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ROLE OF MACROPHAGE MIGRATION INHIBITORY FACTOR IN AGE-RELATED HEARING LOSS

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Abstract—Hearing loss related to aging is the most common sensory disorder among elderly individuals. Macrophage migration inhibitory factor (MIF) is a multi-functional molecule. The aim of this study was to identify the role of MIF in the inner ear. MIF-deficient mice (MIF$^{-/-}$ mice) of BALB/c background and wild-type BALB/c mice were used in this study. Expression of MIF protein in the inner ear was examined by immunohistochemistry in wild-type mice (WT). The hearing function was assessed by the click-evoked auditory brainstem response in both MIF$^{-/-}$ mice and WT at 1, 3, 6, 9, 12, and 18 months of age. Morphological examination of the cochlea was also performed using scanning electron microscopy and light microscopy. MIF was observed in the spiral ligament, stria vascularis, Reissner's membrane, spiral ganglion cells (SGCs), saccular macula, and membranous labyrinth. The MIF$^{-/-}$ mice had a significant hearing loss as compared with the WT at 9, 12, and 18 months of age. In the MIF$^{-/-}$ mice, scanning electron microscopy showed that the outer cochlear hair cells were affected, but that the inner cochlear hair cells were relatively well preserved. The number of SGCs was lower in the MIF$^{-/-}$ mice. MIF was strongly expressed in the mouse inner ear. Older MIF$^{-/-}$ mice showed accelerated age-related hearing loss and morphological inner ear abnormalities. These findings suggest that MIF plays an important role in the inner ear of mice.

Key words: macrophage migration inhibitory factor, hearing loss, cochlea, ear, hair cell, spiral ganglion cells.

INTRODUCTION

Age-related hearing loss, also known as presbycusis, is a common and serious human health concern. Approximately 10% of the population has a significant hearing loss that impairs communication, and this rate increases to 40% in those older than 65 years (Gates and Mills, 2005). Multiple factors, including noise damage, genetic susceptibility, otological disorders, systemic diseases, and exposure to ototoxic agents, affect the inner ear. The impaired central processing of acoustic information is also related to reduced hearing sensitivity, speech understanding in noisy environments, and impaired localization of sound sources in age-related hearing loss (Huang and Tang, 2010; Roth et al., 2011).

Macrophage migration inhibitory factor (MIF) was originally described as a factor that inhibited the random migration of macrophages. MIF is an innate immunity molecule with ubiquitous tissue expression. However, it has now been identified as having multiple functions, such as acting as a pituitary hormone, growth factor, and pro-inflammatory cytokine (Calandra and Roger, 2003).

MIF is strongly expressed in the central nervous system, but its function has not yet been defined (Fingerle-Rowson and Bucala, 2001). MIF is also observed in the peripheral nerves, and plays an important role in the acceleration of peripheral nerve regeneration and in the prevention of Schwann cell apoptosis (Nishio et al., 1999; Nishio et al., 2002). In an experimental animal study, MIF has been shown to be an essential factor in axis formation and neural development in embryos of the African clawed frog (*Xenopus laevis*) (Suzuki et al., 2004). A recent study showed that MIF plays a key instructional role for sensory hair cell differentiation, semicircular canal formation, and statoacoustic ganglion development in zebrafish (*Danio rerio*) (Shen et al., 2012).

After the generation of MIF knockout mice, numerous studies reported various functions of MIF both within and outside the immune system (Calandra and Roger, 2003). MIF is considered an important factor in the pathogenesis of middle ear diseases in both humans and mice, but the role of MIF in the mammalian inner ear has not been fully defined (Kariya et al., 2003, 2008a,b). The purpose of this study was to examine the expression of MIF in the inner ear and to confirm its role in cochlear function in mice.

EXPERIMENTAL PROCEDURES

Animals

BALB/c mice were used in this study. Through targeted disruption of the MIF gene, MIF-deficient mice

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Abbreviations: ABR, auditory brainstem response; MIF, macrophage migration inhibitory factor; SGCs, spiral ganglion cells; SL, spiral ligament; SV, stria vascularis; WT, wild-type mice.

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(MIF$^{-/-}$ mice) were generated from a BALB/c background (Honma et al., 2000). In brief, a gene-targeting vector was generated using a 6.0-kb *Xba*I fragment that contained all of the MIF exons subcloned. A 201-bp *Sac*I fragment consisting of the 3′ region of exon 1 and the 5′ region of intron 1 was replaced with a pMC1-neo poly (A) cassette in a forward orientation relative to MIF gene transcription. A DT-A cassette was also introduced at the 3′ flanking region for negative selection. R1 embryonic stem cells were transfected and subjected to positive selection. Subsequently, they generated germline chimeras with targeted disruption of the MIF gene by the aggregation method, and then generated a mouse strain deficient in the MIF gene. To achieve a pure strain of the BALB/c background on MIF$^{-/-}$ mice, backcrossing was performed more than 10 times. Otoscopic examination was performed for all mice prior to treatment in order to ensure that the tympanic membranes were normal and that no middle ear effusion was present. Mice were deeply anesthetized using intraperitoneal injection of both ketamine (100-mg/kg body weight) and xylazine (10-mg/kg body weight). This study was performed in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals, revised 1996. The Animal Research Control Committee of the Okayama University approved the study.

Immunohistochemistry

Wild-type BALB/c mice (n = 2, 8 weeks old) were deeply anesthetized using an intraperitoneal injection of both ketamine and xylazine. The mice were decapitated, and the temporal bones were fixed in fresh 4% paraformaldehyde in phosphate-buffered saline for 24 h at 4 °C. They were then decalcified in 4% ethylenediaminetetraacetic acid in phosphate-buffered saline for 14 days at 4 °C. Immunohistochemical staining was performed on the paraffin-embedded tissues using a rabbit polyclonal MIF antibody (sc-20121; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA, dilution 1:100) and 3,3′-diaminobenzidine (DAB) reagent (Dako, Glostrup, Denmark) according to the manufacturer's instructions.

Auditory brainstem response (ABR)

MIF$^{-/-}$ mice (n = 5) and wild-type mice (WT) (n = 5) were used in this study. The animals were anesthetized. The stimuli used were clicks generated by a RP2.1 Enhanced Real-Time Processor (Tucker-Davis Technologies, Gainesville, FL, USA) with a plateau of 0.1 ms. A closed system with monaural stimulation was used, and clicks were delivered directly to the outer ear canal by a plastic tube. ABR was evoked with clicks, and was recorded with needle electrodes inserted through the skin (vertex to the ipsilateral retroauricle with a ground at the contralateral retroauricle). Responses were processed through a 300-Hz to 3000-Hz bandpass filter and were averaged 1000 times using a signal processor RA16 (Tucker-Davis Technologies, Gainesville, FL, USA). Stimuli were applied in 10-dB steps with a 21-Hz stimulus repetition rate. ABR thresholds were defined as the lowest sound level at which the response peaks were clearly present in stacked waveforms. ABR thresholds were examined at 1, 3, 6, 9, 12, and 18 months of age.

Electron microscopy

MIF$^{-/-}$ mice (n = 5) and WT (n = 5) were used in this study. The animals were anesthetized, and cochleae were collected. The tissues were fixed with 2% paraformaldehyde and 2% glutaraldehyde in 0.1 M sodium phosphate buffer (pH 7.4). The specimens were post-fixed in 2% osmium tetroxide, dehydrated through a graded ethanol series, and then dried by critical point drying. The morphological findings of stereocilia of cochlear hair cells were investigated using scanning electron microscopy. The numbers of inner and outer hair cells were counted, and the results were expressed as the percentage of remaining hair cells, as described previously (Fetoni et al., 2009; Miller et al., 2012).

Light microscopy

MIF$^{-/-}$ mice (n = 5) and WT (n = 5) at 9 months of age were used in this study. The mice were sacrificed and the temporal bones were collected. The tissues were fixed in fresh 4% paraformaldehyde in phosphate-buffered saline for 24 h, followed by decalcification in 4% ethylenediaminetetraacetic acid in phosphate-buffered saline for 14 days at 4 °C. Cochleae were embedded in paraffin, and were cut at a thickness of 10 μm. Hematoxylin and eosin staining was performed for histopathological study.

The number of spiral ganglion cells (SGCs) in the mid-modiolar section of the cochlea was counted under light microscopy according to the methods of previous studies (Willott et al., 1998; Tang et al., 2014). Briefly, the cochlea was divided into four segments (extreme base ('hook' region), mid-base (basal turn of mid-modiolar section), mid-apex (apical turn of mid-modiolar section, but below the apex), and apex (apex of mid-modiolar section)). The cell density of the SGCs was counted in each segment using a 100-μm × 100-μm eyepiece reticle. The criterion for inclusion of SGCs was a complete nucleus.

Statistical analysis

Data are presented as mean ± standard deviation. For statistical analysis, the non-parametric Mann–Whitney U test was used for comparison between two groups. Significant differences were assumed at a level of $P < 0.05$ (IBM SPSS Statistics; IBM, New York, NY, USA).

RESULTS

Expression of MIF in inner ear

We first examined the expression of MIF in the inner ear of WT. Positive immunostaining for MIF was observed in the spiral ligament (SL), stria vascularis (SV), spiral