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MACROPHAGE MIGRATION INHIBITORY FACTOR MEDIATES VIABILITY AND APOPTOSIS OF PVM/MS THROUGH PI3K/AKT PATHWAY

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Abstract—Macrophage migration inhibitory factor (MIF) plays an important role in hearing function; however, the underlying mechanism remains indistinct. PVM/Ms from the stria vascularis of lateral wall of cochlea in young and aged mice were isolated, and the mRNA and protein expression levels were detected. MIF was knocked down or overexpresssed *in vitro*, and transfection was performed *in vivo*. Cell viability and apoptosis were determined by MTT assay and flow cytometry analysis, respectively. The hearing ability was tested by the auditory brain stem response. The results showed that MIF expression was significantly downregulated in aged mice. In aged mice, the viability of PVM/Ms significantly decreased, but the apoptotic number markedly increased. MIF knockdown in PVM/Ms *in vitro* significantly inhibited cell viability and induced cell apoptosis, but MIF overexpression showed contrasting results. Further studies showed that MIF knockdown in young mice resulted in serious hearing loss, but MIF overexpression in aged mice restored the hearing. Si-MIF inhibited the viability and induced apoptosis of PVM/Ms from young mice, whereas Ad-MIF induced the viability and inhibited apoptosis of PVM/Ms from aged mice. Moreover, MIF effectively altered the expression levels of CDK1, BRAF, p-ERK1/2, p-PI3K, and p-Akt. Furthermore, ERK inhibitor PD98059 or PI3K inhibitor LY294002 significantly reversed the effects of Si-MIF on PVM/Ms from young mice, whereas ERK activator EGF or PI3K activator IGF significantly reversed the effects of Ad-MIF on PVM/Ms from aged mice. Taken together, MIF mediates the viability and apoptosis of PVM/Ms, at least partially, through MAPK and/or PI3K/Akt pathway.

Key words: Macrophage migration inhibitory factor, Perivascular-resident macrophage-like melanocytes, Presbycusis, MAPK, PI3K/Akt pathway.

INTRODUCTION

Presbycusis, also named age-related hearing loss (AHL), is a common neurodegenerative disorder that is characterized by gradual and progressive sensorineural hearing loss (Heman-Ackah et al., 2008). Presbycusis affects 40% of patients between 65 and 66 years of age and 66.8% of people between 73 and 74 years of age (Gopinath et al., 2009). Presbycusis not only affects the quality of life and leads to dependence, isolation, and frustration but also affects the healthy people around the patient (Frisina and Frisina, 1997; Gates and Mills, 2005; Heman-Ackah et al., 2008; Huang and Tang, 2010; Campo et al., 2012). The risk factors for presbycusis include heritability, environment factors, free radical, medical conditions, and damage of mitochondrial DNA (Huang and Tang, 2010). Recent studies also identified several proteins related to the progress of presbycusis (Rüttiger et al., 2007; Wang et al., 2010).

A previous study indicated that macrophage migration inhibitory factor (MIF) is a unique cytokine and has been identified as a critical mediator of host defenses, playing an important role in septic shock, chronic inflammatory diseases, and autoimmune diseases (Lue et al., 2002). A recent study suggested that MIF was observed in the Reissner's membrane, stria vascularis, saccular macula, spiral ligament, spiral ganglion cells, and membranous labyrinth (Kariya et al., 2014). The outer cochlear hair cells of MIF-deficient mice were affected, and these mice also had a significant hearing loss and decreased number of spiral ganglion cells as compared with those of wild-type mice at 9, 12, and 18 months of age (Kariya et al., 2014). MIF was also strongly expressed in the mouse inner ear. Older MIF-deficient mice showed morphological inner ear abnormalities and accelerated AHL (Kariya et al., 2014). However, the underlying mechanism was not clear yet. Because MIF plays an important role in the signal transmission of cell viability and cell growth (Nishihira, 2000; Obikane, 2004), it raises the possibility that MIF plays important roles in the hearing function by regulating cell growth.

It is well accepted that the integrity of the intrastrial fluid-blood barrier is one of the tightest blood–tissue barriers in mammals, which is situated between blood flow and the intrastrial region of the stria vascularis. This region is important to maintain inner ear homeostasis and is an essential driving factor for hearing function (Juhn et al., 1981, 2001; Salt et al., 1987; Takeuchi et al., 2001). A large number of perivascular-resident macrophage-like melanocytes

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Abbreviations: ABR, auditory brain stem response; AHL, age-related hearing loss; CDK1, cyclin-dependent kinase1; DMSO, dimethyl sulfoxide; MIF, migration inhibitory factor; PVM/Ms, perivascular-resident macrophage-like melanocytes.

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(PVM/Ms), perivascular cells that possess both macrophage and melanocyte characteristics, were reported to be found in the intrastrial fluid–blood barrier (Dai et al., 2010; Nenadis et al., 2010). Further study verified that PVM/Ms exert a vital effect in maintaining the integrity of the intrastrial fluid-blood barrier and function (Zhang et al., 2012). Taken together, these investigations may indicate the important role of PVM/Ms in hearing function.

On the basis of the fact that MIF plays an important role in cell viability, we speculated that MIF regulates PVM/Ms growth and thus plays important roles in hearing function. To validate our speculation, PVM/Ms from stria vascularis of lateral wall of cochlea in young and aged mice, respectively, were isolated. MIF was knocked down or overexpresssed both *in vitro*, and *in vivo*. Cell viability and cell apoptosis were performed to explore the effects of MIF on the growth of PVM/Ms. The hearing function was tested to explore the effects of MIF on hearing function. The protein expression levels of cell cycle-related protein CDK1, and BRAF, p-ERK1/2, p-PI3K, and p-Akt were detected by Western blotting. Our results revealed that MIF mediates the viability and apoptosis of PVM/Ms through MAPK and/or PI3K/Akt pathway, which may provide a potential therapy and prevention method for presbycusis.

EXPERIMENTAL PROCEDURES

Animals

Approval for all experiments was obtained from the Ethics Committee of the First Affiliated Hospital of Zhengzhou University. C57BL/6J mice were chosen in our study because of their rapid, high-frequency hearing loss during the first year of life (Hunter and Willott, 1987; Keithley et al., 2004). A total of 64 mice, purchased from the Chinese Academy of Medical Sciences, were used in our study (Beijing, China). Mice aged 2 and 10 months were termed as the young and the aged group, respectively (Fig. 1; $n = 20$ in each group) and PVM/Ms were isolated from mice aged 2 and 10 months respectively Another 24 mice were divided into four groups ($n = 6$ in each group): mice with adenovirus MIF overexpression vector (Ad-MIF), mice with *in vivo* siRNA transfection (Si-MIF), and the corresponding control groups. The mice were housed 2 or 3 per cage and had free access to food and water, with suitable temperature and humidity conditions and a 12/12-h light/dark cycle.

PVM/M isolation and culture

PVM/Ms were isolated from mice aged 2 and 10 months respectively and were cultured as previously described (Zhang et al., 2012; Neng et al., 2013). The stria vascularis of lateral wall of cochlea was firstly isolated. For the production of PVM/Ms, the minced stria vascularis was then cultured on collagen-coated dishes in medium 254 (Invitrogen, Carlsbad, CA, USA), containing 10% FBS, 0.5% gentamicin/amphotericin, and 1% human melanocyte growth factor. The cells were incubated at 37 °C in an atmosphere of 5% CO_2, and the medium was changed every 3 days. Cell clones were formed at about 10 days. With a solution of trypsin-EDTA, the cells were detached from the cell colony and purified. In experiments using ERK inhibitor PD98059 (Alexis Biochemicals, Florence, Italy), PI3K inhibitor LY294002 (Cell Signaling Technology, Beverly, MA, USA; 0.1%), ERK activator EGF (Sigma, St. Louis, MO, USA; 100 ng/ml) and PI3K activator IGF (Sigma; 100 ng/ml), cells were pretreated with the inhibitors separately for 1 h. PD98059 was dissolved in dimethyl sulfoxide (DMSO), and the final concentration of DMSO was never exceeded 0.05% during the treatment stage. LY294002 was dissolved in DMSO. EGF was dissolved in sterile normal saline.

MIF knockdown and overexpresssion *in vitro*

MIF silencing *in vitro* was performed on passage 3 PVM/Ms seeded in 24-well plates, as described previously (Zhang et al., 2012). The PVM/Ms (1×10^5 cells per well) were transfected with Si-MIF (Applied Biosystems, USA). Briefly, 2.5 μL TransIT-TKO reagent and 50 μL serum-free medium were mixed in a sterile tube and incubated for 15 min. Then, 25 nM siRNA was added to the mixture and incubated for another 15 min, which was then added to the 24-well plates for transfection. The recombinant adenovirus Ad-MIF was constructed by homologous recombination after cotransfecting 293 cells with a virus-rescuing vector pAdBHG10 and MIF, as described previously (Lubberts et al., 1999). High titers of recombinant adenoviruses were amplified, purified, titered, stored, and used to infect PVM/Ms cells (Lubberts et al., 1999). After 4 days, the transfection efficiency was determined by Western blotting, and cells were used for cell viability and apoptosis assay.

MIF knockdown and overexpresssion *in vivo*

The *in vivo* siRNA transfection was performed as described previously (Kaur et al., 2011; Zhang et al., 2012). Briefly, animals ($n = 6$) were anesthetized, and a 30-G needle was used to make a single puncture in the anterior-inferior quadrant of the tympanic membrane to allow the exit of air from the middle ear during drug injection. MIF was silenced with a 5-μL solution of siRNA (20 ng/μL) injected through the posterior-inferior quadrant of the tympanic membrane. The siRNA for MIF was as follows: Forward, 5′-GCGTAATACGACTCACTATAGGGAGAGCCACCATGCCTATGTTCATC-3′ and Reverse, 5′-GCGTAATACGACTCACTATAGGGAGAGACTCAAGCCAAGGTGGAAC-3′.The middle ear was filled completely with the solution for 5 days. Scrambled siRNA at the same concentration was given to the control group ($n = 6$). Eight days after the *in vivo* siRNA transfection, the transfection efficiency was determined by Western blotting. Auditory brain stem responses (ABRs) were detected 2, 4, and 6 months after the knockdown of MIF. The construction of adenovirus overexpression vector (Ad-MIF) and the intramyocardial injection of Ad-MIF (5×10^9 IFU/ml) into mice ($n = 6$) were performed as previously described (Ma et al., 2009). The empty recombinant adenovirus vector was used as the control group ($n = 6$). ABRs were detected after 10, 12, and 14 months after overexpression of MIF.