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ALH-L1005 attenuates endotoxin induced inner ear damage

Chul Ho Jang [a,b,*], Yong Beom Cho [a], Cheol Hee Choi [b,c], Yoon Seok Jang [c], Sook Jin Jang [c], Won-Kyo Jung [d], Byung Young Park [e], Min Young Kim [e]

[a] Department of Otolaryngology, Chonnam National University Medical School, Gwangju, South Korea
[b] Research Center for Resistant Cells, Chosun Medical School, Gwangju, South Korea
[c] Department of Bio New Drug Development, Chosun University, Gwangju, South Korea
[d] Department of Biomechanical Engineering, and Center for Marine-Integrated Biomedical Technology (BK21 Plus), Pukyong National University, Pusan, South Korea
[e] AngioLab, Daejeon, South Korea

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ABSTRACT

Objective: To assess whether this compound (ALH-L1005) is conceivably an effective agent in protecting against cochlear damage induced by LPS.
Materials and methods: Tube formation using human umbilical vein endothelial cell (HUVEC) and matrix metalloproteinase (MMP)-9 inhibition assay was performed. 24 guinea pigs were randomly divided into three groups. Intratympanic instillation of LPS ($n = 8$) as negative control, instillation of oxytetracycline 1 h after LPS as positive control ($n = 8$), and intratympanic instillation of ALH-L1005 ($n = 8$) 1 h after LPS were considered experimental group. Evaluation by auditory brainstem response (ABR) measurement, cochlear blood flow, and blood–labyrinth barrier (BLB) permeability were performed. Cochlear hair cells were observed by field emission-scanning electron microscopy (FE-SEM). MMP-9 activation was measured by gelatin zymography.
Results: For HUVEC, the tube formation was suppressed in a dose dependant manner. ALH-L1005 inhibited the MMP-9 activity prominently. It also attenuated the elevation of LPS-induced hearing threshold shift and recovery of CBF. By FE-SEM, cochlear hair cells could be preserved in experimental group. ALH-L1005 significantly reduced the BLB opening compared to LPS group. Active MMP-9 expression could be detected in the LPS group. In contrast to ALH-L1005 group, active MMP-9 expression was not detected.
Conclusion: Our results conclude that ALH-L1005 showed a protective effect in the cochlear lateral wall damage induced by LPS.

1. Introduction

It is well known that various types of otitis media, including otitis media with effusion, chronic otitis media with cholesteatoma can cause sensorineural hearing loss [1]. However, the mechanisms of sensorineural hearing loss associated with otitis media are not well understood. Most of the middle ear inflammatory mediators and toxins from the middle ear enter into the inner ear through the round window membrane [2]. It is known that intratympanic instillation of lipopolysaccharide (LPS) causes profound pathological changes in the inner ear [3–5].

The blood–labyrinth barrier (BLB) serves to protect the inner ear from damage by exogenous molecules. The functional significance of the BLB is to restrict immune cell migration and diffusion of soluble molecules from the systemic compartment of the body into the inner ear [6]. The microvascular endothelial cells are the important component of BLB as well as blood–brain barrier (BBB). Angiogenesis is one of the processes required for the formation and function of all organs and is involved in both physiological and pathological situations. Neovascularization is a multistep process requiring the degradation of the basement membrane, endothelial cell migration, capillary tube formation, and endothelial cell proliferation. Among cytokines, our results, in accordance with those of a recent study, suggest that VEGF expressions may be related not only to angiogenesis but also to BBB disruption after focal cerebral ischemia [7]. The matrix metalloproteinase (MMPs) are a family of zinc binding, Ca^{+} dependant neutral endopeptidases that can act to degrade most components of the extracelluar matrix. Two members of the MMP family, MMP-2 and MMP-9, are subclassified for their preferential abilities to degrade denatured collagens (gelatin) and collagen type IV, the main component of basement membrane [8]. In vitro, VEGF increases both expression and release of MMP-2 and

* Corresponding author at: Department of Otolaryngology, Chonnam National University Hospital, Dongku, Hakdong 8, Gwangju, South Korea.
Tel.: +82 62 2206774; fax: +82 62 2206776.
E-mail addresses: chulsavio@hanmail.net, jchsavio@gmail.com (C.H. Jang).

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MMP-9 [9]. Opening of the blood–brain barrier occurs when matrix metalloproteinases (MMPs), cytokines and free radicals are expressed and activated in a number of neurological diseases [10]. Inhibitors to the MMPs block the disruption of the blood–brain barrier, and have been proposed to treat neuroinflammation [11]. And LPS stimulate angiogenesis directly or indirectly via mediators [12]. In contrast to MMP-2, which is often expressed in a constitutive manner, MMP-9 synthesis and secretion are regulated, and in most cell types it occurs only upon stimulation. Direct evidence for a crucial role of MMP-9 in the breakdown of the BLB has been obtained by previous studies [13,14], suggesting that MMP-9 as a preferential target in the development of bacterial meningitis complicated labyrinthitis or LPS induced labyrinthitis.

Historically, the seed extract of horse chestnut (*Aesculus hippocastanum* L.) was used as a treatment for many ailments, including rheumatism, bladder and gastrointestinal disorders, fever, chronic venous insufficienty, post-operative edema, and topically for clearing skin conditions [15]. The primary active constituent of the leaf extract found in horse chestnut seed extract is aescin. Aescin is actually a mixture of triterpene saponins. Other constituents include bioflavonoids (quercetin and kaempferol), proanthocyanidin A2 (an antioxidant), and the coumarins fraxin and aseculin [16]. Extract from horse chestnut leaves showed the antiproliferative and antiangiogenic properties [17]. To date, there has been rare reports regarding the therapeutic effect of the horse chestnut leave extract on LPS induced labyrinthitis. ALH-L1005 is a compound, extracted from horse chestnut leaves with aqueous ethanol [18]. The purpose of this study was to determine whether this compound (ALH-L1005) is conceivably an effective agent in protecting against cochlear damage induced by LPS.

2. Materials and methods

2.1. Angiogenic tube formation test

To perform tube formation assay, human umbilical vein endothelial cells (HUVEC) were purchased from Lonza (Basel, Switzerland) and cultured in M199 medium supplemented with 10% fetal bovine serum and endothelial cell growth supplement (ECGS, Sigma–Aldrich, St. Louis, MO, USA) at 50 μg/ml in a 37 °C incubator with humidified atmosphere containing 5% CO_2. 200 μl of Matrigel (BD Biosciences, Bedford, MA, USA) was pipetted into the wells of a 48-well plate and allowed to solidify for 1 h at 37 °C. HUVEC were plated on Matrigel-coated wells at a density of 4×10^4 cells/well and incubated for 16 h at 37 °C with medium in the absence or presence of 10, 25, 50 and 100 μg/ml of ALH-L1005 dissolved in distilled water. The formation of capillary-like tubular networks was observed with an inverted microscope and photographed.

2.2. In vitro test for MMP-9 inhibition assay

MMP activities were measured on a spectrofluorometer LS50B (Perkin-Elmer, Waltham, MA, USA) using 2,4-dinitrophenyl-Pro-Leu-Gly-Met-Trp-Ser-Arg (Calbiochem, San Diego, CA, USA) as a substrate for MMP-9. Recombinant human MMP-9 were purchased from R&D Systems (Minneapolis, MN, USA) and used after activation with 1 mM APMA before the assay. MMP (10 nM) and substrate (1 mM) were mixed in 2 ml of reaction buffer (50 mM tricine, pH 7.5, 10 mM $CaCl_2$, 200 mM NaCl). Fluorescence intensity was measured at room temperature using a 280-nm excitation wavelength and a 360-nm emission wavelength.

2.3. In vivo test

Experiments were performed in 24 young male guinea pigs weighing 250–300 g (Samtaco Bio Korea, Osan, Korea) with normal tympanic membranes and Preyer's reflexes. All animal experiments were performed in accordance with the local ethical committee at Research Center for Resistant Cells, Chosun University. The guinea pigs were anesthetized by the intraperitoneal injection of zolletil® (1:1 combination of tiletamine and zolazepam) and xylazine hydrochloride. Ear canals and the tympanic membranes were examined under an operating microscope. Ear infection was excluded by the examination of the external auditory canal and the tympanic membrane. The guinea pigs were randomly divided into three groups (experimental, positive control, and negative control group). To investigate the therapeutic effects of MMPs inhibitor compound (ALH-L1005) in LPS-induced cochlear damage, intratympanic instillation of ALH-L1005 (30 μl 10 mg/ml, $n = 8$) 1 h after LPS instillation (30 μl, 3 mg/ml). Oxytetracycline (OTC) is a well-known broad spectrum MMPs inhibitor. In this study, OTC was included as a positive control. Intratympanic instillation of OTC (30 μl, 1.5 mg/ml, $n = 8$) was done 1 h after LPS instillation, and the intratympanic instillation of phosphate buffered saline (30 μl, $n = 8$) 1 h after LPS instillation was considered as a negative control. Intratympanic instillations were always done in the right ear. The left ear was left untouched and was the normal control.

2.4. Evaluation of auditory function

Auditory brainstem response (ABR) was recorded using an evoked potential system (Tucker-Davis Technologies; TDT, Alachua, FL, USA) and a Samsung computer. Stimuli were digitally synthesized using Siggen© software and presented through an ER-2 insert earphone (Etymotic Research, Elk Grive Village, IL, USA). Acoustic stimuli consisting of click (low frequencies less than 4 kHz) and 4, 8, 16, and 32 kHz tone bursts were produced. Tone bursts consisted of a 3 ms envelope: 1 ms ramp onset, 1 ms plateau and 1 ms decay. The ABR was recorded through Grass® stainless steel needle electrodes placed subcutaneously at the vertex (active), right cheek (inverting) and left cheek (common). The resulting signal was band-pass filtered (100–3000 Hz), amplified (10,000×) and digitized by a TDT Bioamp. Responses were collected and averaged at 30 presentations per second for up to 512 times. The stimulus was presented at 90 dB SPL and progressed downward in 10 dB steps until no response was identifiable. ABRs were assessed preoperatively, 3 days after intratympanic instillation. Analysis of variance (ANOVA) was used to test for differences in ABR thresholds between the three groups. A separate model was used for each frequency. Pairwise contrasts were done if an effect in treatment with ALH-L1005 group was statistically significant ($p < 0.05$).

2.5. Evaluation of cochlear blood flow (CBF)

After recording of ABR, a tracheotomy was performed to ensure free breathing. The CBF measurement was evaluated as previous described [14]. The right tympanic bulla was exposed by a ventral approach. It was opened by a sharp dental excavator. After the middle ear mucosa over the bony wall of the cochlea was removed with a cotton pledget, a 1.0 mm needle probe of a moorLAB™ laser Doppler flowmeter (Moor Instruments, Devon, UK) was placed on the bony lateral wall of the basal turn of the cochlea. CBF output data were sampled every 20 s and were analyzed using the PowerLab® computational data acquisition program (AD Instruments). The CBF ratio of the treated right ear and untreated left ear was calculated and analyzed. Normalized CBF in left ear was used as the baseline. The CBF ratio between each group was analyzed using Student *t*-test. A difference was assumed to be statistically significant when $p < 0.05$.