Original Article

A novel model for prognosis of Meniere's disease using oxidative stress susceptibility of lymphoblastoid cell lines

Ken Hayashi[1,*], Ryo Kobayashi[2], Koichi Kitamura[3], Fumiyuki Goto[4], Kaoru Ogawa[4], Tetsuya Matsumoto[2]

[1] *Department of Otolaryngology, Shinkawa Clinic, Kanagawa, Japan;*
[2] *Department of Microbiology, Tokyo Medical University, Tokyo, Japan;*
[3] *Department of Otolaryngology, Tokyo Medical University, Tokyo, Japan;*
[4] *Department of Otolaryngology, Head and Neck Surgery, Keio University, Tokyo, Japan.*

Summary **The aim of this study was to examine differences of susceptibility to oxidative stress of Epstein-Barr virus (EBV)-transformed lymphoblastoid cell lines (LCLs) established from Meniere's disease (MD) patients and to examine the effect of ATP treatment in the prognosis and treatment MD. LCLs were established from 10 patients with MD and 10 healthy donors by EBV. Cell viabilities were calculated after treatment of H_2O_2 with or without ATP. The relationship between the sensitivity of H_2O_2-treated LCLs to ATP and the staging scale of MD was examined. The nuclear morphological changes of Hoechst 33258-stained LCLs after H_2O_2-treatment were observed under a fluorescence microscope. LCLs from MD were significantly more sensitive ($p < 0.001$) to H_2O_2 than LCLs from healthy donors after 3 h of H_2O_2 treatment. All of the ATP-sensitive LCLs were categorized as Stage 1 or 2, while others categorized as Stage 3 or 4 were not sensitive to ATP. There were significant differences ($p < 0.01$) of cell viabilities after addition of ATP between H_2O_2-treated LCLs classified as Stage 1 or 2 and as Stage 3 or 4 in MD. Both chromatin condensation and swelling of the cell body were observed in H_2O_2-treated LCLs. Our findings indicate that LCLs established from MD patients might be used as a unique model to detect susceptibility to oxidative stress and ATP treatment in MD patients. Also, the difference of the sensitivity of H_2O_2-treated LCLs to ATP might relate to prognosis and treatment of MD. This system may form the basis of tailor-made therapy for MD.**

Keywords: Meniere's disease (MD), lymphoblastoid cell line (LCL), oxidative stress, adenosine 5'-triphosphate (ATP)

1. Introduction

Meniere's disease (MD), described by Prospero Meniere in 1861, is typically characterized by fluctuating hearing loss, episodic vertigo, tinnitus and a sensation of pressure. The histopathological hallmarks of the disease, at the bone level, are endolymphatic hydrops, atrophy and erosion of the endolymphatic sac. Despite a rigorous pathological definition, the etiology of MD, which is usually defined as idiopathic, is ascribed to a variety of causes, such as alterations of ionic homeostasis, vascular disorder, trauma, viral infections and immunological disorder. However, until recently it has been difficult to estimate the prognosis of MD clinically. Current studies have reported that oxidative stress may play a crucial role in the pathogenesis of a variety of inner ear diseases, such as noise-induced hearing loss (*1*), ischemic impairment (*2*) and age-related hearing loss (*3*). Concerning MD, Horner and Guilhaume suggested that oxidative insult was likely to contribute to the pathology associated with endolymphatic hydrops and therefore free radical scavengers might be useful in the treatment of MD patients (*4*). Takumida *et al*. demonstrated that edaravone (3-methyl-1-phenyl-2-pyrazolin-5-one), an

**Address correspondence to:*
Dr. Ken Hayashi, Department of Otolaryngology, Shinkawa Clinic, 1-2-3 Minamiyama, Hadano, Kanagawa 257-0003, Japan.
e-mail: ken.hayashi@jcom.home.ne.jp

inhibitor of reactive oxygen species (ROS), attenuated the formation of endolymphatic hydrops in the guinea pig cochlea (*5*). In a clinical trial, treatment using such radical scavengers was reported to have the potential to become an effective new therapy for patients with MD (*6*). However, the direct effect of oxidative stress for MD inner ear tissue cells is still unknown, because the normal inner ear tissue can not be obtained. Therefore, we focused on Epstein-Barr virus (EBV)-transformed B-lymphocytes (lymphoblastoid cell lines; LCLs) as a cellular model for MD like hypertension (*7*), diabetes mellitus (*8*), Alzheimer's disease (*9*), Huntington's disease (*10*), and bipolar disease (*11*), because we hypothesized that LCLs could be used in place of inner ear cells of patients with MD. In addition, LCLs can be easily established from B-lymphocytes obtained from patients using EBV infection and can be maintained for a long time.

Hydrogen peroxide (H_2O_2) is an intermediate product of the degradation of ROS and a highly reactive molecule. The treatment of cells with H_2O_2 induces oxidative stress *via* an increased production of ROS and may subsequently lead to cell damage or cell death. Extracellular H_2O_2 is able to cross cell membranes and directly alters their intracellular concentration (*12*). The loss of adenosine 5'-triphosphate (ATP) has been reported to be an early step after initiation of H_2O_2-induced oxidative stress in non-neuronal (*13*) and neural systems (*14*). Teepker *et al*. reported that ATP-decline under H_2O_2-induced oxidative stress might point to a relevant ATP consumption related to apoptosis (*15*). In this study, we evaluated the difference of the susceptibility of the LCLs to H_2O_2-induced oxidative stress from patients with MD and healthy donors. We also investigated the ability of ATP treatment to modulate the cell viability of LCLs loaded with H_2O_2 and considered the relationship between the sensitivity of the H_2O_2-treated LCLs to ATP and the staging scale of MD. The aim of this study was to examine whether the difference of the susceptibility of LCLs to H_2O_2-induced oxidative stress and the effect of ATP treatment reflects the prognosis of MD. To our knowledge, this is the first report in which LCLs established from the patients were used as a cellular model for MD.

2. Materials and Methods

2.1. *Materials*

Ten patients with MD as defined by the American Academy of Otolaryngology-Head and Neck Surgery (AAO-HNS) (4 males and 6 females, 54.7 ± 13.7 years old) and 10 healthy volunteers (5 males and 5 females, 53.5 ± 11.4 years old) were studied. Informed consent was obtained from all cases. Sensory hearing threshold was classified on the four-way classification of the American Academy of Otolaryngology (Committee on Hearing and Equilibrium, 1995): Stage 1 (mean threshold < 26 dB), Stage 2 (mean threshold 26-40 dB), Stage 3 (mean threshold 41-70 dB), and Stage 4 (mean threshold > 71 dB). Mean threshold was in each case calculated as the arithmetic mean of the threshold at 4 frequencies (500, 1,000, 2,000, and 3,000 Hz) measured on the same day as the dynamic posturography session.

2.2. *Cell Culture*

Peripheral blood lymphocytes (PBMC) were obtained from patients with MD and normal controls and transformed by Epstein-Barr virus (B95-8 strain) for establishing LCLs as described elsewhere (*16*,*17*). The LCLs were grown in complete medium consisting of RPMI 1640 (Sigma-Aldrich Co., St. Louis, MO, USA) supplemented with 10 % fetal calf serum (FCS).

2.3. *Viability assay*

Cells were suspended at a density of 4.0×10^6 cells/mL in complete medium and seeded at 1.0×10^5 cells per well of a 96-well UV plate (Nunc, Roskilde, Denmark). An equal volume of 0.04 mM H_2O_2 was added to each cell suspension and the mixtures were incubated at 37°C. The adequate concentration of H_2O_2 in this study was 0.02 mM at the final concentration by determining the results of the preliminary experiments and on the basis of the effects of H_2O_2 in HeLa cells (*18*), fibroblasts (*19*), cardiac myocytes (*20*), or human T-lymphoma (*21*). ATP (GE Healthcare, Salt Lake City, UT, USA) was also added as 20× stock solution to 5 mM at the final concentration. Cell viability was determined by trypan blue exclusion. Cells that were treated with H_2O_2 with or without ATP and incubated at 37°C for the times indicated were suspended in an equal volume of 0.4% trypan blue (Invitrogen, Carlsbad, CA, USA). Dead (blue) and live (clear) cells were counted using a hemocytometer. The percentage of viability was defined as the number of live cells divided by the number of live and dead cells. All experiments were performed in triplicate.

2.4. *Morphological examinations of cells*

LCLs exposed to the medium containing 0.02 mM H_2O_2 for 5 h were observed under a phase-contrast microscope. For determination of nuclear morphological change, the cells were additionally incubated with 1 µL of Hoechst 33258 (Dojido, Kumamoto, Japan) (1 mg/mL) for 10 min. After staining, the cells were washed with PBS and analyzed under a fluorescence microscope.

2.5. *Statistical Analysis*

All statistical analysis was performed using Ystat 2004.

xls program for Windows/Macintosh. Descriptive statistics were used to describe the response, and paired *t*-test or unpaired *t*-test was used where appropriate. Continuous data were displayed as the mean ± SD. Statistical significance was accepted when the *p* value was less than 0.05.

3. Results

3.1. *Clinical trial*

The results are summarized in Table 1. AAO-HNS Staging scale identified two patients (Me1 and Me10) with Stage 1, three patients (Me3, Me4, and Me7) with Stage 2, one patient (Me8) with Stage 3, and four patients (Me2, Me5, Me6, and Me9) with Stage 4.

3.2. *Susceptibility of LCLs to killing by H_2O_2*

After treatment with 0.02 mM H_2O_2, the viabilities of LCLs from MD patients and from normal controls were compared (Figure 1). These data showed that LCLs from MDs were significantly more sensitive ($p < 0.001$) to H_2O_2 than LCLs from healthy donors after 3 h of H_2O_2 treatment. These results indicate that control cells are resistant to H_2O_2 treatment, while MD cells are sensitive.

Table 1. Demographic and clinical characteristics

	Age	Gender	AAO-HNS Staging
Me1	40	F	1
Me2	70	F	4
Me3	68	M	2
Me4	38	M	2
Me5	66	M	4
Me6	57	F	4
Me7	66	F	2
Me8	44	F	3
Me9	36	F	4
Me10	62	M	1

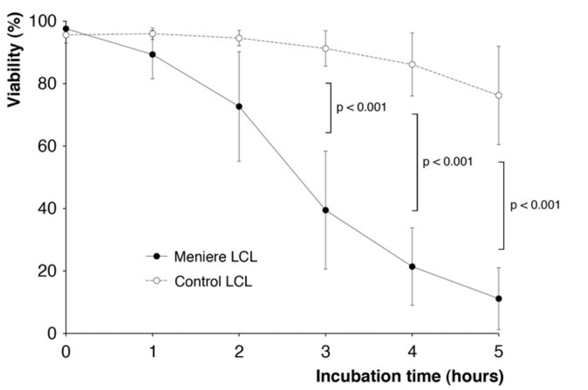

Figure 1. Comparison of the viabilities of LCLs from MDs and healthy donors after H_2O_2 treatment. LCLs from MDs were significantly more sensitive ($p < 0.001$) to H_2O_2 than LCLs from healthy donors after 3 h of H_2O_2-treatment.

3.3. *Effects of ATP addition on H_2O_2 cell damage in LCLs*

Figure 2 showed that the viability after 5 h of 0.02 mM H_2O_2 treatment were 24.6% in Me1 cells, 3.3% in Me2 cells, 27.8% in Me3 cells, 8.2% in Me4 cells, 5.9% in Me5 cells, 0% in Me6 cells, 5.6% in Me7 cells, 9.4% in Me8 cells, 4.3% in Me9 cells, and 22.2% in Me10 cells. Addition of ATP could obviously recover the viabilities of the H_2O_2-treateted LCLs from Me1, Me3, Me4, Me7, and Me10 patients (Figures 2A, 2C, 2D, 2G, and 2J). In contrast, the viabilities of H_2O_2-treated LCLs from Me2, Me5, Me6, Me8, and Me9 patients decreased in a similar manner as when ATP was not added (Figures 2B, 2E, 2F, 2H, and 2I). The viabilities after 5 h of treatment of 0.02 mM H_2O_2 and the addition of 0.05 mM ATP were 89.9% in Me1 cells, 7.2% in Me2 cells, 84.8% in Me3 cells, 61.1% in Me4 cells, 10.0% in Me5 cells, 0% in Me6 cells, 80.1% in Me7 cells, 7.8% in Me8 cells, 4.4% in Me9 cells, and 79.3% in Me10 cells. These data indicate that Me1, Me3, Me4, Me7, and Me10 cells might be sensitive to ATP. On the other hand, Me2, Me5, Me6, Me8, and Me9 cells are not as strongly affected by ATP.

3.4. *The relationship between the sensitivity of H_2O_2-treated LCLs to ATP and the staging scale of MD*

As shown in Figure 3, the ATP-sensitive LCLs (Me1, Me3, Me4, Me7, and Me10) were classified as Stage 1 or 2, while the ATP-insensitive LCLs (Me2, Me5, Me6, Me8, and Me9) were classified as Stage 3 or 4. There were significant differences ($p < 0.01$) in the viabilities of H_2O_2-treated LCLs classified as Stage 1 or 2 and as Stage 3 or 4 in MD after the addition of ATP.

3.5. *Effects of H_2O_2 on membrane integrity and chromatin structure*

Both chromatin condensation and swelling of the cell body were observed after treatment with 0.02 mM H_2O_2 (Figure 4).

4. Discussion

Treatment of cells with H_2O_2 induces oxidative stress, accompanied by lipid peroxidation, DNA and protein damage (*22*), and finally cell death (*23*). In addition, oxidative stress is able to disturb cellular energy metabolism as a result of the decrease of ATP in a variety of cells (*24*). These studies were based on the hypothesis that the susceptibility of individual cells to oxidative stress was different from one person to another. Our results strongly demonstrated that LCLs from patients with MD were significantly more sensitive ($p < 0.001$) to oxidative stress than LCLs

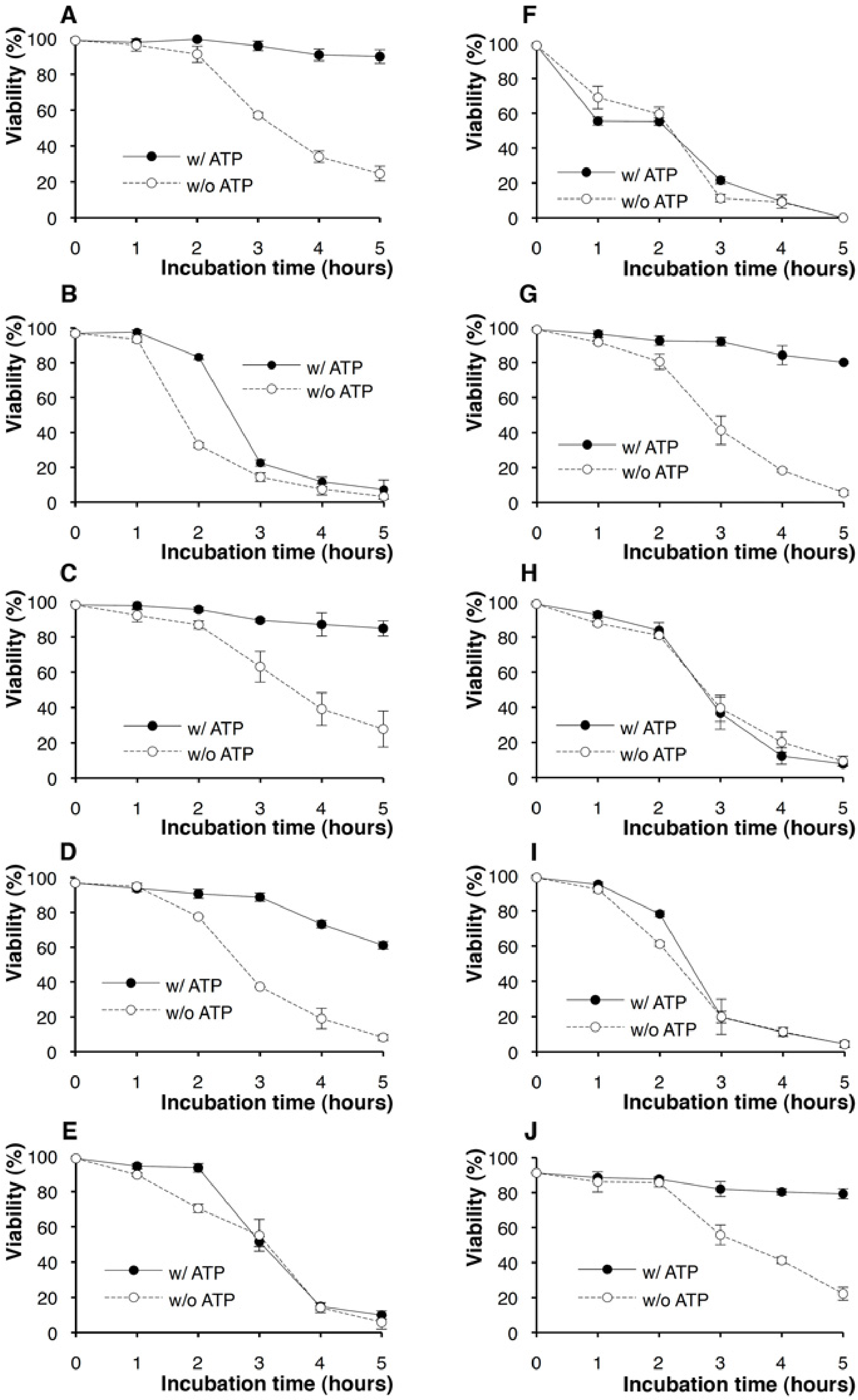

Figure 2. Effect of ATP addition to the viabilities of LCLs after H_2O_2 treatment. Addition of ATP could obviously recover the viabilities of H_2O_2-treated LCLs from Me1, Me3, Me4, Me7, and Me10 patients (**A**, **C**, **D**, **G**, and **J**, respectively). In contrast, the viabilities of H_2O_2-treated LCLs from Me2, Me5, Me6, Me8, and Me9 patients decreased in a similar manner as when ATP was not added (**B**, **E**, **F**, **H**, and **I**, respectively).

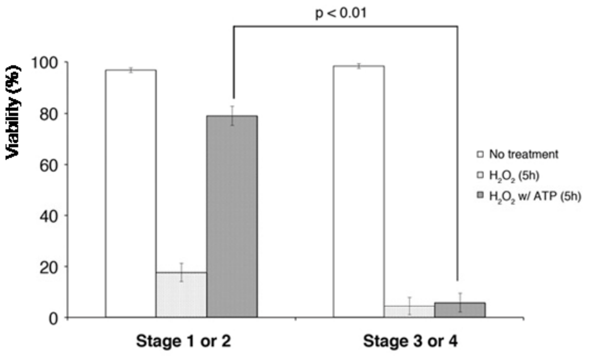

Figure 3. Comparison of the viabilities of H_2O_2-treated LCLs from MD classified on the four-way classification. There were significant differences ($p < 0.01$) of cell viabilities of H_2O_2-treated LCLs classified as Stage 1 or 2 and as Stage 3 or 4 after the addition of ATP.

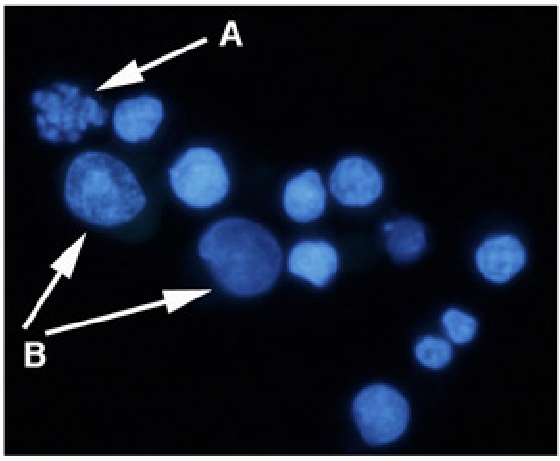

Figure 4. Effects of H_2O_2 on membrane integrity and chromatin structure in LCLs. LCLs treated with H_2O_2 for 5 h showed either chromatin condensation (**A**) or swelling of the cell body (**B**).

from healthy donors (Figure 1). In other words, LCLs from healthy donors were resistant to H_2O_2, while LCLs from MD patients were not. This finding also suggests that patients with MD can be diagnosed by the difference of susceptibility of established LCLs to oxidative stress.

Next, we investigated the effect of ATP treatment on H_2O_2-treated LCLs. At an early stage of cell damage, ATP-depletion and intracellular Ca^{2+} alteration may occur under H_2O_2-induced oxidative stress (*25*). The concentration of extracellular ATP regulates various signaling systems including propagation of intercellular Ca^{2+} signals. To reveal the cause of ATP-depletion after the exposure of cells to H_2O_2, the oxidative inactivation of mitochondrial ATP synthetase was examined (*26*). Lee *et al*. reported that epithelial cells of the inner ear coordinated their ion transport activity through the autocrine and paracrine signal pathway among neighboring cells in the ear *via* ATP (*27*). In addition, ATP is one of the commonly used medications for the treatment of MD in Japan (*28*). Our results demonstrated that the addition of ATP to H_2O_2-treated LCLs clearly recovered the viabilities in Me1, M3, M4, Me7, and Me10 cells, although the cells from Me2, Me5, Me6, Me8, and Me9 did not recover their viability after ATP treatment. Therefore, we thought that Me1, M3, M4, Me7, and Me10 cells were sensitive to ATP treatment, whereas Me2, Me5, Me6, Me8, and Me9 cells, by contrast, were not. Interestingly, as shown in Table 1, all ATP-sensitive cases were classified as AAO-HNS Stage 1 or 2 and all ATP-insensitive cases were classified as AAO-HNS Stage 3 or 4. After the ATP treatment, there was a significant difference ($p < 0.01$) of the viabilities of the H_2O_2-treated LCLs classified as Stage 1 or 2 and Stage 3 or 4 in MD (Figure 3). These results demonstrated that the sensitivity of H_2O_2-treated LCLs to ATP might represent a method for prognosis and treatment of MD. Clinically, some of patients staged 3 or 4 experiences poor control of vertigo, the progressive sensorineural hearing loss and the worsening of tinnitus even after several years treatment. The treatment of MD mainly aims to reduce these symptoms, because all three symptoms, either separately or in combination, cause great distress and have a considerable impact on the patients quality of life (*29*). Therefore, the prognostic expectation of MD is very profound for the quality of life of patients with MD. Additionally, these LCLs established from patients may be used for the drug susceptibility test in MD.

We also investigated morphological changes of the LCLs treated with H_2O_2. Figure 4 showed that after 5 h treatment, H_2O_2-treated LCLs showed either chromatin condensation or swelling of the cell body. Chromatin condensation indicates apoptosis of LCLs and swelling of cells body indicates necrosis. Since apoptosis is a highly regulated and energy-dependent process (*30*), the ATP decline may point to a relevant ATP consumption related to apoptosis. Saito *et al*. reported that prevention of intracellular ATP loss significantly activated caspases and changed the mode of cell death from necrosis to apoptosis, and therefore ATP-dependent apoptosome formation determined whether H_2O_2-induced cell death was due to apoptosis or necrosis (*21*). We previously reported oxidative stress in the form of H_2O_2-induced morphological changes in vestibular hair cells (*31*). This finding suggested oxidative stress caused by H_2O_2 might affect the morphology and survival of inner ear cells.

In this study, 10 EBV-transformed LCLs derived from patients with MD were examined. It is not clear how these cells accurately represent the endogenous condition of inner ear cells, because various genes are expressed in each cell. In spite of these restrictive conditions, our results suggested that patients with MD might be unable to cope with oxidative stress.

In conclusion, LCLs established from MD patients could be used as a unique model to detect the susceptibility to oxidative stress and the effect of ATP treatment in MD patients. The difference of the sensitivity of H_2O_2-treated LCLs to ATP might relate to the prognosis of MD. This system may form the basis of tailor-made therapy for MD.

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