Inhibitory effect of roxithromycin on the local levels of bone-resorbing cytokines in an experimental model of murine osteomyelitis

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Objectives: The purpose of this study was to evaluate the inhibitory effect of roxithromycin on the production of interleukin (IL)-1$\beta$, IL-6 and tumour necrosis factor-$\alpha$ (TNF-$\alpha$) in a murine tibial osteomyelitis model using \textit{Staphylococcus aureus}.

Methods: Cytokine levels in supernatants derived from bone homogenates were measured by enzyme-linked immunosorbent assay for 28 days, after oral administration of roxithromycin at 5 mg/kg/day.

Results: There was no significant difference in IL-6 levels between a group receiving roxithromycin administration and a group not receiving roxithromycin. IL-1$\beta$ and TNF-$\alpha$ levels were significantly lower for the administration group after 7–14 days and after 21–28 days, respectively. However, a significant difference in bacterial counts in bone between the groups was not observed.

Conclusion: These results indicate that roxithromycin suppresses the local expression of IL-1$\beta$ and TNF-$\alpha$, and may exhibit an anti-inflammatory effect in this osteomyelitis model.

Introduction

Some antimicrobial agents given to cure infection can also modify the host immune response. Macrolides in particular have potential ‘non-antibiotic’ anti-inflammatory activity.\textsuperscript{1} Although the precise mechanisms of these effects are not clear, there is increasing evidence that 14- and 15-membered macrolides can act in inhibiting inflammatory cytokines, including interleukin (IL)-1$\beta$, IL-6 and tumour necrosis factor-$\alpha$ (TNF-$\alpha$).\textsuperscript{2–5} These cytokines have long been recognized as bone-resorbing cytokines, and are copiously produced within the bone at the site of infection in patients with osteomyelitis.\textsuperscript{6} However, there are few reports concerning the effects of macrolides on the local production of these cytokines in osteomyelitis.\textsuperscript{7} Thus, we sought to determine the levels of IL-1$\beta$, IL-6 and TNF-$\alpha$ in bone during osteomyelitis after administration of roxithromycin, a 14-membered macrolide and an erythromycin derivative, using an experimental \textit{Staphylococcus aureus} tibial osteomyelitis model in mice.

Materials and methods

Preparation of bacteria

The bacterium used in this study was \textit{S. aureus} E-31461 (clinical isolate, supplied by Eisai Co. Ltd, Tokyo, Japan). This organism has an MIC > 100 mg/L of roxithromycin. A bacterial suspension adjusted with trypto-soya broth (Nissui, Tokyo, Japan) to 10$^8$ cfu/mL was prepared. After a 3-0 braided silk suture (AZWELL Inc., Osaka, Japan) was tumbled in the suspension for 45 min, it was dried in a vacuum desiccator for 90 min and cut to a length of 3 mm. The bacterial count for \textit{S. aureus} per 3 mm length of silk suture was found to be 4.8 ± 0.6 ($\times10^5$) cfu.

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**Mouse model of staphylococcal osteomyelitis**

The experimental protocol was approved by the Ethical Committee of the institution, and was based on previous descriptions of this model. Briefly, the proximal tibia of an ICR female mouse (5 weeks old; weight ∼25 g) was exposed with a 4–5 mm incision and a hole was drilled using a 23 g hypodermic needle. A 3 mm length of 3-0 braided silk suture seeded with *S. aureus* was inserted into the hole and the incision was closed (infected group). For the control subjects, a 3-0 braided silk suture was tumbled in a sterile broth, and thereafter handled in the same manner as the infected sutures (uninfected group).

**Administration ofroxithromycin for osteomyelitis**

Roxithromycin (Eisai Co. Ltd) was suspended in 0.5% carboxymethyl cellulose sodium salt (Wako Pure Chemical Industries, Ltd, Osaka, Japan) and adjusted to a concentration of 0.5 mg/mL. The dose was 5 mg/kg and was administered orally once daily immediately after bacterial inoculation using a stomach tube (roxithromycin administration group).

In the non-administration group, the same dose of carboxymethyl cellulose, but containing no roxithromycin, was given.

**Measurement of cytokine levels in bone**

For the roxithromycin administration and non-administration groups and the uninfected group (control), groups of five mice were killed humanely at 1, 3, 5, 7, 14, 21 and 28 days after the insertion of a silk suture. The IL-1β, IL-6 and TNF-α levels in tibiae were determined by enzyme-linked immunosorbent assay (ELISA).

The removed tibiae were immediately homogenized with 20-fold 1/15 M phosphate-buffered saline (pH 7.4). Part of the prepared homogenate was used for bacterial counts in bone. The remainder was centrifuged (1000 g, RA53G, KUBOTA; at 4°C, 10 min), and the resultant supernatant was stored frozen at −84°C until used for the determination of IL-1β, IL-6 and TNF-α levels. For the measurement of cytokines, the ELISA kits of mouse IL-1β, IL-6 (Genzyme, Cambridge, MA, USA) and TNF-α (Assay Designs, Inc., Ann Arbor, MI, USA) were used according to the manufacturers’ instructions.

**Bacterial counts in bone**

Bacterial counts in bone were measured in the roxithromycin administration and non-administration groups. The homogenate of tibiae prepared as described above was serially diluted with 0.9% (w/v) NaCl and plated on to brain–heart infusion agar for determination of cfu.

**Statistical analysis**

The results of bacterial counts and cytokine levels by ELISA in bone were expressed as means ± S.E.M. These data were analysed by Student’s *t*-test, and the difference was judged significant at *P* < 0.05.

**Results**

**Bacterial counts in bone**

In the roxithromycin non-administration group, the bacterial counts in bone were 0.9 ± 0.5 and 5.8 ± 4.3 (×10^5) cfu/tibia 1 and 28 days after bacterial inoculation, respectively. The bacterial counts in bone in the roxithromycin administration group were 1.1 ± 0.5 and 8.6 ± 4.8 (×10^5) cfu/tibia at 1 and 28 days, showing no significant differences in the bacterial counts between the presence and absence of roxithromycin.

**Suppressive effect of roxithromycin on cytokine levels**

The levels of IL-1β, IL-6 and TNF-α in infected bone with and without roxithromycin were considerably greater than those in uninfected bone (control) with some variations in timing (Figure 1). The roxithromycin administration group showed significantly lower IL-1β levels in bone than the non-administration group at 7 and 14 days. The TNF-α levels in the roxithromycin administration group did not differ from those in the non-administration group up to 14 days, but were significantly lower at 21 and 28 days. In contrast, the IL-6 levels in the roxithromycin administration and non-administration groups did not differ significantly at any point of measurement.

**Discussion**

In this investigation, the levels of IL-1β, IL-6 and TNF-α in bone were determined by ELISA for a period of time up to 28 days after administration of roxithromycin. From the results, an inhibitory effect on IL-1β and TNF-α was observed between 7–14 days and 21–28 days after administration, respectively. However, the inhibitory effect on IL-6 was not found in the roxithromycin administration group. In this osteomyelitis model, a resistant strain of *S. aureus* in which the MIC of roxithromycin exceeded 100 mg/L was used to exclude as much as possible the direct antibacterial effects of roxithromycin. Since the bacterial counts in bone did not differ between the roxithromycin administration and non-administration groups, the inhibition of IL-1β and TNF-α by roxithromycin may be largely due to effects other than direct antibacterial effects.

For the inhibitory effects of roxithromycin on IL-1β, IL-6 and TNF-α, *in vitro* results on monocytes and macro-
phages\(^5\) stimulated with lipopolysaccharide (LPS) have been reported. These reports stated that roxithromycin inhibited the production of IL-1\(\beta\), IL-6 and TNF-\(\alpha\) in a dose-dependent manner. The minimum concentration of roxithromycin exhibiting the inhibitory effects on the production of cytokines varies between reporters, from 0.05\(^2\) to 3.1 mg/L,\(^3\) which are lower than therapeutic blood levels. Regarding in vivo results, Suzaki \textit{et al.}\(^2\) induced pneumonia by the inhalation of LPS in mice after pre-treatment with roxithromycin at 2.5 mg/kg/day for 5–12 weeks. They determined the IL-1\(\beta\) and TNF-\(\alpha\) levels in a lung extract and reported that the production of these cytokines was inhibited in mice receiving roxithromycin for 7 weeks or more. Konno \textit{et al.}\(^4\) conducted the same experiment except that the dose was doubled to 5 mg/kg/day, and they stated that the production of TNF-\(\alpha\) was inhibited after 21 days of administration. In our murine tibial osteomyelitis model, an experiment was conducted with roxithromycin at a dose of 5 mg/kg/day and inhibition was observed for TNF-\(\alpha\) after 21 days, similar to the result reported by Konno \textit{et al.}\(^3\) These facts suggest that the in vivo inhibitory effects of roxithromycin on IL-1\(\beta\) and TNF-\(\alpha\) are affected by the period of administration as well as the dose.

Although the precise mechanism of suppressive effects of macrolides on the production of cytokines is not clear, it has been suggested that the interaction between macrolides and phagocytes may be important. In fact, macrolide antibiotics are able to accumulate within phagocytes, reaching intracellular concentrations far higher than those attained in extracellular fluids (>10- to 200-fold the extracellular concentration), and are able to modify cell activities.\(^9\) It is possible that roxithromycin binds to receptors, such as immunophilin, which are the intracellular receptors of immunosuppressive agents FK506 and rapamycin, and the complexes interfere with cytokine gene transcription,\(^1,10\) resulting in inhibition of cytokine secretion. In addition, it is likely that long-term administration, which would result in greater cellular accumulation, triggers mechanisms other than those obtained at lower concentrations.\(^1\) Although the reason why the inhibitory effects of roxithromycin on IL-6 production were not observed in the present experiment remains unclear, an early increase after infection and a subsequent, relatively rapid decrease in IL-6 levels in our experimental model may have prevented the manifestation of the effects of roxithromycin.

In conclusion, the results obtained in the present study indicate that roxithromycin may exhibit an anti-inflammatory effect by suppressing the local production of IL-1\(\beta\) and TNF-\(\alpha\) in this osteomyelitis model.

\textbf{References}


