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Original Article

The reduction of L-cystine to L-cysteine in the supernatant of A549 cell culture causes imipenem inactivation☆

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ABSTRACT

In the course of measuring the intracellular antibacterial activity of antibiotics using a human alveolar epithelial cell line A549, we discovered that the antimicrobial activity of several carbapenems (CPs) decreased in the supernatant of the cells cultured with fetal calf serum (FCS)-free RPMI1640 medium (RPMI). Further investigation revealed A549 culture supernatant inhibited the antibacterial activity of CPs but did not inactivate other types of antibiotics. CE-TOFMS and LC-TOFMS metabolomics analysis of the supernatant revealed the presence of L-cysteine (Cys), which is not an original component in RPMI. Cys is known to hydrolyze and inactivate CPs in a time- and concentration-dependent manner. In this study, the inactivating effects of A549 culture supernatant on the imipenem (IPM) were examined. Antimicrobial activity of 100 μg/mL IPM decreased to 25% with two-fold dilution of A549 supernatant incubated for 3 h. L-Cystine (CS), the Cys oxide, and an original component in RPMI did not inactivate IPM. However, the inactivating effects of A549 supernatant on IPM corresponds with the Cys concentration and depends on the CS content of the culture medium. Addition of FCS to the culture medium decreased the Cys concentration and reduced inactivation of IPM in a dose-dependent manner. Our data suggest that IPM were inactivated by Cys reduced from CS, and this CS-to-Cys conversion must be considered when evaluating the antimicrobial activity of CPs in cell culture. Further studies are needed to understand if the same inactivation occurs around the cells in the human body.

1. Introduction

Various types of cells, including cell lines, are often used in experiments to evaluate the interaction among human cells, bacteria, and antibiotics. This type of experiment is usually applied for intracellular pathogens, e.g. *Legionella pneumophila* [1,2] and *Chlamydophila pneumoniae* [3], however, recent studies have shown that various cell types could readily internalize *Staphylococcus aureus* [4] and *Pseudomonas aeruginosa* [5], and the cell surface or intracellular survival of the bacteria may causes the persistent infection. Beta-lactam antibiotics including carbapenems (CPs) were often clinically used for the case of *S. aureus* and *P. aeruginosa* infection. Thus in vitro experiments, CPs are also used to evaluate cell-associated or intracellular antimicrobial activity in spite of their poor intracellular uptake [5–8] and to kill extracellular bacteria [9]. In the course of measuring the intracellular antibacterial activity of antibiotics using A549, a human alveolar epithelial cell line, we discovered that the antimicrobial activity of CPs, but not other types of antibiotics, was decreased in cell culture supernatant and collected A549 supernatant to understand the reduction in the antibacterial activity of CPs [10]. In subsequent experiments, metabolomics analysis of the culture supernatants revealed they contain L-cysteine (Cys), which is not an original component in RPMI1640 medium [11]. RPMI1640 contains L-cystine (CS), the Cys oxide, but not Cys. Other investigators have reported Cys could hydrolyze CPs in a time- and concentration-dependent manner [12–14].

Abbreviations: CPs, carbapenes; FCS, fetal calf serum; RPMI, FCS free RPMI 1640 medium; Cys, L-cysteine; CS, L-cystine; IPM, imipenem; EBSS, Earle's balanced salt solution; AA-EBSS, EBSS supplemented with amino acids adjusted to the concentrations in RPMI; AAFR, amino acid-free RPMI; CSFR, L-cystine-free RPMI.

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Here, we examined the inactivating effects of the A549 culture supernatant on the imipenem (IPM). The aim was to reveal how Cys derived from A549 cells causes IPM inactivation, and, thus, we examined the correlation between the IPM inactivation in A549 supernatant and the concentration of Cys and influence of CS and fetal calf serum (FCS) in the medium.

2. Materials & methods

2.1. Decline in the antimicrobial activity of IPM with A549 cell culture

IPM (32 μg/mL) was incubated with A549 cells in FCS-free RPMI1640 (RPMI) or RPMI supplemented with 10% FCS (RPMI/10% FCS) for 1, 2, and 3 h at 37 °C with 5% CO_2. The culture supernatant samples were collected, immediately frozen and stored at −80 °C, and the antimicrobial activity of IPM was tested later. In all experiments, the antimicrobial activity of IPM in the samples was determined by the agar diffusion microbiological bioassay with *Micrococcus luteus* (ATCC 9341) and the data are shown as the % of control, which was fresh RPMI. The detection limits of the bioassay were 0.5 μg/mL.

2.2. Decline in antimicrobial activity of IPM with A549 cell culture supernatant

A549 cells were preincubated in RPMI for 30 min, 1, 2, and 3 h at 37 °C with 5% CO_2 and the supernatant was collected. Undiluted and two-fold diluted supernatants were supplemented with 32 μg/mL IPM and incubated for 2 h to evaluate IPM inactivation. By the medium changing to RPMI/5%, 10% FCS, or amino acid-free RPMI (AAFR), the influence of amino acids and FCS in the preincubation medium on IPM inactivation could be investigated.

2.3. Inactivation of IPM by Cys derived from A549 cells

CE-TOFMS and LC-TOFMS metabolomics analysis (Human Metabolome Technologies, Yamagata, Japan) of A549 culture supernatants was performed [15]. Seventeen candidate substances in the supernatant were selected and assessed for IPM inactivation. Subsequent experiments revealed that the supernatant contained Cys, which is not an original component of RPMI, and inactivates IPM in the supernatant. To reveal the relationship between the concentration of Cys and IPM inactivation in A549 supernatant, subsequent experiments were performed.

A549 cells were preincubated in RPMI, RPMI/10% FCS, Earle's balanced salt solution (EBSS), and EBSS supplemented with amino acids adjusted to the concentrations in RPMI (AA-EBSS) for 1, 3, 6, 8, and 12 h at 37 °C with 5% CO_2. Cys in the culture supernatant was measured by a colorimetric assay using Ellman's reagent [16]. To assess the IPM inactivation in A549 culture supernatant, diluted supernatant was mixed with an equal volume of 200 μg/mL IPM for a final IPM concentration of 100 μg/mL, incubated for 3 h, and diluted 10 times to stop the reaction. IPM inactivation in two-fold diluted supernatants was investigated as above.

2.4. The influence of CS and FCS in the medium on IPM inactivation

RPMI contains 0.0652 g/L (208.1 μM) L-cystine•2HCl (CS) but does not contain Cys. Subsequent experiments were performed to understand the influence of CS and FCS on Cys concentration and the inactivation of IPM in A549 supernatant. First, to understand the influence of amino acids, CS, and FCS, A549 cells were incubated in amino acid-free medium AAFR supplemented with or without 0.0652 g/L (208.1 μM) CS, which is the same amount in RPMI, and 5, 10, and 20% FCS. Second, to understand the effect of CS, cells were incubated in CS-free RPMI (CSFR) supplemented with or without 0.25×, 1×, and 4× the original amount of CS. A549 supernatants were collected at 3 h and 6 h. Cys concentration and IPM inactivation of the supernatant were investigated as above.

2.5. Statistical analysis

All data are expressed as mean ± standard deviation. Differences between experimental groups were determined by analysis of variance followed by Student's *t*-test. A *P* value less than 0.05 was considered significant.

3. Results

3.1. Decline in antimicrobial activity of IPM with A549 cell culture

A549 cells were incubated in RPMI supplemented with 32 μg/mL IPM to understand the influence of the cells on IPM activity. The antimicrobial activity of IPM declined in A549 cell culture in a time-dependent manner and was below the limit of detection after 3 h (Fig. 1). Reduction of IPM activity was more pronounced in cells cultured in FCS-free medium (RPMI) than in RPMI supplemented with 10% FCS (RPMI/10% FCS) at all time points.

3.2. Decline in antimicrobial activity of IPM with A549 cell culture supernatant

To investigate how A549 cells inactivate IPM, A549 supernatant was collected and preincubated in RPMI for 30 min, 1, 2, and 3 h, and a two-fold dilution was incubated with IPM for 2 h. Cell-free culture supernatant preincubated for 30 min inactivated IPM, and the effect was enhanced by preincubation time and concentration of supernatant (data not shown). The inactivating effects of the supernatant preincubated for 3 h with A549 cells with or without FCS and amino acids are presented in Fig. 2. A549 undiluted and two-fold diluted supernatant inactivated IPM, but the undiluted supernatant had a significantly stronger effect at all time points. IPM inactivation decreased by the addition of 5–10% FCS to the preincubation medium in a concentration-dependent manner. On the other hand amino acid-free RPMI (AAFR) preincubation supernatant did not exhibit IPM inactivation.

3.3. IPM inactivation by Cys derived from A549 cells

To reveal the relationship between the concentration of Cys and IPM inactivation effect of A549 supernatant, A549 cells were preincubated in RPMI, RPMI/10% FCS (Fig. 3), EBSS, and EBSS/AA (Fig. 4), the collected supernatant samples were diluted two-fold, and tested. Cys in A549 FCS-free RPMI culture supernatants increased in a time-dependent manner and reached a plateau value of nearly 260 μM. The concentration of Cys in RPMI/10% FCS supernatant was about half of that in RPMI at each time point (Fig. 3). EBSS supernatants did not contain detectable Cys and AA-EBSS contained about the same level of Cys as RPMI supernatants at 8 or 12 h (Fig. 4). FCS-free RPMI A549 culture supernatant inactivated IPM even at 1 h. The antimicrobial activity of 100 μg/mL IPM decreased to 25% with a two-fold dilution of supernatant and the effect increased in a time-dependent manner consistent with the concentration of Cys (Fig. 3). RPMI/10% FCS (Fig. 3) and EBSS/AA (Fig. 4) supernatants also inactivated IPM according to the Cys concentration, but were significantly weaker except for EBSS/AA at 8 h and 12 h than that of RPMI. EBSS supernatant did not inactivate IPM. In other experiments, we verified that concentration-adjusted Cys solutions from commercially available reagents also had the

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