Biofilm formation of the pathogens of fatal bacterial granuloma after trauma: Potential mechanism underlying the failure of traditional antibiotic treatments

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ORIGINAL ARTICLE

Biofilm formation of the pathogens of fatal bacterial granuloma after trauma: Potential mechanism underlying the failure of traditional antibiotic treatments

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Abstract

The pathogen of a new type of disease – fatal bacterial granuloma after trauma (FBGT) – was found to be Propionibacterium acnes (P. acnes). Although in vitro studies showed that the pathogenic P. acnes are sensitive to conventional antibiotics, treatments of FBGT patients with these antibiotics were ineffective. The underlying mechanisms were not clear. Since P. acnes are able to form biofilm on orthopaedic biomaterials in vitro, and pathogenic P. acnes of acnes vulgaris was known to form biofilm in vivo, we hypothesize that the pathogens of FBGT are also able to form biofilm during the pathogenesis, which may be 1 of the reasons for antibiotics tolerance of FBGT. Biofilm forming capacity of the pathogens of FBGT were examined with XTT reduction method, as well as with scanning electron microscope. The effect of long-term subminimal inhibitory concentration (MIC) lincomycin on the biofilm forming ability of the pathogens was also tested. Our results show that both the type strain (NCTC737) and the pathogenic P. acnes of FBGT can form biofilm in vitro. These data demonstrated the biofilm formation of the FBGT pathogens in vitro, and its acceleration by lincomycin, which may be 1 of the major mechanisms for the failure of antibiotic treatment.

Introduction

Fatal bacterial granuloma after trauma (FBGT), a new type of granulomatous disease, was recently reported by our research group [1]. The major clinical presentations of FBGT patients were the development of lesions after slight trauma to the face, followed by spreading dark-red plaques that did not ulcerate [1]. The lesions showed a histolytic granuloma in histopathology [1]. After this first report of FBGT cases, 4 new patients were also diagnosed as FBGT according to history, clinical manifestation and histopathology and were treated in our department. Traditional high-dose antibiotics treatment proved inefficient in treating these 4 FBGT patients, and 3 patients died of diffusive brain inflammation 1 to 6 months after diagnosis, while the only cured patient showed recurrence twice in 4 y.

In order to understand the pathogenesis of FBGT, we isolated the pathogens of FBGT from these 4 patients before antibiotic treatment and cultured them successfully in vitro. The pathogens were found to be Gram-positive rods, and were identified as P. acnes with API 20A systems (bioMerieux, France), 16sRNA sequencing, and DNA-DNA hybridization (unpublished observations from our laboratory). Two FBGT patients who died of brain inflammation were autopsied, and 16s rRNA gene sequence of P. acnes was amplified and detected from the affected brain tissue but not from normal parts of the brain of these 2 patients. 16s rRNA gene sequence was also detected from the skin lesions. During the amplification, P. acnes specific primers and bacterial universal primers were both used. Analysis with basic local alignment tool (BLAST) revealed that the 16s rRNA gene sequence of the
isolated pathogens from skin lesions and the affected brain tissue was the same in both patients, supporting P. acnes as the pathogen of FBGT (unpublished observations from our laboratory).

Despite the fact that high-dose antibiotics in combination failed in treating FBGT patients, our in vitro studies showed that the pathogens of FBGT in culture were sensitive to many conventional antibiotics, such as lincomycin, doxycycline and gentamicin, but were resistant to metronidazole [2]. The mechanisms underlying the resistance of FBGT patients to P. acnes-sensitive antibiotic treatments were unclear. Bacterial biofilm is a common cause of persistent infections [3], and increased tolerance of the bacteria in biofilm to antibiotics is a serious problem for the clinical management of infectious diseases [4]. Wild-type P. acnes strains are known to be able to form biofilm on orthopaedic biomaterials in vitro [5], and the failure of hip replacement surgery is often found to be due to P. acnes biofilm formed on the implanted material [6]. Studies also revealed the in vivo biofilm formation of pathogenic P. acnes of acnes vulgaris [7,8]. With regard to the similar biological characteristics of different strains of P. acnes, we hypothesize that the pathogenic P. acnes of FBGT is also able to form biofilm during the pathogenesis of the disease, and antibiotic treatment may affect the biofilm formation. The biofilm would protect the bacteria from the attack of sensitive antibiotics, making the treatment less efficient.

In the present study, we examined the biofilm forming capacity of the FBGT pathogens on different materials, as well as the effects of long-term sub-minimal inhibitory concentration (MIC) lincomycin on the biofilm forming ability of the FBGT pathogens. Our data demonstrated the in vitro biofilm formation of the FBGT pathogens, and showed the characteristics of the biofilm and its acceleration by lincomycin, which may be 1 of the mechanisms for the failure of antibiotic treatment of FBGT.

Materials and methods

Isolation and culture of pathogens of FBGT

Skin lesions of 4 FBGT patients were biopsied before antibiotic treatment. In order to avoid potential contamination with bacteria from the skin microbiota, the epidermis and dermis layers were carefully removed from the lesion and the underlying subcutaneous tissue was cut into small pieces, and cultured in tubes with 5 ml reformed brain heart infusion (BHI) broth (Oxoid, UK) supplemented with 1% sheep blood, and was maintained at 37°C in an anaerobic incubator (BUG-BOX, USA), according to our previously established protocols [9]. Successful anaerobic culture of bacteria from the subcutaneous tissue of the lesions was usually achieved 7–14 d after inoculation in BHI broth. The broth-grown bacterium appeared pure upon visual inspection of colonies after subsequent growth on BHI agar plates supplied with blood. All these isolates proved to be P. acnes with 16s RNA sequencing, and DNA-DNA hybridization methods, as previously described [10,11]. Accordingly, these strains were encoded as Fc, Fg, Fh, Fl1 and Fl2 (Fl1 and Fl2 were isolated from the first and second recurrence of the recurrent FBGT patient, who was treated by us successfully 4 y earlier [1]. The type strain of P. acnes (NCTC737, bought from ATCC, USA) was also used as control. All these P. acnes strains were grown on BHI agar at 37°C under strict anaerobic conditions. For each strain, a colony was chosen and inoculated into BHI broth, and cultured in an anaerobic incubator for up to 48 h. The bacteria were harvested after centrifugation at 3000 × g for 1 min and density was adjusted to 1 × 10^8 cells/ml with BHI broth according to Mac turbidimetric method. Lincomycin (Northern China Pharmaceutical Company, China) and doxycycline (Xian Pharmaceutical Company, China) were dissolved in sterile PBS to make 1.024 g/ml stock solution, which was filtered through a 0.22-μm filter and kept at −20°C for later use.

Biofilm formation

Biofilm formation of P. acnes strains was examined as previously described [12]. The bacteria suspension was adjusted to 1 × 10^8 cells/ml and inoculated into sterile, flat-bottom 96-well polystyrene microtitre plates (Costar, Corning Inc., USA) and cultured at 37°C in an anaerobic workstation (Bug-Box, USA). This density of cells was selected because previous experiments in our laboratory demonstrated that optimal biofilm formation occurs at this density. Each well contained 200 μl of the suspension, and the plates were not moved during the culture process. The broth in the wells was discarded gently at different time intervals after inoculation, and the wells were washed gently with sterile phosphate buffered solution (PBS) 3 times in order to remove planktonic bacteria. Residual PBS was removed by blotting with sterile paper towels, and 200 μl fresh BHI broth was added into each well. The quantity of biofilm was determined with 2,3-bis (2-methoxy-4-nitro-5-sulphophenyl)-5-(phenylamino)carbonyl-2H-tetrazolium hydroxide (XTT; Sigma, MO, USA) dye reduction method [12]. Briefly, phenazine methosulfate (PMS; Sigma, MO, USA) was diluted with sterile PBS and filtered through a 0.22-μm filter, and stored at 4°C. XTT was diluted with BHI and...
filtered through a 0.22-μm film before use. The plates with fresh BHI broth were agitated for 30 s, and 50 μl of XTT-PMS mixture (final concentrations: 200 mg/ml for XTT and 19.1 mg/ml for PMS) was added into each well, and the plates were incubated for 1.5 h at 37°C under anaerobic conditions. OD490 nm was then read with an Absorbance Microplate Reader (Model 680, Bio-Rad, USA). BHI broth without bacteria was used as blank control. Biofilm formations were tested at different time intervals (at 24 h, 48 h, 72 h, 96 h, 120 h and 144 h) with the XTT assay and concurrently assessed by light microscopy. For each time interval, the experiments were repeated 3 times, and similar results were obtained.

Scanning electron microscope (SEM) observation of P. acnes biofilm

A glass slide was cut into 1 × 1 cm² plates and sterilized by autoclave. The plates were then put into 6-well plates, and 4 ml of the bacterial suspension (1 × 10⁸ cells/ml) was added into the wells and cultured anaerobically at 37°C. The suspension was sucked out after 12 h, 48 h or 72 h, and the plates were washed with sterile PBS gently to remove planktonic bacteria. For fixation, 4 ml of 3% glutaraldehyde was added into the wells and kept at 4°C for 24 h. Plates were further fixed in 1% osmium tetroxide for 1.5 h. Samples were then dehydrated through an ethanol series in buffer: 35%-50%-70%-80%-95%-100% for 15 min each. Immediately after the completion of dehydration, all samples were dried in a critical-point dryer. Later, the samples were mounted on stubs and coated with 22 nm Aurum in a sputter coater. Samples were viewed on the Hitachi S-520 scanning electron microscope (Hitachi, Japan) by an experienced technician.

Quantity of P. acnes biofilm on different materials

Slides made of stainless steel, polyethylene, or frosted glass were cut into small plates of 1 × 1 cm² and autoclaved. The stainless steel was the smoothest among the 3 surfaces, followed by polyethylene and frosted glass in order of increasing roughness. Both stainless steel and frosted glass are hydrophilic, while polyethylene is hydrophobic. The sterile plates were put into 6-well plates, and 4 ml bacterial suspension of 1 × 10⁸ cfu/ml was added and cultured for 96 h, according to our preliminary results. Following biofilm formation the plates were taken out with sterile forceps and gently washed with PBS 3 times to remove any non-adherent bacteria. The plates were then placed in BHI, and bacteria retained on them were dislodged by mild ultrasonication (5 min) in a 150 W ultrasonic bath operating at a nominal frequency of 50 Hz followed by rapid vortex mixing (30 s). The suspension was then added into 96-well plates, and XTT/PMS methods were used to quantify the biofilm on the surface. Testing of each isolates was repeated 4 times.

Minimal inhibition concentration (MIC) for planktonic bacteria and minimal bactericidal concentration (MBC) for the bacteria in biofilm of FBGT pathogens

The MIC of the planktonic P. acnes isolates for lincomycin and doxycycline were measured with micro-broth dilution test [13]. Briefly, serial 2-fold dilutions of antibiotics were performed from a 4 × stock solution in BHI broth, and the concentration of P. acnes was adjusted to 2 × 10⁶ cells/ml. In 96-well plates, 100 μl of BHI broth, 50 μl of the antibiotic dilutions and the P. acnes suspension were mixed and incubated at 37°C for 24 h. The lowest concentration showing inhibition of bacteria growth was considered as the MIC of the bacterial strain of the antibiotics. Type strain NCTC737 of known MICs was used as control. The MIC and MBC for the P. acnes biofilm (sessile MIC and sessile MBC) were also measured with reformed micro-broth dilution test. The 96-h-old biofilm was chosen according to previous results, because at this time point the biofilm formed can be regarded as mature. After biofilm formation at 96 h, the medium was aspirated, and non-adherent cells were removed by gently washing the wells with sterile PBS 3 times. After the residual PBS was removed by blotting with sterile paper, lincomycin and doxycycline were added into the wells in serially double-diluted concentrations (1024 to 0.125 mg/l), respectively; both antibiotics were prepared in BHI medium directly from stock solutions. The total volume in each well was 200 μl, and the plates were incubated for a further 24 h at 37°C. A series of antibiotic-free wells was also included to serve as control. The MIC and MBC for the type strain NCTC737 were determined at 50% inhibition in viable cells (cfu/cm²) [14]. Sessile MBCs were determined as the lowest concentration of antibiotic giving a 99.9% reduction in viable bacteria density (cfu/cm²) compared to bacterial density (cfu/cm²) on the antibiotic-free control. Testing of all the isolates was repeated 4 times.

The influence of long-term sub-MIC antibiotics on the MIC and biofilm forming capacity of FBGT pathogens

F1 and F2 were cultured with BHI supplemented with 50%-MIC lincomycin or doxycycline for 15 or...
30 generations separately (3 d for 1 generation). These generations were chosen because the 2 strains were treated with the antibiotics separately for 45 d or 90 d, a period that is long enough to simulate the clinical process of antibiotic treatment. The 2 anti-biotic-treated strains were named 15th and 30th for FL1 and FL2, respectively. We also measured the MIC results of the 15th and 30th strains for lincomycin and doxycycline with the broth dilution method, as described above. In order to observe the influence of antibiotics on the biofilm forming ability, biofilm of strains treated with 50% -MIC lincomycin or doxycycline at 15 or 30 generation were formed and quantified with XTT reduction method.

**Statistical analysis**

Data are expressed as mean ± standard deviation (SD). Multiple comparisons of biofilm formation were made with 1-way analysis of variance (ANOVA) followed by a post hoc analysis (LSD test) when there was significant difference among the time points. Statistical significance was set at $p < 0.05$.

**Results**

**Biofilm formation as determined by XTT dye reduction method**

XTT dye reduction analysis showed that all the tested P. acnes strains started to form biofilm after 24 h in culture, as reflected by the OD490 nm (Figure 1). Quantities of biofilm increased over time in culture and reached a peak at 96 h ($p < 0.05$, $n = 96$, in 3 independent experiments) (Figure 1). After 96 h in culture, the OD490 nm values were maintained at high levels for 24 h in all the tested strains, and there was no significant difference between the OD values of 96 h and 120 h ($p > 0.05$). However, compared with other time points, there was a significant difference ($p < 0.05$). There was no significant difference between the 4 pathogens and the type strain at all the testing time points ($p > 0.05$, $n = 96$).

**Scan electron microscopy (SEM) observation of P. acnes biofilm**

No significant difference was found in the morphology of the biofilm formed by NCTC737 and the 5 FBGT pathogens (Figure 2). SEM observations showed that biofilm began with small microcolonies 12 h after the inoculation of the bacterial suspension. At 12 h, some bacteria were found attaching to the surface of the glass slide (Figure 2A), but the bacteria did not cover the slip completely, with no 3-dimensional structure observed. At 48 h, 80–90% of the slides were covered with bacteria, and 3-dimensional structure was observed in part of the field (Figure 2B), which indicates the beginning of biofilm formation. At 96 h, 3-dimensional structures were found to fully cover the slips (Figure 2C). Many holes were seen among the mushroom-like bacterial cluster, and the function of these holes was believed to be to transport nutrition and water across the biofilm [15]. Thus, the biofilm at 96 h had the major features of mature biofilm, and was regarded as mature biofilm.

**Effects of different substrata on P. acnes biofilm formation**

Biofilm formation of FBGT pathogens and NCTC737 on the surface of the 3 materials (stainless steel, polyethylene, and frosted glass) are shown in Figure 3. According to our previous results, the biofilm of the P. acnes strains was mature 96 h after culture, and so we chose 96-h-old biofilm on the 3 materials to observe the difference of substrata on P. acnes biofilm formation. At 96 h in culture, significant differences were observed among the 3 groups: the frosted glass group had the highest adherence, and stainless steel had the lowest adherence ($p < 0.05$, $n = 9$). This suggests that the biofilm formation ability is positively related to the roughness of the surface of the material. Stainless steel and glass are moderately hydrophilic, whereas polystyrene is hydrophobic [16]. It seems unlikely that the biofilm quantity of the isolates with different substrata was related to the hydrophobicity/hydrophilicity, since stainless steel and frosted glass have similar hydrophilicity [16] but showed different effects on biofilm formation in the present studies.

![Figure 1. Kinetics of P. acnes biofilm formation in wells of microtitre plates as determined by the colorimetric XTT-reduction assay. NCTC737: type strain of P. acnes. Fc, Fg, Fh, and Fl1: pathogens from 4 FBGT patients. The data show analysis of variance (ANOVA) for repeated measure data. *$p < 0.05$ vs all the other time points except 120 h.](image-url)
Antibiotics susceptibilities of P. acnes biofilm

We also measured the susceptibilities of different P. acnes in plankton or biofilm strains to 2 antibiotics – lincomycin and doxycycline (Table I). We observed that all the 6 strains were sensitive to lincomycin and doxycycline except Fg, which was resistant to doxycycline. The sessile MICs of the P. acnes strains were higher than those of planktonic bacteria, which suggests that the FBGT pathogens in biofilm are resistant to both lincomycin and doxycycline, which were mainly used in treating FBGT patients. This further shows that the FBGT pathogens form biofilm in vitro.

The influence of long-term sub-MIC antibiotics on the MIC of FBGT pathogens

Table II shows the MIC results of FL-1 and FL-2 strains at 1st, 15th and 30th generations. The MIC of doxycycline for FL-1 was not changed at the 15th or 30th generation, while the MIC of doxycycline for FL-2 was not changed at the 15th generation (0.125 mg/l), but was increased to 0.25 mg/l at the 30th generation. The MIC of lincomycin for FL-1 was increased from 0.125 mg/l to 0.25 mg/l at the 25th generation, and to 0.5 mg/l at the 30th generation, while the MIC of lincomycin for FL-2 was not changed at the 15th generation (0.5 mg/l), but was increased to 1.0 mg/l at the 30th generation. These results suggest that the MIC of P. acnes can be significantly affected by long-term treatment with lincomycin, but not with doxycycline. Therefore, lincomycin, but not doxycycline, was chosen for the subsequent biofilm forming ability test.

The influence of long-term sub-MIC lincomycin on the biofilm forming capacity of FBGT pathogen

Long-term sub-MIC lincomycin on the biofilm forming of the FBGT pathogen Fl-1 and Fl-2 was observed at 96 h after inoculation in the 30th generation strains. Control strains were not treated with lincomycin during culture for 15 or 30 generations. As shown in Figure 4, biofilm forming in Fl-2 was significantly greater ($p<0.05$, $n=9$) than Fl1 both in the absence or presence of lincomycin at all
the 3 different generations. At the 1st generation, there was no difference between control and lincomycin groups for both Fl1 and Fl2. At the 15th generation, biofilm formation was greater in lincomycin groups than control groups for both Fl1 (p < 0.05, n = 9), and Fl2 (p < 0.05, n = 9). At the 30th generation, biofilm formation was also greater in lincomycin groups than control groups for both Fl1 (p < 0.05, n = 9) and Fl2 (p < 0.05, n = 9). These results suggest that long-term sub-MIC lincomycin culture increases the biofilm forming ability of FBGT pathogens.

Discussion

According to our previous clinical records for the treatment of FBGT patients, P. acnes-sensitive antibiotics in combination were inefficient. This was inconsistent with the observations that the pathogens from these FBGT patients were sensitive to most of these antibiotics in in vitro studies [1,2]. We postulated that the pathogens can form biofilm in vivo, which may lead to the difficulty in the treatment with antibiotics. The major findings of the present study are: 1) all the pathogens isolated from 4 FBGT patients can form biofilm in vitro; 2) the biofilm formation of the pathogens is related to the smoothness of the substratum surface, but not to the hydrophobicity/hydrophilicity of the materials; 3) the speed of biofilm formation can be enhanced by long-term sub-MIC lincomycin treatment. These results suggest that biofilm formation may be 1 of the mechanisms underlying the failure of sensitive antibiotic treatment of these FBGT patients.

In the present study, biofilm formation of the FBGT pathogens in culture was demonstrated by both SEM and antibiotic susceptibility tests. P. acnes strains isolated from the skin lesion of FBGT patients and the type strain NCTC737 can form biofilm in vitro. No difference was found in biofilm quantity and quality in culture among the different strains. This suggests that the pathogen of FBGT may be able to form biofilm in vivo, since P. acnes is reported to form biofilm in vivo [7,8]. In addition, the study of the complete P. acnes genome [17] supports the existence of P. acne biofilm, because the genome of P. acnes contains clusters of genes involved in polysaccharide capsule biosynthesis of a glycolyx polymer, which accounts for adherence to a surface in biofilm formation [18].

It is known that P. acnes can produce biofilm-related medical device infections, and such devices are mostly stainless steel or polystyrene [19]. Polystyrene is hydrophobic, while stainless steel is moderately hydrophilic, and glass has a hydrophilic surface, which is a good positive control for stainless steel [20]. Thus, we chose these 3 kinds of materials as substrata in the present study. It is reported that

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Fl1(generation)</th>
<th>Fl2(generation)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1st</td>
<td>15th</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>0.125</td>
<td>0.125</td>
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<tr>
<td>Lincomycin</td>
<td>0.125</td>
<td>0.25</td>
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</tbody>
</table>

Table I. Antibiotics susceptibilities of different P. acnes strains under planktonic or biofilm (SMIC and SMBC data), as measured by XTT reduction method (mg/l). The MICs for planktonic P. acnes strains were measured after the inoculation of the bacteria suspension into the wells (0 h culture). SMIC and SMBC were determined on 96-h-old biofilms at 50 and 99.9% inhibition, respectively.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Planktonic</th>
<th>SMIC</th>
<th>SMBC</th>
<th>Planktonic</th>
<th>SMIC</th>
<th>SMBC</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCTC737</td>
<td>0.5</td>
<td>512</td>
<td>1024</td>
<td>0.25</td>
<td>1.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Fc</td>
<td>0.25</td>
<td>256</td>
<td>512</td>
<td>0.5</td>
<td>2.0</td>
<td>8.0</td>
</tr>
<tr>
<td>Fg</td>
<td>16</td>
<td>1024</td>
<td>1024</td>
<td>0.125</td>
<td>0.5</td>
<td>1.0</td>
</tr>
<tr>
<td>Fh</td>
<td>0.25</td>
<td>512</td>
<td>512</td>
<td>0.25</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Fl1</td>
<td>0.125</td>
<td>256</td>
<td>512</td>
<td>0.125</td>
<td>1.0</td>
<td>2.0</td>
</tr>
<tr>
<td>Fl2</td>
<td>0.125</td>
<td>256</td>
<td>512</td>
<td>0.5</td>
<td>4.0</td>
<td>16.0</td>
</tr>
</tbody>
</table>

Table II. Long-term influence of antibiotics on the MIC of the pathogens isolated after the first and second recurrence (mg/l). FL1 and FL2 were the strains isolated from the skin lesion of the recurred FBGT patient after the first and second recurrence. The doxycycline and lincomycin affected strains were named as 15th and 30th for FL1 and FL2, respectively.
the hydrophobicity/hydrophilicity may influence the biofilm formed by E. coli on the surface of substrate materials such as polystyrene and stainless steel [21]. However, there are contrary results being reported: hydrophobicity of the support material did not influence the initial development and the microbial composition of anaerobic biofilm [20]. Therefore, we tested whether the hydrophobicity/hydrophilicity of the materials can affect the biofilm formed by P. acnes. Our result suggests that, at least in the pathogens of FBGT, the roughness of the substrata is more important than hydrophobicity/hydrophilicity in biofilm forming. These results contribute to our understanding of P. acnes biofilm.

The pathogens cultured after each recurrence of 1 FBGT patient proved to be the same strain. It provided a good chance to study the influence of long-term antibiotic use on the biofilm forming ability of the pathogen. We found that biofilm forming abilities of the same strain cultured with or without long-term sub-MIC lincomycin are different – long-term culture with sub-MIC lincomycin can accelerate the biofilm of the strains. The same result was obtained from the bacteria cultured after the second recurrence, suggesting that long-term sub-MIC lincomycin may be helpful in biofilm formation and therefore may be responsible for the recurrence. Sub-MICs of antibiotics are able to modify the physicochemical properties and the architecture of the outer surface of S. epidermidis [22], thus inhibiting bacterial initial adhesion to abiotic substrates [23]. However, other studies have demonstrated that initial adherence to a surface and subsequent biofilm formation can be independent phenomena [24–26]. Thus, the effect of sub-MIC antibiotics on initial bacterial adherence may not be directly extrapolated to biofilm formation [27]. The architecture of the outer surface of long-term sub-MIC lincomycin influencing P. acnes biofilm changed little with SEM observation, which helps to explain that the influence of sub-MIC antibiotics on initial bacterial adherence may not be directly extrapolated to biofilm formation. Even though we do not know exactly the influence of sub-MIC lincomycin on the initial adhesion of P. acnes biofilm, long-term culture with lincomycin still can accelerate biofilm formation of the P. acnes strains.

The best time point for P. acnes biofilm formation was at 96 h after inoculation when a bacterial density of $1 \times 10^8$ cfu/ml was used. At 96 h after inoculation, the biofilm presents the characteristics of mature biofilm (clear 3-dimensional structure with many nutrition-transporting holes among it), and can be regarded as mature biofilm [15]. Ramage et al. reported that P. acnes can form biofilm on orthopaedic biomaterials [5]; their observation time is 0.5–18 h after inoculation. According to our results, 18 h is not enough for the maturation of P. acnes biofilm. Because the generation time for P. acnes was approximately 5.1 h [28], much slower than other anaerobic bacteria, it is almost impossible to form biofilm in 24 h. Also, biofilm has a type of living status, and it represents a protected mode of growth that allows cells to survive in hostile environments [29]. At earlier time points after inoculation, the nutrients in the medium are still sufficient, a reasonable environment for the bacteria to form biofilm. Therefore, in our opinion, P. acnes biofilm should be studied 72–96 h after inoculation.

Theoretically, the best way to test our hypothesis is to conduct clinical studies on FBGT patients: 1) to examine whether the pathogens form biofilm in vivo before any antibiotic treatment; 2) whether interventions, for example, ultrasound [30], electric current [31] and liposomes [32] that can inhibit the biofilm formation can improve the conditions of FBGT patients. However, this clinical study is not feasible for us at this stage. Due to the difficult diagnosis and low incidence of FBGT, there have been no new live patients found in our hospital since these 4 FBGT patients involved in the present study, apart from 3 who died before they arrived at our hospital. Our group will conduct further in vivo studies when new patients are available.

In conclusion, the present study showed that pathogens of FBGT patients can form biofilm in vitro, and long-term P. acnes-sensitive antibiotics (such as lincomycin) treatment may enhance biofilm formation. This may be 1 of the major reasons for the failure of the antibiotic treatment of FBGT patients. Future treatment strategies of FBGT should include interventions and antibiotics that can inhibit biofilm formation of the pathogens.
Acknowledgements

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