Microbes and Infection 16 (2014) 237–243

www.elsevier.com/locate/micinf

Original article

Acquired resistance of *Listeria monocytogenes* in and escaped from liver parenchymal cells to gentamicin is caused by being coated with their plasma membrane

Masakazu Kaneko, Yoshiko Emoto, Masashi Emoto*

Laboratory of Immunology, Department of Laboratory Sciences, Gunma University Graduate School of Health Sciences, Maebashi, Gunma 371-8511, Japan

Received 26 June 2013; accepted 9 November 2013
Available online 22 November 2013

Abstract

After systemic infection, a majority of *Listeria monocytogenes* invade liver parenchymal cells (LPC), replicate therein and spread to neighboring cells, suggesting that 3 different types of *L. monocytogenes* exist in the liver: *L. monocytogenes* being unable to invade LPC, residing in LPC, and escaped from infected LPC. Although listeriolysin O (LLO) participates in escape of *L. monocytogenes* from macrophages and *L. monocytogenes* is susceptible to gentamicin (Gm), it remains elusive whether LLO participates in invasion/escape of *L. monocytogenes* into/from LPC, and whether *L. monocytogenes* in/escaped from LPC are susceptible to Gm. In the present study, we examined whether LLO is involved in invasion/escape of *L. monocytogenes* into/from LPC and whether *L. monocytogenes* in/escaped from LPC are susceptible to Gm. Invasion/escape of *L. monocytogenes* were found in LPC lines regardless of LLO expression, and *L. monocytogenes* in/escaped from LPC lines showed resistance to Gm. *L. monocytogenes* escaped from LPC lines were coated with their plasma membrane and the acquired resistance to Gm was abrogated by saponin. Our results indicate that invasion/escape of *L. monocytogenes* into/from LPC occur independently of LLO, and suggest that the acquired resistance of *L. monocytogenes* in/escaped from LPC to Gm is caused by being coated with their plasma membrane.

Keywords: *Listeria monocytogenes*; Hepatocyte; Gentamicin; Drug resistance

1. Introduction

Listeria monocytogenes is a Gram-positive facultative intracellular bacterial pathogen, which can replicate in professional phagocytes such as macrophages (Mϕ) [1]. After being phagocytosed by Mϕ, *L. monocytogenes* escapes from the phagosome into the cytosol by means of listeriolysin O (LLO), which is a 58-kDa pore-forming hemolytic protein encoded by *hly* gene, replicates inside the cytosol, and spreads to neighboring cells by formation of filamentous polymeric F-actin [2–9].

Liver is a major target organ of *L. monocytogenes* [10]. A vast majority of *L. monocytogenes* systemically infected are trapped in the liver immediately after infection [10]. Liver parenchymal cells (LPC) are a habitat of *L. monocytogenes* [11,12] and hence, the majority of *L. monocytogenes* trapped in the liver invade LPC [12]. Because LPC are not professional phagocytes, *L. monocytogenes* promptly replicates in LPC without being attacked by immune system and spreads to neighboring cells [13,14]. Thus, in infected liver there are at least 3 different types of *L. monocytogenes* as follows: (i) *L. monocytogenes* which does not invade LPC, (ii) *L. monocytogenes* which resides in LPC, and (iii) *L. monocytogenes* which escaped from infected LPC.

Gentamicin (Gm) is an aminoglycoside antibiotic which binds to bacterial ribosome 30s subunit and induces misreading of wide range of RNAs [15]. Because, *L. monocytogenes* is susceptible to Gm [16], Gm is generally, though in most cases in combination with other antibiotics, used for the treatment against listeriosis at minimal inhibitory

* Corresponding author. Tel./fax: +81 27 220 8935.
E-mail address: masashi.emoto@gmail.com (M. Emoto).

1286-4579/$ - see front matter
http://dx.doi.org/10.1016/j.micinf.2013.11.005

concentration (MIC) to avoid its side effect [17–19]. Yet, many investigators have reported that *L. monocytogenes* not only in Mϕ but also other mammalian cells show resistance to Gm [7,20–27], which is caused by inability of Gm to pass through plasma membrane [7,24,28], although this issue has been claimed by Drevets et al. [29] and Ohya et al. [30]. It is thus possible that *L. monocytogenes* not only in LPC, but also escaped from LPC also show resistance to Gm.

In the present study, we examined whether LLO participates in invasion/escape of *L. monocytogenes* into/from LPC, and compared the susceptibility of the 3 different types of *L. monocytogenes* in the liver to Gm. We found that invasion/escape of *L. monocytogenes* into/from LPC occurred independently of LLO, and that the 3 types of *L. monocytogenes* in the liver showed different susceptibility to Gm. Our results indicate that *L. monocytogenes* not only in LPC but also escaped from LPC show resistance to Gm and suggest that the acquired resistance of *L. monocytogenes* escaped from LPC to Gm is caused by being coated with the plasma membrane of LPC.

2. Materials and methods

2.1. Cell lines

HepSV40 derived from Simian virus 40 large T antigen transgenic mice [31] and AII derived from p53 knockout mice [32] were used in this experiment (Fig. S1). Both LPC lines were incubated in antibiotics-free RPMI 1640 (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan) containing 10% fetal calf serum (Bio West, Nuaillé, France) (designated as complete medium (CM)).

2.2. Bacteria and infection

The parental wild-type strain of *L. monocytogenes* used in this experiment was EGD. The Δ*hly* mutant strain was kindly provided by Dr. Kawamura (Kyoto University Graduate School of Medicine). *L. monocytogenes* (strains EGD and Δ*hly*) recovered from infected livers were grown in tryptic soy broth (Difco Laboratories, Detroit, MI) at 37 °C for 18 h and aliquots were frozen at −80 °C for later use. The final concentrations of viable bacteria were enumerated by plate counts on tryptic soy agar (Difco Laboratories). HepSV40 (2×10^5 cells) and AII (5×10^4 cells) were incubated with 2×10^6 colony-forming unit (CFU) and 5×10^5 CFU of *L. monocytogenes* (strains EGD and Δ*hly*), respectively.

2.3. Determination of MIC of Gm

L. monocytogenes (strains EGD and Δ*hly*) were cultured in tryptic soy broth containing different concentrations of Gm (0–5 μg/ml) at 37 °C for 48 h. The lowest concentration of Gm inhibiting the growth of *L. monocytogenes* was regarded as MIC. The growth of both strains was found in the presence of 1 μg/ml of Gm, whereas that was undetectable in the presence of ≧2 μg/ml of Gm. Thus, the MIC of Gm to both strains of *L. monocytogenes* was 2 μg/ml.

2.4. Determination of optimal time for killing L. monocytogenes by Gm

L. monocytogenes (strains EGD and Δ*hly*) were cultured in CM containing 10 μg/ml of Gm at 37 °C, and the CFU were determined 10, 15 and 20 min after the culture. Viable *L. monocytogenes* of both strains became undetectable 20 min after the culture.

2.5. Determination of CFU in infected LPC lines and their culture supernatants

HepSV40 and AII were incubated with *L. monocytogenes* (strains EGD and Δ*hly*) in CM at 37 °C for 1 h. After removing their culture supernatants, the LPC lines were further incubated in CM containing 10 μg/ml of Gm for 30 min. After washing with CM containing 10 μg/ml of Gm followed by CM, the LPC lines were further incubated in CM for 48 h. For determination of CFU in infected LPC lines, the LPC lines were washed 3 times with CM, plated on tryptic soy agar after sonication, and the CFU were enumerated by plate counts. For determination of CFU in culture supernatants of infected LPC lines, the culture supernatants of infected LPC lines were collected and the CFU were enumerated by plate counts on tryptic soy agar.

2.6. Flow cytometry

HepSV40 and AII were stained with Vybrant® Dio cell-labeling solution (Dio) (Life Technologies™, San Francisco, CA) at 4 °C for 20 min according to the manufactur's instructions. The stained LPC lines were intensively washed with CM and then incubated in CM containing 10 μg/ml of Gm for 30 min. The LPC lines were washed with CM and then incubated with *L. monocytogenes* (strains EGD and Δ*hly*) in CM at 37 °C for 1 h. After removing their culture supernatants, the LPC lines were incubated in CM containing 10 μg/ml of Gm for 30 min. After washing with CM containing 10 μg/ml of Gm followed by CM, the LPC lines were further incubated in CM for 48 h, and their culture supernatants were collected and centrifuged. The sediments were suspended in CM and acquired by FACSCalibur® (BD Biosciences, Mountain View, CA). *L. monocytogenes* gated by forward scatter (FSC) and side scatter (SSC) was analyzed with Flow Jo software (version 7.6.5; Tomy Digital Biology, Tokyo, Japan).

2.7. Saponin treatment

L. monocytogenes (strains EGD and Δ*hly*) appeared in culture supernatants of HepSV40 and AII after infection were collected, centrifuged, and treated with or without PBS (−) containing 0.5% saponin (Sigma Aldrich, Steinheim, Germany).