the peak in 2-3 days and then was recovered (63ears/72ears,87%). There was no difference in the hearing threshold according to concentration ($p<0.05$) (Fig.1). Some guinea pigs showed no recovery in the hearing threshold (9 ears/ 72 ears,13%).

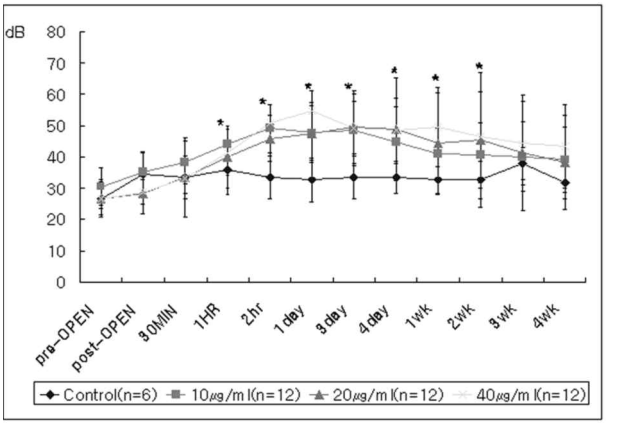

Figure 1. Change of hearing threshold after application of PAI-1 at different concentrations. All groups were elevated and turned to decrease after average 2.3 days compared with control ($p<0.05$). *$p<0.05$

Change of Endocochlear Potential

The initial value of intracochlear EP was 1.0 ± 0.1 mV. The voltage decreased over the interval from 30 min to 120 min after the administration of PAI-1, when compared to the control group (Fig. 2).

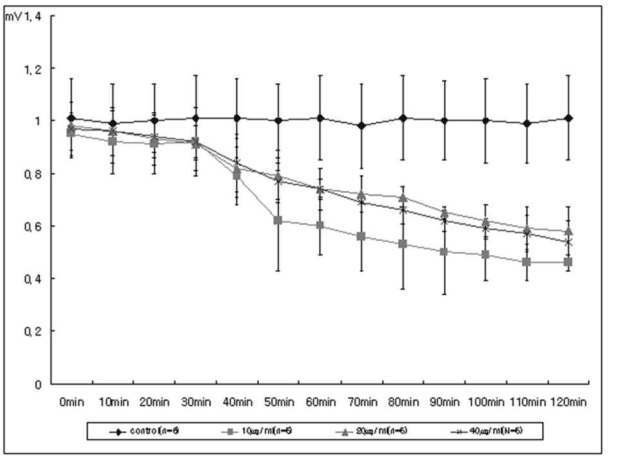

Figure 2. Change of endocochlear potential after application of PAI-1 at different concentrations. There was decrease of EP after 30 minutes in all concentrations, but there was no change in control group ($p<0.05$).

Light microscopic findings

There were no morphological changes in the control or in the transient hearing loss group, but thinned stria vascularis of lateral walls of cochleae and compressed organs of Corti were observed in the permanent hearing loss group (Fig. 3).

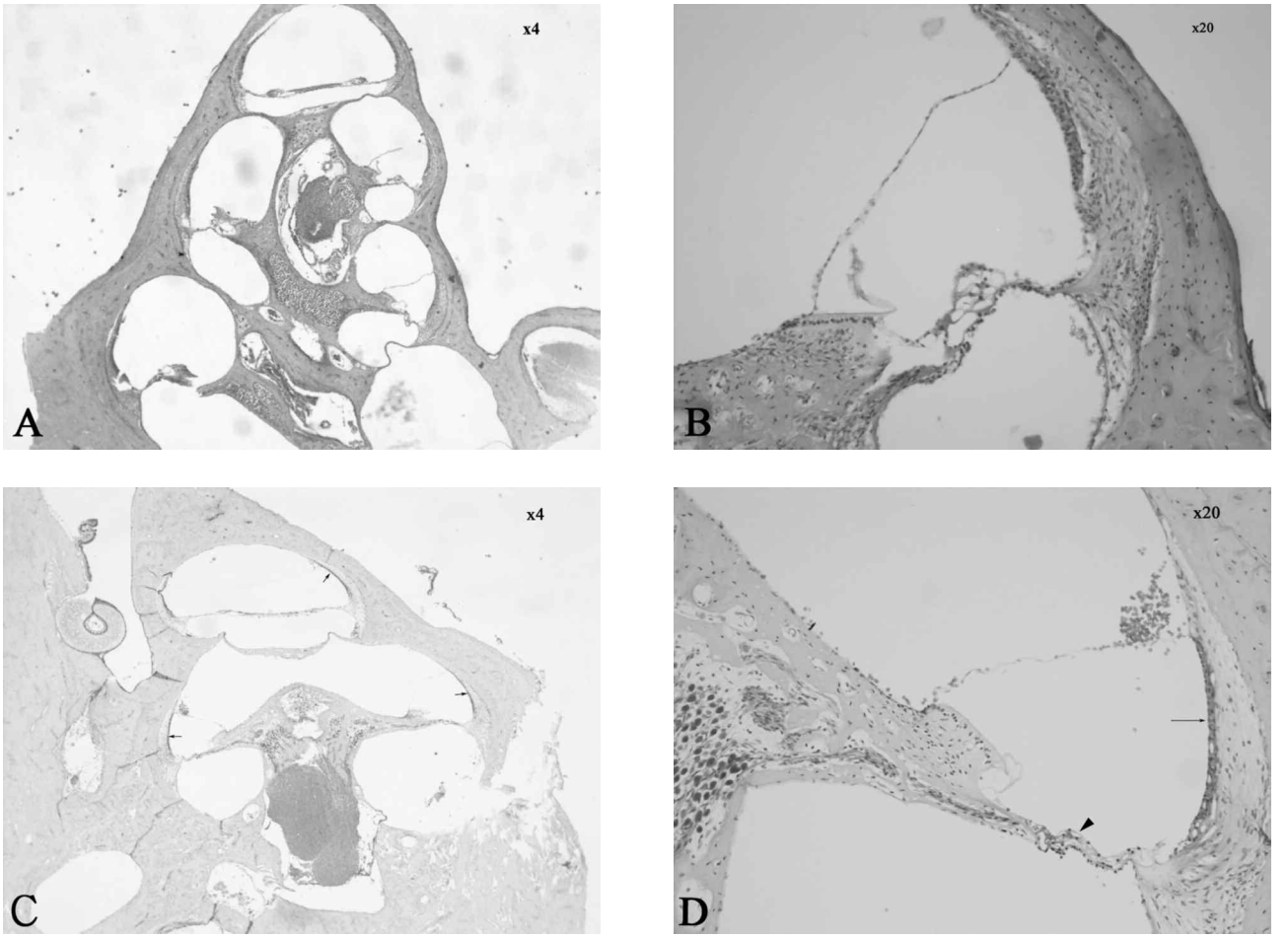

Figure 3. Light microscopic findings of the cochlea in guinea pig. **(A,X4 and B,X20)** Normal cochlear findings in control and in transient hearing loss group **(C,X4 and D,X20)**. In permanent hearing loss group, thinned stria vascularis (arrow) of lateral wall and compressed hair cells in organ of Corti (arrow head) were observed.

Transmission electron microscopic findings

Normal marginal cells, intermediate cells, basal cells and endothelium of capillary were seen in the control and in the transient hearing loss group. Loss of intermediate cells or vacuolized cells was observed in the permanent hearing loss group (Fig. 4).

Scanning electron microscopic findings

Regularly arranged inner and outer hair cells were seen without deformity in the control and in the transient hearing loss group. In the permanent hearing loss group, the outer hair cells in the third row were damaged most severely, followed by the second and first row hair cells. The stereocilia of damaged hair cells appeared to be either deleted, fused or splayed out (Fig. 5).

Discussion

It is well-known that decreased blood supply in the cochlea affects the function of auditory nerves, hair cells and the basement membranes. It is believed that the capillary bed of stria vascularis, spiral eminence and basement membrane are responsible for the blood supply of cochlea, although there is no clear connection between cochlea and circulatory system [9].

An interpretation of our results is that PAI-1 may cause the decrease of the blood supply to the organ of Corti and stria vascularis, leading to the elevation of hearing threshold as other ototoxic agents do. Hearing loss occurred transiently and recovered at the average of 2.3 hrs in most of study groups. PAI-1 may have an influence on the control of appropriate amount of

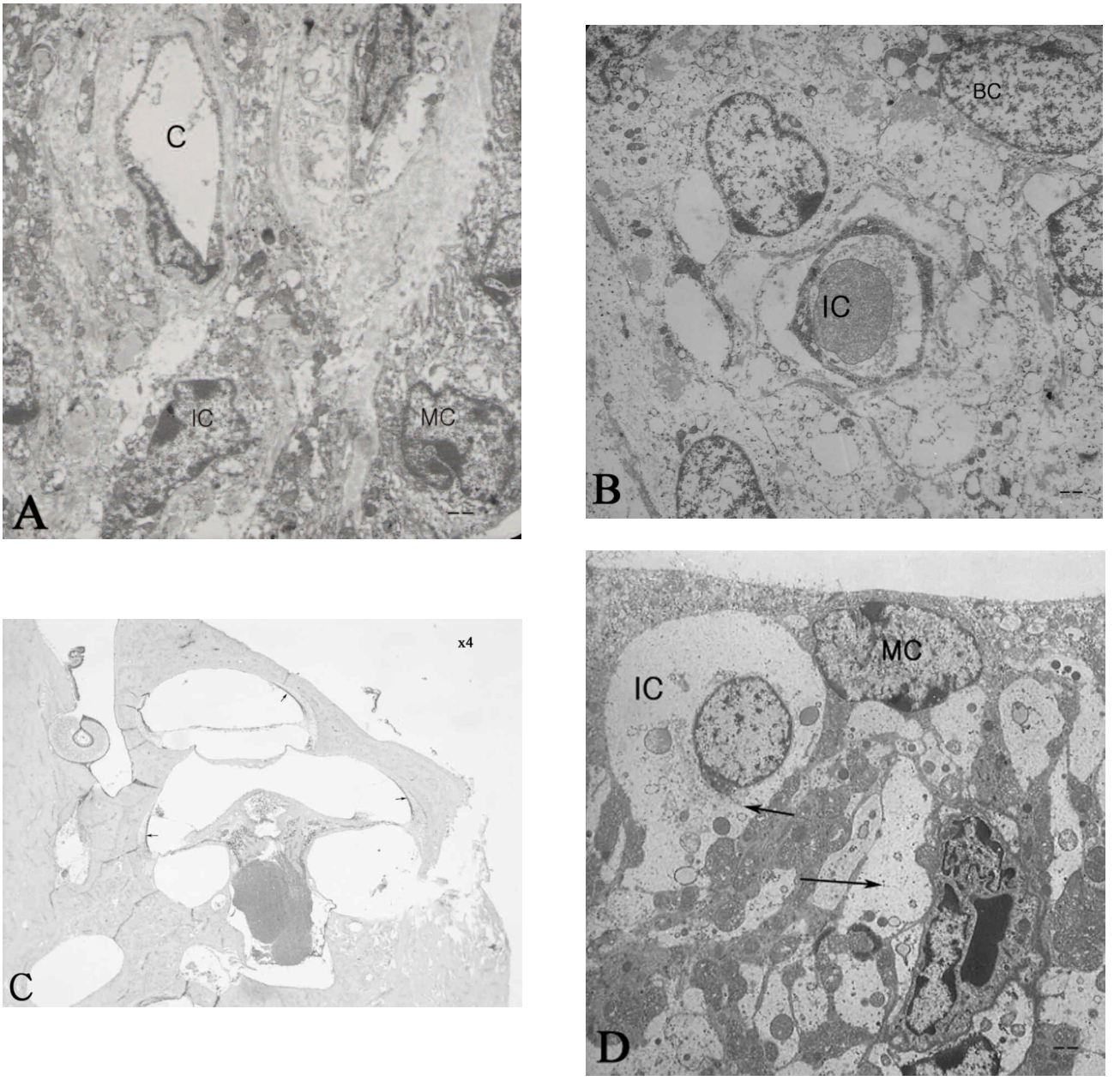

Figure 4. Transmission electron microscopic findings of stria vascularis in guinea pig. **(A, B)** Marginal cell, intermediate cell, basal cell and capillary were observed in control group (bar= 1.7 ㎛). **(C)** Absence of intermediate cells and slightly thinned marginal cells. **(D)** Vacuolized intermediate cells (arrow) and normal capillaries were seen (bar = 2.5 ㎛).
MC: Marginal cell; IC: intermediate cell; BC: basal cell; C: capillary.

cochlear blood flow transiently regardless of concentration. It is probable that the pathologic findings of the permanent hearing loss group were due to the effect of PAI-1. We do not know as yet the reason why PAI-1 causes a permanent change (13%), but genetic individual outer hair cells' vulnerability would be possible. No experimental procedure error was observed, and no control animals had permanent

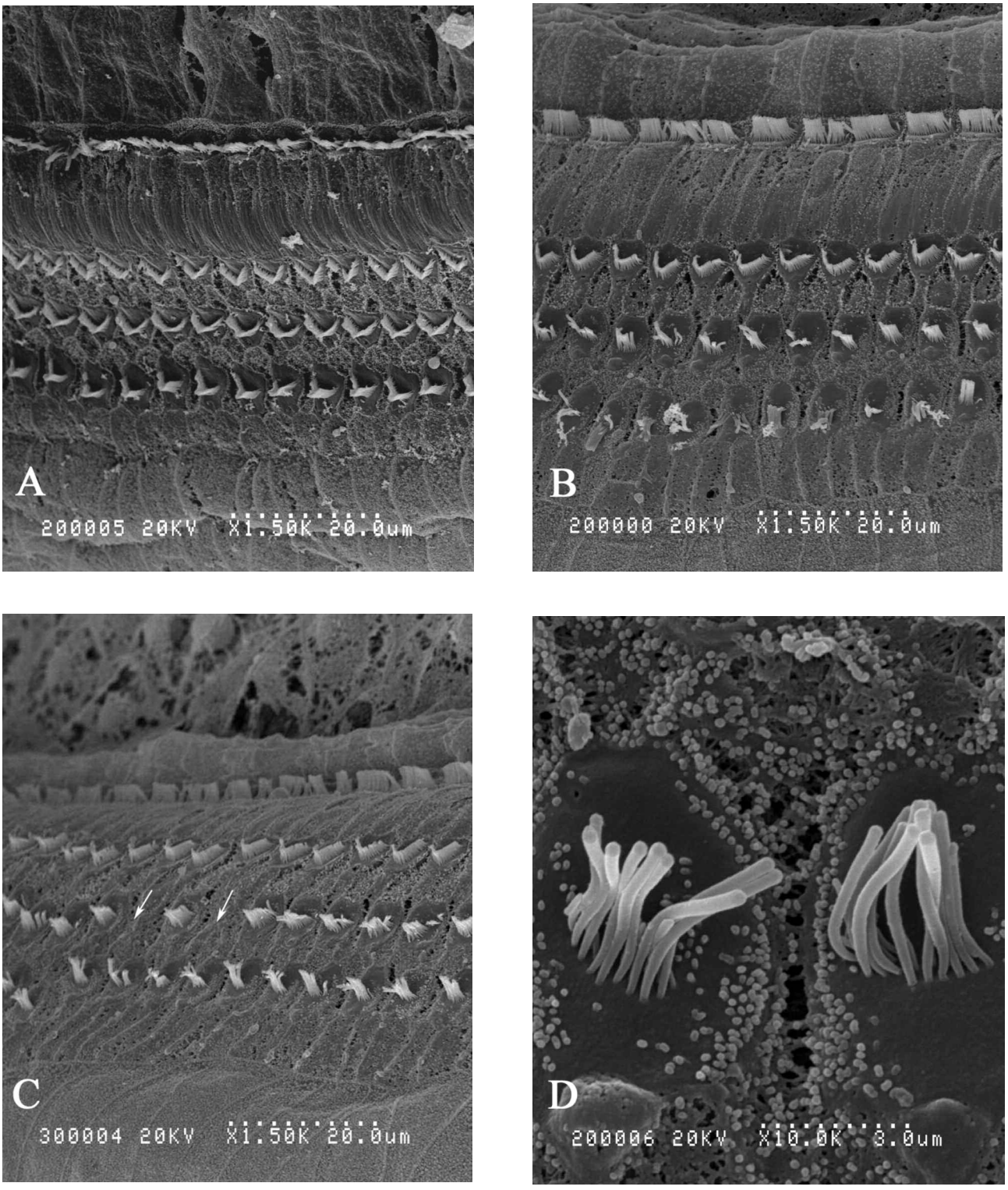

Figure 5. Scanning electron microscopic findings of organ of corti in guinea pig. **(A)** Normal arranged hair cells were seen in control group. **(B)** The outer hair cells were damaged, particullary severe in order to outer layer. **(C)** Stereocilia of outer hair cells were deleted (arrow) or **(D)** Curved to outside or fused to inside.

hearing loss.

Molecular-weight substances not exceeding 500 kD are reported to pass through the round window membrane (RWM) into the inner ear within a short period of time [10,11]. It seems that PAI-1 of 50 kD needs about 30 min to pass through RWM to affect the cochlea. Another possibility is that the permanent hearing loss group had a very rapid absorption of PAI-1 via RWM and a high transmitted dose of the molecule resulted in permanent hearing loss. The approach using gelfoam has several advantages. First, the problem of general toxicity can be avoided, and second, the material can be directly transported to and non-invasively to the normal cochlea. Lastly, this approach can avoid confusing results owing to the influence of systemic effects. Additionally, such material can be cleared out from the middle ear via the Eustachian tube spontaneously within several hours. However one possible explanation for the permanent hearing loss group is slow drainage of solution via the Eustachian tube. It is commonly accepted that the EP is generated in the stria vascularis and is dependent on the blood supply in the stria vascularis. Previous studies reported that elevated EP values were due to the secretion of highly concentrated K+ ions by marginal cells in the stria vascularis [12,13]. Damaged marginal cells lead to disturbed inflow of K+ into the endolymph as a result of decreased function of Na+-K+ ATPase [14]. Dominant white spottingW/Wv mice are well-known mutants that lack strial intermediate cells in their cochlea and manifest hereditary sensorineural hearing loss. Strial intermediate cells, which are mesenchymal cells situated between the marginal and basal cells of the stria vascularis, play an important role in the ion transport of the cochlear lymph as well as in the secretion of melanocytes [15,16]. Our findings therefore may suggest that the loss or vacuolized intermediate cells in the stria vascularis is an important relation to elevated hearing threshold, decreased EP value and thinned stria vascularis.

EM findings show that injured hair cells of the cochlea have deleted, fused, splayed or bent stereocilia when affected by noise or by some ototoxic agents [17,18]. In our study, compressed hair cells were observed in the LM findings. Weak, injured inner hair cells were seen and the outer hair cells were damaged most severely in the third row, followed by the second and first rows in the SEM findings.

We could not find direct pathologic foci such as occlusion of blood vessels or thrombi. But it is thought that PAI-1 affects the microcirculation of cochlea and causes morphological changes and hearing threshold changes functionally in the permanent hearing loss group. Recently, PAI-1 was used experimentally for the treatment of acute pulmonary thromboembolism in PAI-1 knockout mice [19], and clinically in recombinant tissue plasminogen activator(rtPA) treatment for patients with sudden hearing loss and/or chronic hearing loss [20]. Also elevated PAI-1 levels may contribute to glaucoma pathogenesis by an unknown mechanism [21]. Our results suggest that further investigation is necessary to prove the effect of systemic administration of PAI-1 in cochlea. Furthermore, we believe it is necessary to conduct a comparative study of cochlear blood flow before and after the administration of PAI-1 using a technique such as Laser Doppler flowmetry. Establishing a causal relationship between the elevated PAI-1 level and inner ear diseases will greatly enhance possible therapies.

Conclusion

In our study, after a sufficient amount of PAI-1 gelfoam was administered for a sufficient period of time, permanent hearing loss appeared in some animals, whereas the majority of the transient hearing loss group recovered their hearing. The recovery pattern in the transient hearing loss group seems very similar to that of inner ear diseases such as sudden deafness, Bell's palsy and vestibular neuronitis.

We suggest that the changes in the cochlea by PAI-1 may explain the pathophysiology of some inner ear diseases.

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