Establishment of a Real-Time, Quantitative, and Reproducible Mouse Model of *Staphylococcus* Osteomyelitis Using Bioluminescence Imaging

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Osteomyelitis remains a serious problem in the orthopedic field. There are only a few animal models in which the quantity and distribution of bacteria can be reproducibly traced. Here, we established a real-time quantitative mouse model of osteomyelitis using bioluminescence imaging (BLI) without sacrificing the animals. A bioluminescent strain of *Staphylococcus aureus* was inoculated into the femurs of mice. The bacterial photon intensity (PI) was then sequentially measured by BLI. Serological and histological analyses of the mice were performed. The mean PI peaked at 3 days, and stable signals were maintained for over 3 months after inoculation. The serum levels of interleukin-6, interleukin-1 β , and C-reactive protein were significantly higher in the infected mice than in the control mice on day 7. The serum monocyte chemotactic protein 1 level was also significantly higher in the infected group at 12 h than in the control group. A significantly higher proportion of granulocytes was detected in the peripheral blood of the infected group after day 7. Additionally, both acute and chronic histological manifestations were observed in the infected group. This model is useful for elucidating the pathophysiology of both acute and chronic osteomyelitis and to assess the effects of novel antibiotics or antibacterial implants.

One of the most serious problems in the orthopedic field is infectious osteomyelitis, which causes progressive inflammation and destruction of bone tissue (19). Treatment of infectious osteomyelitis is challenging, because the pathogenic organisms and their drug sensitivities are variable. This problem is compounded by increasing numbers of drug-resistant bacterial strains, implant-associated infections, and elderly patients with compromised immune systems (18). Although progress has been made, controlling infectious osteomyelitis is still difficult. Therefore, experimental studies are warranted to develop more effective treatment options. A number of animal models of osteomyelitis have been reported (13, 26, 33, 35). However, many of them require sacrifice of the animals, and thus, they are limited in their availability for real-time assessments of the severity of infection and the efficacy of treatments. As a result, the pathophysiology of osteomyelitis remains poorly understood.

A recent development in optical imaging, bioluminescence imaging (BLI), permits the noninvasive sequential monitoring of cell growth and gene expression *in vivo*. This method allows real-time monitoring of implanted cells in live animals (16, 27). Inoculated bacteria that emit a constant bioluminescent signal can be detected through the tissues of a live animal using an ultrasensitive, cooled, charge-coupled device (CCD) camera. This approach has proven useful in studies in the fields of oncology (38), endocrine disruptors (28), metabolism (37), hematopoietic cells (41), regenerative medicine (16, 27), immunology (6, 21), and infections (2, 5, 11, 14, 20, 24, 34, 42).

Previous models of infectious diseases required sacrifice of animals to quantify the bacteria by culturing tissue specimens. In contrast, the BLI technique monitors bacterial growth throughout the course of infection in real time without sacrificing animals. To

our knowledge, there is no previous model in which infectious processes in bone from the acute to the chronic phases were evaluated using BLI, as well as the kinetics of immune cells and the levels of cytokines/chemokines in serum. The purpose of the present study was to establish a real-time, quantitative, and reproducible mouse model of osteomyelitis using the BLI technique.

MATERIALS AND METHODS

Bioluminescent bacteria. A bioluminescent strain of *Staphylococcus aureus*, Xen-29, was obtained from Caliper LS Co. (Hopkinton, MA) and cultured in Luria Bertani medium (Sigma-Aldrich Co., St. Louis, MO) at 37°C under ambient aeration with gentle agitation. The bacteria were selectively grown in medium containing 200 μ g/ml kanamycin. *S. aureus* Xen-29, derived from the parental strain ATCC 12600, has a stable copy of a modified *Photobacterium luminescens luxABCDE* operon, encoding enzymes responsible for the luminescent reaction. Bacterial bioluminescence requires no substrate to be added to generate the light and will constitutively emit a bioluminescent signal as long as the organism is viable. The bacterial samples were frozen and stored at -80°C . The samples were thawed at 4°C for 1 h prior to each experiment. Typically, bacterial viability was maintained at 4°C for approximately 5 h after thawing.

Mouse osteomyelitis model. Eighteen BALB/c adult male mice (12 weeks old; 20 to 25 g) purchased from Sankyo Labo Service (Shizuoka,

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FIG 1 Correlation between bacterial number and bacterial photon intensity *in vitro*. Photon emission of the bacterial bioluminescent signals of *S. aureus* strain Xen-29 was captured as false-color photon count images and quantified by a BLI system. To examine the sensitivity of the BLI, a CCD-based macroscopic detector was used to quantify the bacterial PI (photons/s/cm 2 /sr) at various bacterial numbers (7.8×10^5 to 1.0×10^8 CFU per well). Bioluminescent signals were detected from colonies of bioluminescent *S. aureus in vitro* (A and B), and there was a significant correlation between the number of bacterial CFU and the bacterial PI *in vitro* ($R^2 = 0.998$) (C).

Japan) were used in this study. The mice were maintained in our animal facility under specific-pathogen-free conditions. The mice were anesthetized with an intraperitoneal injection of 50 mg of pentobarbital/kg of body weight, and the skin on the left knee was shaved and sterilized with povidone iodine. A skin incision was made over the left knee, and the distal femur was exposed through a lateral parapatellar arthrotomy with medial displacement of the quadriceps-patellar complex. The distal end of the femur was perforated using a high-speed drill with a 0.5-mm sharp steel burr (Fine Science Tools Inc., Foster city, CA). Then, a channel was created using a 23-gauge (external diameter, 0.6 mm) needle, through which the bioluminescent strain of *S. aureus* (1.0×10^8 CFU) in 1 μ l of medium was inoculated into the medullary cavity of the femur using a Hamilton syringe. Phosphate-buffered saline (PBS) was administered to the control group using the same technique. The burr hole was closed with bone wax, the dislocated patella was reduced, and the muscle and skin openings were closed by sutures. The animals were placed on a heating pad and carefully monitored until recovery. The observation of spontaneous forelimb movement and the drinking of water were the criteria used to determine that the animals had recovered from the anesthesia.

To measure and analyze the bacterial bioluminescent signal by BLI,

the mice were anesthetized via inhalation of aerosolized isoflurane mixed with oxygen. The animals were laid on their backs and imaged for 5 min. All experiments were approved by the Animal Care and Use Committee of Keio University.

BLI. A Caliper LS-Ivis Lumina cooled CCD optical macroscopic imaging system (Summit Pharmaceuticals International Co., Tokyo, Japan) (30) was used for the BLI. Photon emissions of the bacterial bioluminescent signal were captured, converted to false-color photon count images, and quantified with Living Image version 3.0 software (Caliper LS Co., Hopkinton, MA). The bacterial photon intensity (PI) was expressed as photon flux in units of photons/s/cm 2 /sr. To quantify the bacterial luminescence, regions of interest (ROIs) were defined in the bacterial plates or inoculated areas and examined. To evaluate the luminescence expression of the bacteria, we first examined whether various numbers of bacteria correlated with the bacterial PI *in vitro* and *in vivo*. To analyze the time course of the infection *in vivo*, the bacterial PI in an ROI was sequentially measured on days 1, 3, 7, 14, and 21 after the operation.

Serological evaluation. Blood samples were collected from the infected and the control mouse groups by retro-orbital bleeding before surgery (day 0) and on days 0.5 (12 h), 1, 3, 7, 14, and 21 after the operation.

A

B

FIG 2 Correlation of bacterial number and bacterial photon intensity *in vivo*. (A) During *ex vivo* imaging, the bacterial bioluminescent signal was detected only in the medullary cavity of the femur and not in the surrounding tissue. (B) Different amounts of bacteria (1.0×10^8 to 6.0×10^8 CFU per inoculation) were inoculated into the femurs, and the bioluminescence in the ROIs was monitored by the BLI system. Significant correlation was observed between the inoculated bacterial number and the bacterial PI *in vivo* ($R^2 = 0.999$).

To measure inflammatory cytokines and chemokines, the sera of both groups were serially diluted, and interleukin-6 (IL-6), IL-1 β , C-reactive protein (CRP), and monocyte chemoattractant protein 1 (MCP-1) were measured by using enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN; Kamiya Biomedical Co., Seattle, WA). Detection was carried out according to the manufacturers' instructions.

Flow cytometry. Peripheral blood samples from the infected and the control mice were subjected to double immunofluorescence staining and analyzed by flow cytometry on days 1, 3, 7, 14, and 21 after the operation. Fluorescein isothiocyanate (FITC)-anti-CD11b (clone M1/70) and phycoerythrin (PE)-anti-Ly-6C (Gr-1) (clone RB6-8C5) antibodies (Abs) were purchased from BD Biosciences (San Diego, CA). To block the non-specific binding of Abs to Fc receptors, the isolated cells were incubated

with an anti-CD16/32 monoclonal Ab (MAb) (clone 2.4G2; 1:250) at 4°C for 20 min. The cells were then stained with a mixture of fluorochrome-labeled MAbs at 4°C for 20 min, washed, and incubated with 7-aminoactinomycin D (1:500; BD Biosciences, San Diego, CA) at 4°C for 5 min. Flow cytometry was performed on a FACSCalibur (BD Biosciences, San Diego, CA), and the data were analyzed with FlowJo software (Tree Star, Ashland, OR). Murine granulocytes were defined as SSC^{high} ($1 \times$ side scatter is 0.15 M NaCl plus 0.015 M sodium citrate) CD11b⁺ cells (29). These cells also expressed Gr-1 (not shown).

Histological analysis. Femur specimens were collected and analyzed histologically on days 3, 7, 21, and 28 after the operation in both groups. The mice were sacrificed, and the femurs were removed and separated from the soft tissues. The samples were fixed in 4% paraformaldehyde and

FIG 3 Time course changes of bacterial photon intensity in the mouse osteomyelitis model. *S. aureus* strain Xen29 (1.0×10^8 CFU) in $1 \mu\text{l}$ of medium was inoculated into the medullary cavity of the femur. The bacterial PI from an ROI was then sequentially measured on days 1, 3, 7, 14, and 21 after surgery ($n = 6$ for each time point). The mean bacterial PI in the infected group peaked on day 3 ($7.2 \times 10^5 \pm 1.0 \times 10^5$ PI) and remained at a high level until approximately day 7 ($5.2 \times 10^5 \pm 0.7 \times 10^5$ PI). Shown are means $\pm$ standard errors of the mean (SEM).

demineralized with EDTA. The samples were then embedded in paraffin, cut into $5\text{-}\mu\text{m}$ -thick sections, and stained with hematoxylin and eosin or Gram stain.

Statistical analysis. Correlations between the bacterial CFU and the bacterial PI *in vitro* and *in vivo* were analyzed by linear regression. Changes in the bacterial PI in the infected group were analyzed with Student's *t* test. One-way analysis of variance (ANOVA) and the Fisher *post hoc* test were used to compare the levels of IL-6, IL-1 β , CRP, and MCP-1 in serum and the proportion of granulocytes in the peripheral blood between the two groups. Correlation between the bacterial PI and the serum CRP levels was determined using Pearson's correlation coefficient. SPSS II software (IBM-SPSS, Tokyo, Japan) was used, and a *P* value of less than 0.05 was considered significant in all the statistical analyses.

RESULTS

Correlation between bacterial number and bacterial photon intensity *in vitro*. A bioluminescent signal that was sufficient to yield a significant value over background was detected by the BLI system from a single colony of the bioluminescent strain *S. aureus* Xen-29 cultured in Luria Bertani medium (Fig. 1A and B). To examine the sensitivity of the BLI, we used a CCD-based macroscopic detector to measure the PI of bacterial samples with 7.8×10^5 to 1.0×10^8 CFU/well. A minimum of 7.8×10^5 CFU of bacteria was sufficient to produce a detectable signal above the background noise. This quantitative bioluminescence analysis re-

vealed that there was a significant correlation between the number of bacterial CFU and the bacterial PI *in vitro* ($R^2 = 0.998$) (Fig. 1C). To confirm that only live *S. aureus* naturally emitted the luminescent signals, colonies of *S. aureus* bacteria fixed with 4% paraformaldehyde were visualized with the BLI system. No signal was detected from the fixed bacteria (data not shown).

Correlation between bacterial number and bacterial photon intensity *in vivo*. To visualize the infected site *ex vivo*, immediately after the intrafemoral inoculation of *S. aureus*, the infected femur was removed and separated from the soft tissues, and the exposed femur was monitored by the BLI system. The bacterial bioluminescent signals were detected only in the medullary cavity of the femur and not in the surrounding tissue (Fig. 2A). To examine whether the number of inoculated bacteria correlated with the bacterial PI *in vivo*, we performed inoculations with different numbers of bacteria (1.0×10^8 to 6.0×10^8 CFU per inoculation) and measured the bacterial PI. As shown in Fig. 2B, there was a significant correlation between the number of inoculated bacteria and the bacterial PI ($R^2 = 0.999$).

Time course of bacterial photon intensity in the mouse OM model. Immediately after the inoculation of *S. aureus* (1.0×10^8 CFU) in $1 \mu\text{l}$ of medium into the femur, stable luminescent signals were observed in all the animals. Sequential analyses of the bacte-

FIG 4 Serological evaluation of control and infected groups. Blood samples from the site of retro-orbital bleeding were collected from the mice before surgery (day 0) and on days 0.5 (12 h), 1, 3, 7, 14, and 21 after the operation in the infected and the control groups ($n = 3$ each). The serum IL-6 (A), IL-1 β (B), and MCP-1 (C) levels were measured with ELISA kits. Shown are means $\pm$ SEM.

rial luminescence revealed that the mean bacterial PI in the infected group peaked on day 3 ($7.2 \times 10^5 \pm 1.0 \times 10^5$ PI) and remained at a high level until approximately day 7 ($5.2 \times 10^5 \pm 0.7 \times 10^5$ PI) (Fig. 3). Notably, the strong bacterial bioluminescent signal was detected only at the injection site of the femur, and the surrounding tissue was free of infection for 3 months after surgery (data not shown). These observations indicated that this novel mouse model is reproducible and suitable for evaluating the pathophysiology of both acute and chronic osteomyelitis.

Serological evaluation. During the early phase of infection, the mean serum IL-6 and IL-1 β levels in the infected group were elevated. The serum IL-6 level was significantly higher in the infected group than in the control group on day 7 ($P < 0.05$) (Fig. 4A). The mean serum level of IL-1 β in the infected group was significantly higher on days 7 and 14 ($P < 0.05$) (Fig. 4B). On day 0.5 (12 h), the mean level of MCP-1 was significantly higher in the infected group than in the control group ($P < 0.001$) (Fig. 4C). The mean serum CRP level increased quickly in both groups and remained at 20 ng/ml for 3 days, after which the level remained significantly higher in the infected group on days 7, 14, and 21 ($P < 0.001$) (Fig. 5A). There appeared to be a direct correlation between the bacterial PI and the serum CRP level in the samples obtained on days 14 and 21 ($n = 3$ each), the chronic phase of infection ($r = 0.85$; $P < 0.05$) (Fig. 5B).

Flow cytometry. Flow cytometric analyses using anti-CD11b and anti-Gr1 MAbs showed the presence of granulocytes in the peripheral blood in both groups (Fig. 6). The proportion of SSC^{high} CD11b⁺ granulocytes in the peripheral blood was significantly higher in the infected group than in the control group on days 7, 14, and 21 ($n = 4$ each) (Fig. 6C).

Histological analysis. On day 21, the femur bone marrow from the sham-treated mice contained the normal cellular components of bone marrow: megakaryocytes, erythroid cells, and myeloid cells. In contrast, on day 3, bacterial colonies were detected in the medullary cavity of the infected mouse femur, along with a marked infiltration of neutrophils. The bacterial colonies were Gram positive. New bone formation started beneath the periosteum on day 7. By day 21, new bone formation and trabecular bone resorption by osteoclasts were present. Manifestations of chronic osteomyelitis, such as sequestrum, new bone formation, and fibrosis, were prominent on day 28 (Fig. 7).

DISCUSSION

Osteomyelitis is a serious infectious disease characterized by progressive bone destruction and formation (1, 19). In most cases, chronic osteomyelitis requires the administration of antibiotic drugs for prolonged periods and sometimes surgical procedures. Recently, the incidence of serious nosocomial infection due to

FIG 5 Serum CRP levels. (A) Correlations of bacterial photon intensity and the serum CRP level. The serum CRP levels in the infected and control groups were also measured with ELISA kits ($n = 3$ each). Shown are means $\pm$ SEM. (B) The bacterial CFU and bacterial PI are correlated. The serum CRP concentration was examined in the same samples from mice on days 14 ($n = 3$) and 21 ($n = 3$). A direct correlation between the bacterial PI and the serum CRP concentration was observed on both days ($r = 0.85; P < 0.05$).

multiple-drug-resistant strains of bacteria has risen. Thus, the treatment of osteomyelitis has become more difficult (32, 44). Additional sources of rapidly spreading infections include orthopedic implants, such as those used in fracture fixations, arthroplasty, and spinal surgery (8, 39). A number of infection models have been created to study the diagnosis and treatment of osteomyelitis. For example, some investigators have attempted to implant staphylococci intravenously or directly into the bone. Although they successfully produced bone infections, these lesions were not progressive enough to simulate human osteomyelitis (31). Scheman et al. (35) established a reproducible model of chronic osteomyelitis in rabbits by injecting sodium morrhuate and *S. aureus* directly into the tibial metaphysis. Experimental models using small animals, such as rats and mice, allow easy handling and are cost-effective; in particular, mice are especially useful for understanding the pathophysiology of osteomyelitis because various genetically modified mice are commercially avail-

FIG 6 Proportion of granulocytes in the peripheral blood. (A and B) Peripheral blood samples were analyzed by flow cytometry on days 0.5 (12 h), 1, 3, 7, 14, and 21 after the operation in the infected and the control groups. Flow cytometric analyses of SSC-H^{high} CD11b⁺ granulocytes in the peripheral blood of the control (A) and infected (B) mice on day 21 are shown. (C) The proportions of SSC-H^{high} CD11b⁺ granulocytes in the peripheral blood on days 7, 14, and 21 were significantly higher in the infected group than in the control group ($n = 4$ each). Shown are means and SEM.

able. In recent papers, tibia infection mouse models have been used to evaluate implant-associated osteomyelitis (20, 36). However, because the mouse has tibial curvature with a short medullary cavity and scant surrounding soft tissue, the preparation of this model is technically difficult and often associated with incidental tibial fractures or leakage of the inoculated bacteria. In comparison, our novel osteomyelitis model using the mouse femur is easy and reproducible, because the medullary cavity is straight, with a long, thick cortex and adequate soft tissues surrounding the bone.

In previous animal models of osteomyelitis, the animals had to be sacrificed to quantify the bacterial burden and to assess the extent of infection and inflammation (7). Experiments using such models are very time-consuming, and there is an increased possibility of technical errors during sampling. Furthermore, since the animals have to be sacrificed at certain time points, it is impossible to monitor the same animal throughout the course of the infection. In contrast, recently developed BLI techniques enable us to monitor sequential gene expression patterns and the viabilities of the implanted cells or inoculated bacteria throughout the course of diseases without sacrificing the animal. Moreover, appropriately prepared animals can be selected at the outset of the experi-

FIG 7 Changes over time in the histology of femurs from infected and control mice. Shown are hematoxylin and eosin staining of longitudinal sections of the uninfected and infected femurs on days 3, 7, 21, and 28 after bacterial inoculation. The middle and right images show higher-power views of the white-boxed areas of the left images and black-boxed areas of the middle images, respectively. The inset of the infected femur (day 3) indicates Gram-stain-positive bacteria. *, necrotic area with bacterial colonies; arrows, osteoclasts; **, sequestrum. Both acute and chronic manifestations of osteomyelitis were observed in this model. Bars = 100 μ m.

ment, because the bacterial bioluminescence is visible and can be quantified immediately, thus enabling the accurate evaluation of a treatment and avoiding unnecessary follow-ups. Several studies have shown the advantages of *in vivo* BLI for the real-time monitoring of bacterial infections and their treatment (2, 5, 11, 14, 20, 24, 34, 42). In our mouse osteomyelitis model, sequential analysis of the bacterial luminescence revealed that the bacterial signal peaked on day 3 after the inoculation and then plateaued until day 7 and could be visualized for over 3 months. This time course is similar to that in the previous osteomyelitis models (2, 20), in which the innate immune system contributes to inhibiting the growth of the bacteria at the early phase (20). Recently, Bernthal et al. (2) established a mouse model of implant-associated infection

as a preclinical screening tool. However, a limitation of their model is the use of the SH1000 *S. aureus* bioluminescent strain, in which the *lux* genes are contained in a plasmid. The plasmid is stable for only the first 3 days of broth culture, so it is difficult to estimate bacterial numbers by the bioluminescent signals of the strain after 3 days. Thus, their model represents only acute, not chronic, infection. In our model, the *lux* genes were inserted into the *S. aureus* chromosome and the bioluminescent signals were maintained for a longer period. In addition, leakage of the inoculated bacteria to surrounding tissues is often observed in implant-associated osteomyelitis and joint infection models and can be a major cause of skin ulcers. Such models are poorly reproducible, with short, unstable emission of the bacterial luminescence. In

contrast, the pinhole created in the femur for bacterial inoculation in our model closed spontaneously and rapidly enough to keep the bacterial infection contained inside the medullary cavity of the femur. In fact, a strong bacterial bioluminescent signal was detected for over 3 months after inoculation in our model.

In the present study, the mean serum IL-6 and IL-1 β concentrations in the infected group were significantly higher than those in the control group. Marriott et al. (22) demonstrated that osteoblasts express IL-6 during bacterial bone infection in a mouse model and in human bone tissues. IL-6 (12, 17) and IL-1 β (4, 25), which are produced by stimulated monocytes/macrophages, stimulate osteoclasts and lead to bone resorption. Yoshii et al. reported that the local levels of IL-6 and IL-1 β in the infected bone were elevated in the early postinfection period in a staphylococcal osteomyelitis model. They suggested that the elevated IL-6 and IL-1 β levels induced by infection may be related to bone damage mainly in the early phase of infection (43). Our results also demonstrated that the mean serum IL-6 and IL-1 β concentrations in the infected group were significantly higher at the time pathological changes, such as new bone formation beneath the periosteum, appeared (approximately on day 7). The serum CRP levels in both groups were elevated during the first 3 days (days 0.5, 1, and 3), after which the high level of CRP in the infected group was prolonged through day 7. The elevated CRP level in the early phase might have been caused by the surgical invasion. However, the elevated level was sustained in the infected group for a longer period. The CRP level is one of the most valuable markers for evaluating infectious processes in the clinical field (10, 15, 40). In this study, there was a high correlation between the CRP level and bacterial PI *in vivo* during the chronic phase, suggesting that our model is useful for real-time, noninvasive monitoring of the chronic inflammatory processes in osteomyelitis. In contrast, the mean serum level of MCP-1 was significantly higher in the infected group on day 0.5 (12 h). Cultured osteoblasts produce high MCP-1 levels in response to *S. aureus*, leading to a proposal that MCP-1 causes the inflammation that results in progressive bone destruction (3, 9). Marriott et al. (23) reported that increased MCP-1 is the pivotal inflammatory chemokine during *S. aureus*-associated osteomyelitis *in vivo*. We also demonstrated here that the proportion of granulocytes in the peripheral blood was significantly higher in the infected group than in the control group after day 7, suggesting that granulocytes are induced by chemokine activities during the early phase and that systemic infection due to osteomyelitis was maintained during the chronic phase. Thus, the present osteomyelitis model mimics the infectious processes in humans well.

Additionally, the histological study also demonstrated the reproducibility of the present model. The histological analysis showed new bone formation beneath the periosteum at the early phase and trabecular bone resorption by osteoclasts and fibroblast proliferation during the chronic phase, demonstrating the pathological features of chronic osteomyelitis.

In conclusion, we have successfully visualized and quantified bacterial growth in a mouse osteomyelitis model using *in vivo* BLI. We were able to monitor the infectious processes throughout the course of the disease in both the acute and chronic phases without sacrificing the animals. To our knowledge, this is the first report describing a real-time, quantitative, and reproducible model for both acute and chronic osteomyelitis of the mouse femur with kinetics of immune cells and serum cytokine/chemokine levels.

This novel, quantitative, and reproducible model can be used to clarify the pathology and kinetics of osteomyelitis and to evaluate novel *in vivo* therapeutic strategies, including the development of new antibiotics and bacterium-resistant implants, before performing studies in larger animals and human subjects.

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