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Linezolid and vancomycin decrease the therapeutic effect of methylene blue-photodynamic therapy in a mouse model of MRSA bacterial arthritis

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Abstract

We previously reported that photodynamic therapy (PDT) using intra-articular methylene blue (MB) could be used to treat arthritis in mice caused by bioluminescent methicillin-resistant *Staphylococcus aureus* (MRSA) either in a therapeutic or in a preventative mode. PDT accumulated neutrophils into the mouse knee *via* activation of chemoattractants such as inflammatory cytokines or chemokines. In the present study, we asked whether PDT combined with antibiotics used for MRSA could provide added benefit in controlling the infection. We compared MB-PDT alone, systemic administration of either linezolid (LZD) alone or vancomycin (VCM) alone or the combination of PDT with either LZD or VCM. Real-time non-invasive imaging was used to serially follow the progress of the infection. PDT alone was the most effective, while LZD alone was ineffective and VCM alone showed some benefit. Surprisingly the addition of LZD or VCM reduced the therapeutic effect of PDT alone ($P < 0.05$). Considering that PDT in this mouse model stimulates neutrophils to be antibacterial rather than actively killing the bacteria, we propose that LZD and VCM might inhibit the activation of inflammatory cytokines without eradicating the bacteria, and thereby reduce the therapeutic effect of PDT.

Introduction

Antibiotic therapy is still a mainstay of treatment for microbial infections in the orthopedic field. However, intractable microbial infections can arise due to the use of artificial implant material such as metal or resin, combined with the widespread occurrence of multidrug-

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resistant bacterial strains. New therapeutic modalities as an alternative to traditional antibiotics are therefore needed.

In recent years, photodynamic therapy (PDT) has been examined as an alternative approach to treat local microbial infections, and there have been an increasing number of reports of testing of PDT for combating microbial infections both in pre-clinical models (1, 2) and in clinical trials (3). PDT or photodynamic inactivation involves the combination of a non-toxic dye or photosensitizer (PS) excited with harmless visible light that produces reactive oxygen species such as singlet oxygen and hydroxyl radicals that selectively destroy microbial cells.

We have previously reported a series of studies (4) designed to answer the question of whether PDT can be used to treat a model of bacterial arthritis in the mouse knee caused by methicillin-resistant *Staphylococcus aureus* (MRSA). We first showed that systemic injection of Photofrin followed by red light illumination of the knee failed to produce any benefit (5). Next we studied intra-articular injection of Photofrin and found a biphasic dose response with increasing light fluence whereby a moderate amount of light produced a benefit but lower or high doses of light did not (6). This observation was explained by the ability of high dose PDT to not only kill bacteria, but also to kill neutrophils in the knee that could control the infection by host-defense. An in vitro study then found that methylene blue (MB) or toluidine blue-O were the best antibacterial PS as they maximized bacterial killing while minimizing damage to neutrophils (7). We recently demonstrated that PDT using intra-articular MB could accumulate a large number of neutrophils into the joint *via* an activation of chemoattractant factors and at the correct dose could exert a therapeutic effect on bioluminescent MRSA infection monitored by in vivo imaging (8). Furthermore, we demonstrated that PDT in a pre-conditioning regimen could strongly inhibit subsequent bacterial growth and suppress the development of infection, therefore PDT could as a non-specific vaccination approach (4).

Based on these results, we hypothesized that a combination of PDT using MB with antibiotic therapy using anti-MRSA antibiotics such as linezolid (LZD) or vancomycin (VCM) (9) could achieve a more favorable therapeutic effect in the MRSA arthritis model. This hypothesis was tested in the present communication with surprising results.

Materials and Methods

This study was conducted under a protocol approved by the Subcommittee on Research Animal Care (IACUC) of Massachusetts General Hospital, Boston MA, and the Institutional Review Board for the Care of Animal Subjects at the National Defense Medical College, Japan.

Murine intractable MRSA arthritis model using bioluminescent MRSA and resin microparticles

A mouse model of intractable MRSA arthritis was employed using stable bioluminescent MRSA Xen31 (*lux*-MRSA, Caliper Life Sciences, Alameda, CA) (10) combined with resin microparticles (MP) made of polystyrene (Copolymer Microsphere Suspensions 7000φ3.2 μm, Thermo Scientific Particle Technology, Fremont, CA) as described previously (8). Eight to 9-week-old male C57BL/6 mice (Charles River Laboratories, Wilmington, MA) were anesthetized by intraperitoneal (i.p.) injections of a cocktail composed of 100-mg/kg ketamine and 10-mg/kg xylazine and the left knees were shaved. 10 μL of PBS suspension containing *lux*-MRSA (1×10⁸ CFU) and MP (2.5 % volume) was intra-articularly injected into the left knee joint through the midline of the patellar ligament using a syringe with a 29

G needle. The day on which the *lux*-MRSA and resin microparticles were inoculated into the left knee joint was defined as day 0.

Photodynamic therapy using methylene blue

Twenty-four hours after the inoculation (at1d), 10 uL of PBS solution of MB (100 uM) was injected into the joint followed by immediate irradiation of the left knee with a 1-cm diameter spot using a xenon light source with a band-pass filter (wavelength of 660+/- 15 nm, LumaCare, Newport, CA). The fluence rate was fixed at 100 mW/cm² using a power meter (model DMM 199 with 201 standard head; Coherent, Santa Clara, CA) and a fluence of 50 J/cm² was used.

Antibiotics

Linezolid (LZD, Waterstone Technology, Carmel, IN) or vancomycin hydrochloride (VCM, Fisher BioReagents, Fair Lawn, NJ) were used as anti-MRSA antibiotics. Each antibiotic was dissolved in phosphate-buffered saline (PBS) in an adequate concentration for each administration (2 mg/mL for LZD and 6 mg/mL for VCM). Prior to the *in vivo* experiments, we confirmed in an *in vitro* experiment that minimum inhibitory concentration (MIC) of both LZD and VCM on the *lux*-MRSA was less than 4 µg/mL.

Experimental groups

Six different groups were established for the evaluation ($n = 5$ each). (1) a no treatment (NT) group, which receive neither antibiotic therapy nor PDT; (2) a LZD alone group, which received systemic administration of LZD by 2 intra-peritoneal injections per day (approximately 100 uL) on days 1, 2 and 3 in the amount of 10 mg/kg in each 12 hours (20 mg/kg per day); (3) a VCM alone group, which received 2 i.p. injections of VCM on days 1, 2 and 3 in the amount of 33 mg/kg in each 12 hours (66 mg/kg per day); (4) a LZD + PDT combination group, which received PDT at day1 and systemic administration of LZD with the same regimen as the LZD group; (5) a VCM + PDT combination group, which received PDT at day 1 and systemic administration of VCM with the same regimen as the VCM group; and (6) a PDT alone group, which received PDT at day 1 in the regimen described above.

The time-course series of the MRSA arthritis in each group was assessed by measuring the bioluminescent intensity of the *lux*-MRSA in the infected knee joint using the methods described previously (8) and the intensity was indicated as relative luminescence units (RLUs). Integral values of the time courses of bioluminescent intensity from 2h to 7d were calculated and expressed as the area under the curve (AUC). AUC in each group were statistically compared to AUC in the PS-IR- group as described previously (10).

Statistical Analysis

All the data are expressed as means $\pm$ SE. Mean values in each group were used for statistical analysis. One-way repeated-measures analysis of variance (ANOVA) test followed by Dunnett's post *hoc* test was used for data analysis of time courses. One-way ANOVA test followed by Dunnett's post *hoc* test was used for data analysis of RLUs at day 7. SPSS ver.16 was used for each data analysis. *P*-values < 0.05 were considered statistically significant.

Results and Discussion

Figure 1 shows representative bioluminescence images from individual mice taken from each of the six experimental groups over the time course of the study (7 days). The control (no treatment) mice showed a long-lasting infection that was still robust at day 7. LZD

treatment alone appeared to have no effect on the progress of the infection. VCM treatment alone had some therapeutic effect and the infection was reduced on days 5 and 7. PDT alone clearly had the best effect with the infection effectively disappearing on the day after PDT and remaining gone for the whole 7 days. Surprisingly both the combination therapy groups (PDT + LZD and PDT + VCM) did worse than either therapy used alone. PDT+LZD showed a robust infection still present on day 7, while PDT+ VCM had a lower but significant infection present on day 7. It can be seen that none of the three PDT groups showed a marked reduction in bioluminescence immediately after PDT. This immediate reduction is typically seen when the PDT is having a direct bacterial killing effect (11–14) and the lack of it suggests that a different mechanism is at work.

Figure 2 shows the quantification of the mean bioluminescence signals from the mice in the six groups. Figure 2A plots the mean RLU values ($n=5$) from the 6 groups of mice, and it can be seen at day 7 that the mean value for PDT alone is significantly lower than the mean value for all the other five groups ($P<0.05$).

Fig 2B plots the mean integrated AUC values of the time courses of the six groups. It can be seen that while the AUC for VCM alone is significantly lower than the AUC for NT ($P<0.05$), the AUC for PDT alone is significantly lower than PDT+VCM ($P<0.05$). The corresponding values for LZD were less remarkable with LZD alone showing no significant benefit over NT, and the combination of LZD+PDT being worse than PDT alone but not significantly so.

We hypothesized that the combination of PDT with antibiotic therapy could exert a better therapeutic effect on MRSA arthritis than either treatment used alone. However our results showed that PDT alone showed a higher therapeutic effect than the combination therapy with LZD or VCM. The surprising finding is therefore that antibiotic therapy with LZD and VCM inhibits the therapeutic effect of PDT by an unknown mechanism.

Our previous reports showed that the antibacterial effect PDT using methylene blue in the intractable MRSA arthritis model depended on the accumulation of neutrophils into the infectious knee joint rather than a direct ROS-mediated killing of bacterial cells (4). This accumulation of neutrophils occurred *via* an activation of chemoattractant factors after PDT such as inflammatory cytokines and chemokines (8). There are reports showing that the antibiotics LZD and VCM are able to inhibit inflammatory cytokines, e.g. interleukin 1β (IL- 1β), tumor necrosis factor α (TNF- α), or interleukin 6 (IL-6) that are related to initial inflammatory reaction (15–18). Therefore, the combination of LZD or VCM with PDT might inhibit the most important key factor that mediates the therapeutic effect of PDT in this model, namely neutrophil infiltration into the knee, resulting in the reduction of the PDT effect.

When an anti-MRSA antibiotic such as LZD or VCM is used on a severe systemic microbial infection such as sepsis, it can prevent a “cytokine storm” by inhibiting a global release of inflammatory cytokines as well as inhibiting bacterial growth (19). In other words, antibiotic therapy could treat the infection by inhibiting bacterial growth as well as saving the patient’s life by inhibiting the inappropriate and overwhelming release of cytokines throughout the system.

However, in a local microbial infection such as pyogenic arthritis, neutrophils play a most important role in host defense mechanism (20–22), and chemoattractant factors such as inflammatory cytokines or chemokines are beneficial because they increase the chemotaxis and accumulation of neutrophils in the region of the infection. We previously showed that PDT using MB exerted a strong therapeutic effect *via* activation of the chemoattractant factors and an accumulation of neutrophils into the knee joint rather than by directly killing

the bacteria themselves (8). Therefore, LZD or VCM, which inhibit chemoattractant factors such as inflammatory cytokines, might be useful for a treatment of systemic microbial infection, while PDT alone might be useful for a local microbial infection. Furthermore, since combined therapy of PDT with anti-MRSA antibiotics was less effective than PDT alone, PDT in such situations as joint infections should be performed independently and could be followed by antibiotics after several days if needed.

We reported a previous study (23) that showed that PDT could indeed be beneficially combined with antibiotics in a mouse model of localized infection. That model used an excisional wound infected with *Pseudomonas aeruginosa* treated with PDT mediated by a cationic fullerene. It was shown that by combining the PDT with a sub-therapeutic regimen of tobramycin, that additional benefits could be achieved in both bacterial burden in the wound and also increased mouse survival by lessening the chances of mice developing sepsis.

Therefore it is too early to make a general rule about whether antibiotics can or cannot be beneficially combined with antimicrobial PDT in all infectious diseases. The choice is likely to rest upon whether the outcome of the infection model is heavily impacted by host defenses such as neutrophils. If this is the case then the addition of antibiotics, particularly those like LZD and VCM that suppress inflammation, may be ill-advised. On the other hand if the infection model is one where the outcome is largely governed by the bacterial burden and the likelihood of the bacteria spreading systemically and causing sepsis is real, the addition of antibiotics to an antibacterial PDT regimen may be beneficial. Further study will be needed to confirm the distinction between these two scenarios in animal models, and to explore whether similar distinctions might apply to humans.

Conclusion

The addition of anti-MRSA antibiotics, linezolid and vancomycin, reduced the therapeutic effect of PDT using methylene blue in a murine bacterial arthritis model. This observation may be explained by the action of LZD and VCM in reducing inflammatory cytokines, thereby abrogating the PDT-induced activation of cytokines and reducing the accumulation of neutrophils into the joint.

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Figure 1.

Representative bioluminescent images of individual mice followed over a time course (1–7 days) series in the no treatment (NT) group, the linezolid alone (LZD) group, the vancomycin alone (VCM) group, the LZD + PDT group, the VCM + PDT group and the PDT alone group.

Figure 2.

A: Mean ($\pm$ SD) bioluminescent intensity values in the six groups ($n = 5$ each) measured over the course of the study. * $P < 0.05$; ** $P < 0.01$ (compared to the value at day 1 in each group); + $P < 0.05$; ++ $P < 0.01$ (compared to NT). **B:** Comparison of the area (mean $\pm$ SD) under the RLU curve (AUC) of the data indicated in Fig 2A. $n = 5$ each. * $P < 0.05$.