Original Article

Rothia mucilaginosa’s cellular proliferation inhibiting ability and microfilament-destructive effects on original cells in the lower respiratory tract

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Abstract: The present study aimed to explore the effect of cytotoxicity of Rothia on its virulence. Rothia mucilaginosa (RM) and the original H1975 cells of the respiratory tract were chosen as counterparts for coculture in this study, and virulent Streptococcus pneumoniae (SP) and nonvirulent Escherichia coli (E. coli) were used as the positive and negative controls, respectively. To determine the ability of RM to inhibit cellular metabolic processes, induce apoptosis, and damage cellular construction, several experiments such as cell counting to assess cell proliferation, cell flow cytometry to detect apoptosis, and light and confocal microscopy to observe cellular destruction were conducted. The results indicated that RM possessed a weaker ability to inhibit cellular proliferation and induce apoptosis than SP, but the effects of RM were much stronger than those of E. coli. Furthermore, morphologically, among the three types of bacteria, RM showed the most obvious destructive effects on cellular structure, with evident damage to the cellular microfilament system. First, we found that RM had an exact influence on cellular function and structure. Second, RM may be classified as a type of bacteria that triggers infections in immunocompetent individuals, rather than merely as an opportunistic infectious agent. Third, more attention should be paid to Rothia-related infections in clinical practice, especially to lower respiratory infections such as community-acquired pneumonia (CAP). Finally, the present study is based on our previous microbiota analysis. Therefore, these findings suggest that an independent culture method is reliable in determining the causes of infection.

Keywords: Rothia mucilaginosa, cytotoxicity, coculture, pneumonia, lower respiratory tract

Introduction

Lower respiratory tract (LRT) infections is the most common concern that always draws attention from both clinical practitioners and researchers [1]. In clinical practice, community-acquired pneumonia (CAP) is a major cause of such infections and significantly contributes to morbidity and mortality. Based on the current knowledge, Streptococcus, Haemophilus, Staphylococcus, Mycoplasma, and Chlamydo- philia act as dominant infectious agents in CAP [2]. Our previously published research on the microbiota present in the LRTs of patients with CAP, which was based on bacterial 16s rDNA sequencing, identified a bacterial genus known as Rothia whose 16s rDNA was significantly abundant compared with the corresponding bacterial communities found in healthy individuals and patients with hospital-acquired pneumonia (HAP) as shown in Figure 1 [3, 4]. Although the ability of pathogen to cause infections always depends on bacterial pathogenicity and virulence rather than the local colonial quantity, a noticeable expansion of a single genus, particularly in the LRT of patients with CAP (Figure 1), also attracted our attention. This result suggested that Rothia, which was rarely mentioned in CAP, may be a potential pathogenic agent for such infections [5].

Rothia, which comprises a series of bacteria featured with aerophilic Gram-positive coccobacillus, is classified as part of the normal flora.
Figure 1. Published research comparing Rothia’s expansion in patients with community-acquired pneumonia (CAP), healthy individuals, and patients with hospital-acquired pneumonia (HAP). A. Heatmap of the bacterial distribution of sputum samples in all three categories (patients with community-acquired pneumonia, patients compared with healthy individuals, and patients with hospital-acquired pneumonia). Abbreviated genus names corresponding to 80 genera, which appeared in patients with CAP, are listed at the bottom of the heatmap. Subjects are listed to the left of the figure. Intensity of the coloration of cells in the heatmap indicates the percentage of each genera appearing in every subject. Features of healthy individuals are showed in the lower area (N01~N18), those of patients with CAP in the middle area (C01~C45), and those of patients with HAP in the upper area (H01~H44). Cells representing Rothia are emphasized by a red square. B. Ratio of genera existing simultaneously in healthy individuals, patients with CAP, and patients with HAP. These genera have more tendencies to be normal residents in human respiratory tracts. The whole ratios of all these genera vary slightly from healthy individuals to patients with CAP except that some genera’s ratio, such as Rothia, rise obviously. However, their whole ratio in patients with HAP was significantly compacted by other genera. The proportion representing Rothia are emphasized by red arrows.
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in the oral cavity and respiratory tract [6]. Rothia mucilaginosa (RM) is a species of the Rothia genera that shares common features with other Rothia species [7]. RM is a Gram-positive bacterium that colonizes as a commensal organism in the oral cavity and respiratory tract [8]. Recent and previous clinical case reports have indicated that this bacterium has low virulence and only causes opportunistic infections in immunocompromised patients or those with end-stage wasting diseases [9, 10]. Cases of infections in immunocompetent patients are rarely reported; therefore, the potential of RM as a pathogen remains unclear [11].

CAP is a common LRT infection that occurs in immunocompetent individuals [12]. Unlike patients with HAP, patients with CAP have no history of previous antibiotic use, indicating that LRT microbiota cannot be significantly disturbed. Further, according to our latest research on LRT microbiota, the microbiota of patients with CAP is very similar to that of healthy individuals. Nevertheless, there still exists a significant increase in Rothia in patients with CAP. Therefore, it is believed that Rothia is more likely to act as a potential CAP-causing pathogen rather than an imbalanced colonial commensal organism influenced by an external factor. In fact, the CAP microbiota has demonstrated significant Rothia abundance, which brings our immediate attention to further investigate the virulence of Rothia.

Although microbiota analysis involved a type of high-throughput and culture-independent method that can efficiently provide an overview of the local bacterial community in the LRT, it lacks the ability to indicate the virulent mechanism of a single species. In the present study, we chose RM as a Rothia representative and original alveolar epithelial cells in the LRT (H1975) as the infected host. In addition, nonvirulent Escherichia coli DH5α (E. coli) and virulent Streptococcus pneumoniae (SP) were selected as the negative and positive controls, respectively, to act on H1945 cells. The objective of our study was to determine the ability of RM to inhibit cell metabolism while directly injuring the cell structure. We believe that the findings of the present study unveil more obvious cytotoxicity of RM on original cells of LRT than that of nonvirulent bacteria.

Materials and methods

Design

In the present study, LRT original H1975 cells were selected as epithelial cell models to interact with RM in a coculture system in vitro. In addition to RM, virulent SP and nonvirulent E. coli were selected as the positive and negative controls, respectively. The entire research study was divided into two parts to evaluate the cytotoxic influences of RM on both cell validity and cellular structure. These two aspects can be proven by a series of cellular functional and morphological experiments. Cell Counting Kit-8 (CCK-8) and flow cytometry were used to assess the inhibitory and harmful effects of cytotoxicity on cell metabolism and demonstrate the bacteria's negative impacts on cellular viability and apoptosis. Moreover, the gross cell structure and skeleton were directly observed by light and confocal microscopy to demonstrate the destructive effects of RM on these cellular structures. In the above-mentioned experiments, all the selected bacteria, including RM, SP, and E. coli, were mixed with the cells, and the observations were conducted when the coculture was terminated at different time points (0, 30, 60, 90, 120, 150, 180, and 210 min). The NCI-H1975 cells were purchased from Shanghai Yu Bo Biotech Co., Ltd. (YB-026).

Determination of the RM growth curve and quantification of bacteria

After being thawed from -80°C storage, RM was initially streak cultured on a 5% CS lysogeny broth (LB) plate. After incubation for 12 h, single colonies were visible on the plate, and one single colony was separated and transferred into a 5 mL liquid LB medium and shaken overnight at 37°C for primary amplification. Then, 200 μL of bacterial liquid was inoculated in a fresh 200 mL LB medium and shaken overnight at 37°C for primary amplification. Then, 200 μL of bacterial liquid was inoculated in a fresh 200 mL LB medium for shaking. Every 2 h beginning at 0 h (the time at which the liquid was added), 1 mL of liquid was withdrawn, and optical density (OD) values were measured at precise time points. The recorded OD values were used to draw the growth curve (Figure 2). In addition, the SP growth curve was drawn in a similar method, whereas the E. coli’s growth curve had been previously documented [13].
Bacterial quantification was achieved using the plate colony counting method. For each type of bacteria studied, the bacterial liquid was shaken overnight and amplified in 200 mL of the liquid medium, and then 10 mL of bacterial liquid was extracted to measure the OD value (λ = 600 nm). Another 100 μL of bacterial liquid was mixed in an Eppendorf (EP) tube with 900 μL of sterilized LB medium and was thus diluted by 10 times the bacteria liquid. The dilution grade was recorded as 10^{-1}. Subsequently, 100 μL of the diluted bacterial liquid was extracted again and transferred to a new EP tube with 900 μL of sterilized LB medium, and the liquid was further diluted in a similar manner to 10^{-2}. The previous steps were repeated until the dilution grade reached 10^{-7}. From the bacterial liquid of each grade, 50 μL were transferred and tiled on LB plates for incubation until separate colonies were observed. The plates with extremely high and low quantities of colony-forming units (CFUs) were discarded, whereas those with a CFU between 20 and 400 were used to record the colony number. By counting the CFUs and corresponding OD values, the bacteria number within a specific volume of the LB liquid could be estimated [14].

CCK-8 allows the sensitive colorimetric assay to determine cell proliferation and cytotoxicity. WST-8, a highly water-soluble tetrazolium salt, was reduced by dehydrogenase activities within cells to yield a yellow-colored formazan dye, which was soluble in the tissue culture medium. The amount of formazan dye generated by
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In this study, the reaction between the bacterial cellular coculture medium was performed on a 96-well culture plate. These wells were divided into five groups: three coculture groups of RM, SP, and E. coli; one group of equal cells counting to determine the standard curve of full cell quantity; and one group of fold-diluted cell counting to create a standard curve of descending cell quantity. H1975 cells were cultured using a cell medium (RPMI 1640 + 10% FBS) in each well under conditions of 200 μL in volume and 1 × 10^5 cells/well. These cells were initially cultured in 3.6% CO_2 for 12 h; thereafter, RM, SP, and E. coli were added to the wells at a MOI of 100:1 at different time points (0, 30, 60, 90, 120, 150, 180, and 210 min). After the coculture was complete, the supernatant was discarded, and the CCK-8 solution was added for another 4 h of incubation. Then, the OD value (λ = 450 nm) of each well was measured, and the results of the OD reduction are shown in Figure 3.

Cellular apoptosis detection by FITC Annexin V/propidium iodide (PI) flow cytometry

FITC Annexin V and PI, which provide a rapid and convenient assay for apoptosis and dead cells, were used to assess the cellular apoptosis-inducing ability of RM [15-17]. The coculture system with a MOI of 100:1 was established using the abovementioned method on a 96-well cell culture plate. The only difference was that the cell count in each well was increased to 5 × 10^5 cells. When the culture was completed, the supernatant was discarded, and the cell-bacteria mixture was trypsinized and resuspended in a sterilized phosphate-buffered saline (PBS). Low-speed centrifugation at 1,500 rpm for 5 min was used to separate the cells and bacteria. The harvested cells were tagged with FITC Annexin V and PI using the kit (Thermo Fisher Scientific, Waltham MA, USA) according to the manufacturer’s instructions. Subsequently, the tagged fluorescence was detected by a cytometry machine, and the results were displayed in four-quadrant bivariate plots (Figures 4, 5).

Observation of cellular morphology changes under optical and confocal microscopy

Changes in cellular morphology, such as the destructive effects of the bacteria on cellular structure, are additional keys to determine the cytotoxicity of RM. Therefore, the cellular morphological changes were observed at different time points in the bacteria/H1975 cocultured cells using both optical and confocal microscopy (Figures 6, 7).

Prior to observation by light microscopy, the bacteria and cells were cocultured using the abovementioned method on a 12-well culture plate. There were 5 × 10^5 cells in each well, and...
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Figure 4. FITC Annexin V/PI-labeled cells in the four-quadrant figures indicating the apoptotic and dead cells detected by flow cytometry. This figure shows the natural degeneration of H1975 cells from 0 to 210 minutes. In the following figures, “R0” to “R210” represents the apoptotic rate of H1975 cells at different time points cocultured with RM. “S0” to “S210” represents cells with SP, and “E0” to “E210” represents cells with E. coli.

the quantity of bacteria was 100 times that of the cells (MOI of 100:1). The time points were set from 0-90 min at intervals of 30 min. The supernatant was discarded, and the cells were directly observed using an optical lens (Leica, DM1L, 400 x; Leica Microsystems, Wetzlar, Germany).

For confocal microscopy, the cocultured protocol was similar to that of light microscopy. The time points were set from 0-180 min at intervals of 30 min. After reaching the time points, the cells at the bottom of the plate were washed three times with PBS. Triton X-100 was added for 4 min to permeabilize the unfixed cell membranes, and the cells were then washed three times with PBS, and microfilament F-actin was stained using rhodamine phalloidin (Molecular Probes, Eugene, OR, USA) at a concentration of 2 U/mL and a temperature of 37°C for 40 min. After 30 min of staining, another cell-fluorescent dye-DAPI (4',6-Diamidine-2'-phenylindole dihydrochloride)-was added for core staining at a concentration of 5 μg/mL and a temperature of 37°C for 10 min [18, 19]. When all the processing
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procedures were performed, the stained cells were observed by confocal microscopy (ZEISS, LSM 700; Leica Microsystems).

Statistical analysis

The data are presented as mean ± standard error of the mean (SEM). Statistical analyses were performed using the GraphPad Prism 5.0 software (GraphPad Software Inc., La Jolla, CA, USA). One-way analysis of variance was used to compare the data of CCK-8 and the UR% + LR% in flow cytometry analysis, followed by the least significant difference post-hoc test. P values of < 0.05 were considered statistically significant.

Results

Growth curves of RM and SP

The present study was based on RM’s growth property. Figure 2 presents the growth curves of RM and SP. The logarithmic phase of RM initiated at 4 h from the beginning of the incubation phase, and the growth curve entered a stationary phase at 14 h. The logarithmic phase of RM was milder than that of a typical bacterial growth curve, and it lasted for an extended period. Figure 2 also presents the growth curve of SP, wherein it exhibited a sharp shape over a short time. All the bacteria evaluated in the present study were removed during the logarithmic phase and added to the cell medium for coculture.

CCK-8 assay showing the cell proliferation inhibiting ability of different bacteria

The results of the CCK-8 assay are presented in Figure 3. The curves with black squares and hollow triangles were the standard curves that indicated the full cell counts and 2-fold-diluted cell proliferation, respectively. In Figure 3, the curve with hollow circles represented the cells that were cocultured with SP; this result was quite similar to the 2-fold curve exhibiting a sharply descending shape. Conversely, the growth curve of E. coli was similar to the curve documenting full cell counts. The growth curve of RM, which is indicated by black circles, is in the middle area of Figure 3. Virulent SP exhibited potent inhibitory effects on the cells. Although the growth curve of RM did not descend as sharply as that of SP, the inhibiting effect was also obvious. In addition, the OD value decreased with fold changes in dilution, and at the final fold of dilution, the OD value for E. coli was the highest among all the groups. RM is between the group of E. coli and SP. The difference in OD values in each group was significant (P < 0.01).

LR% + UR% in the flow cytometry test showing the cell apoptosis-inducing ability of different bacteria

Figure 4 presents a series of four-quadrant images indicating the apoptotic and dead cells detected by flow cytometry. In each figure, the dead cells labeled by PI are located in the upper right quadrants (UR), and the FITC Annexin V-labeled apoptotic cells are located in the lower right quadrants (LR). The living cells are concentrated in the lower left quadrants. Thus, the total LR% + UR% represents damaged cells at different stages. In other words, higher LR% + UR% reflects stronger cytotoxicity possessed by the various bacteria. Furthermore, Figure 5 shows that the LR% + UR% was significantly lower in the RM group than in the E. coli group, whereas it was still significantly higher than that in the SP group at 30, 60, 90, 120, 150, 180, and 210 min (P < 0.01).
Figure 6. Morphological observation under light microscopy at different time points for H1975 cells cocultured with bacteria (400 ×). The figures in the left column are the observations of H1975 cells cocultured with RM at different time points. The middle column represents cells co-cultured with SP, while the right column represents cells cocultured with *E. coli*. 
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Morphological observations disclosing destructive effect of RM on cellular structure

Morphological observations under light microscopy described the changes in the cellular structure following the cell-bacteria coculture. Figure 6 shows a ladder of progressive destruction on the cells as time progresses. The top to bottom images were listed over time; the right to left columns record different cocultured bacteria, including RM (left), SP (middle), and *E. coli* (right). Based on the different columns, the cells in the RM column demonstrated the most obvious destructive effects. In the left column of Figure 6, it was evident that the cell structure quickly became shapeless. The cell margin became blurring at 30 min, and the visible cellular structures mostly merged with the background at 180 min. Conversely, the changes in cells were milder in the SP (middle) and *E. coli* (right) columns than in the RM column; cells margins were still visible up to 180 min.

Various causes can be responsible for collapse of the cellular construction. Based on the findings of light microscopy, RM possessed a strong ability to damage the cells. By means of confocal microscopy, the cellular microfilaments were stained using rhodamine phalloidin (Figure 7), and at initiation of the coculture of RM and H1975 cells, the cellular microfilament was depolymerized and degenerated into a dotting pattern, and at 180 min, the microfilament's degeneration was so evident that no microfilament structures could be observed.
that microfilament structures could not be observed and were completely replaced by the stained dotted patterns.

Discussion

CAP is a common infection that occurs in immunocompetent individuals [20, 21]. According to the conclusion drawn by our previously published study, CAP can be directly caused by Rothia, or this genus may serve as an important coinfection factor [4, 22]. Given that the microbiota of normal individuals and patients with CAP are quite similar overall, the presence of Rothia associated with changes in CAP microbiota is noteworthy.

Determining the bacterial growth curve and observing the cell morphology under light microscopy constituted the very foundation that assisted us in deciding the right time to obtain bacteria from the liquid culture media and ensure the most appropriate MOI with which the bacteria can be mixed with H1975 cells. All the bacteria were isolated at their logarithmic growth periods and were cocultured with H1975 cells at a MOI of 100:1.

CCK-8 is a commonly used method to detect cell proliferation ability. With a decrease in the said ability, an OD value of λ = 450 nm would also decrease. Unlike the OD value tendencies observed for the coculture media of SP and E. coli, the growth curve of RM is somewhere in between, which indicates that RM has a stronger influence on inhibiting cell proliferation than E. coli and is as competent as the typical virulent SP. The CCK-8 assay revealed that RM has a relatively strong effect on inhibiting cell proliferation.

As a member of oral commensal bacteria, Rothia mucilaginosa’s pathogenicity was rarely reported and most case report showed that it only caused opportunistic infections in children and elder population [23, 24]. A newly published article shows that Rothia mucilaginosa is the first commensal oral bacterium found to produce enterobactin which may be attributed to pathogenic gut bacteria and help Rothia mucilaginosa to prevalent exist in oral cavity [25]. So, there is still lacking of the systemic research upon its pathogenicity. In addition to the ability to inhibit cell proliferation, the induction of apoptosis also plays a crucial role in cell toxicity. In the present study, flow cytometry was performed to detect the ability of RM to induce cell apoptosis and result in cytotoxicity, both alone and in comparison with the typical potent virulent SP and nonvirulent E. coli. Based on the results shown in Figures 4, 5, the quantity of cells distributed in the UR and LR areas was also between the values of SP and E. coli, which indicated that although the ability of RM to initiate cellular apoptosis was weaker than that of SP, it was obviously stronger than that of E. coli and thus provided additional evidence supporting the fact that RM might not only be involved in cellular metabolism but also positively induce cellular apoptosis.

From a morphological perspective, observations of the general cellular structure with optical microscopy as well as advanced observations of the cellular skeletal structure using confocal microscopy demonstrated how SP had a powerful ability to damage cellular structures. Figure 6 presents the basic changes observed under optical microscopy when examining a mixed culture of H1975 cells and different types of bacteria. Under conditions of MOIs of 100:1 and coculture for 180 min, RM showed the most obvious destructive effects on H1975 cells. Further, the confocal images (Figure 7), which exhibited a fracture in the skeleton of H1975 cells, suggested that RM had an obvious destructive impact on the cell structure and thus also contributed to cellular cytotoxicity.

Taken together, the following conclusions can be drawn. First, RM can directly influence the origin of LRT cells function by both inhibiting cellular metabolism and inducing apoptosis. Although the present study has demonstrated that this ability is stronger in RM than in nonvirulent bacteria and is not as potent as that in common pathogens, including SP, such effects still contribute to the cytotoxicity of RM.

Second, based on the morphological experiments, the effects of the destructive ability of RM on cell structure were much stronger than those of the negative and positive control bacteria (E. coli and SP, respectively), suggesting that direct damage to the cell skeleton integrity is a significant mechanism of action associated with the cytotoxicity of RM.
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Third, in response to the previously proposed hypothesis noted in our published study, the evidence in support of the cytotoxicity of RM suggested that culture-independent microbiota analysis should be reliable when identifying the potential pathogens.

Finally, the ability of RM to inhibit cellular apoptosis is weaker than the ability of SP. Nevertheless, RM may possess greater destructive ability on the cellular skeleton than SP. These findings strongly suggest that Rothia, which was considered an opportunistic pathogen in immunocompromised individuals, causes LRT infection in healthy individuals and that it is a virulent bacterium that must be explored by clinical practitioners.

The limitation of this study is as follow: It is only a descriptive study that shows the cytotoxic effect of Rothia mucilaginosa by bacteria-cell interactions. Although the bacteria’s inhibitive and destructive effect upon the respiratory original cells was proven, the mechanism needs further researches.

Conclusion

The findings of the present study demonstrated that RM possesses a strong ability to inhibit cellular metabolism and damage cellular structure. This study suggests that RM is a potential CAP-causing pathogen. RM was selected as the target of the present study based on our previous LRT microbiota analysis, together indicating that microbiota analysis is a useful method for identifying new potential pathogens.

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Disclosure of conflict of interest

None.

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