Protective effect of teriparatide against vancomycin-induced cytotoxicity in osteoblasts

Kentaro Tsuji a, Soichiro Kimura b,c,*, Kazuhiro Tateda b, Hiroshi Takahashi a

a Department of Orthopaedic Surgery, Toho University School of Medicine, 6-11-1 Omori-nishi, Ota-ku 143-8541, Tokyo, Japan
b Department of Microbiology and Infectious Diseases, Toho University Faculty of Medicine, 5-21-16 Omori-nishi, Ota-ku 143-8540, Tokyo, Japan
c Division of Infection Prevention and Control, Faculty of Pharmaceutical Sciences, Shonan University of Medical Sciences, 16-10 Kamishinano, Totsuka-ku, Yohokaha 244-0806, Kanagawa, Japan

Abstract

Background: Intrawound vancomycin powder is effective in preventing surgical site infection after spine surgery. In a previous study, vancomycin-induced cytotoxicity in osteoblasts was investigated in vitro, and vitamin D3 was verified to be a candidate drug aiding recovery from vancomycin-induced cytotoxicity. The treatment practices involving osteogenesis-promoting drugs vary widely. Teriparatide, an anabolic agent, highly promotes bone formation by inducing osteoblast activation, increasing bone formation and mineral density, and preventing vertebral fractures. Hence, teriparatide may be administered in combination with vancomycin.

Methods: MC3T3-E1 cells were cultured in minimum essential medium supplemented with 10% fetal bovine serum at 37°C in a humidified incubator containing 5% CO2. The experimental concentrations of vancomycin (2500, 5000, and 7500 μg/mL) were determined based on previous reports and our preliminary experiments. Teriparatide (100 ng/mL) was administered concomitantly to prevent cytotoxicity in osteoblasts, using pulsed vancomycin for 24 h (measured at 1, 3, and 7 days). Cell numbers and morphological changes in cells treated with vancomycin or vancomycin plus 100 ng/mL teriparatide were measured. Osteoblast differentiation was assessed using alkaline phosphatase staining, alkaline phosphatase activity, and alizarin red S staining.

Results: Teriparatide showed a recovery effect when vancomycin (7500 μg/mL) was administered only for 24 h. Microscopic examination revealed that teriparatide had a protective effect on osteoblasts exposed to 7500 μg/mL vancomycin. Addition of teriparatide led to the recovery of alkaline phosphatase staining and alizarin red S staining.

Conclusion: Vancomycin-induced cytotoxicity in osteoblasts could be inhibited by administering teriparatide concomitantly with vancomycin.

© 2022 The Authors. Published by Elsevier B.V. on behalf of The Japanese Orthopaedic Association. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/).

Keywords: Infection, Vancomycin, Cytotoxicity, Teriparatide, Osteoblasts

1. Introduction

The use of intrawound vancomycin (VCM) powder to prevent surgical site infections (SSIs) in spine surgery has been widely examined and proven to be considerably beneficial [1–3]. However, VCM powder is also associated with adverse effects at high concentrations [4–6]. High local antimicrobial concentrations may lead to non-union after spine surgery; moreover, the implant may break under these high concentrations, causing pain and poor alignment [6]. Furthermore, a high local VCM concentration may have cytotoxic effects on osteoblasts [7,8]. In a previous work, Tsuji et al. investigated VCM-induced cytotoxicity in osteoblasts in vitro [9]. Although high local antimicrobial concentrations cause toxic side effects, minimal local concentrations may not effectively prevent SSIs. Thus, the use of candidate concomitant drugs may be a strategy to prevent high-dose VCM-induced cytotoxicity in osteoblasts. Vitamin D3 is a candidate concomitant drug that aids recovery from VCM-induced cytotoxicity [9]. Teriparatide has also been used to treat osteoporosis. Correlating the
increase in bone mineral density (BMD) with the use of vitamin D3 and teriparatide, vitamin D3 increased lumbar spine BMD by 2.3%, and teriparatide increased it by 10.0% in 12 months [10,11]. Teriparatide is considered to have high bone formation ability. Teriparatide is a bone anabolic agent that induces osteoblast activation, increases bone formation and mineral density, and prevents vertebral fractures [12,13]. Therefore, teriparatide may be a candidate concomitant drug for preventing VCM cytotoxicity. We investigated whether teriparatide administration confers a protective effect on osteoblasts when administered with continuous VCM. To demonstrate this, we determined the number of cells and examined their morphology in groups treated with pulsed VCM and VCM + PTH. In addition, to confirm the restorative effect of PTH on cell differentiation and maturation, experiments were conducted on alkaline phosphatase (ALP), mineralization, and gene expression.

2. Materials and methods

2.1. Cell culture

MC3T3-E1 cells derived from mouse calvaria were provided by RIKEN BRC, which participated in the National Bio-Resource Project of the MEXT/AMED, Japan. MC3T3-E1 cells have a proliferation rate similar to that of other osteoblast cell lines, and their matrices are mineralized in a similar manner [14,15]. These cells were cultured to 70% confluence, detached, and cultured in flesh flasks.

2.2. Vancomycin concentrations for in vitro experiments

VCM (Shionogi & Co, Ltd., Osaka, Japan) was dissolved in 5% minimum essential medium; unmodified medium was used as the vehicle control. The VCM concentration used was the same as that administered clinically, ranging from 0.5 to 6 g. Topical application of 2 g VCM in a case of scoliosis was reported to be associated with approximately 1500 µg/mL of VCM in the fluid [3]. In other words, its clinical concentration is approximately 500–5000 µg/mL.

2.3. Teriparatide concentration

Teriparatide was purchased from Sigma Chemical Industry (St. Louis, MO, USA). The concentrations of teriparatide used in this study were based on previous reports [16,17]. Wang et al. [16] used teriparatide concentrations ranging from 50 to 200 ng/mL. We used a teriparatide concentration of 100 ng/mL. Before the experiment, teriparatide was dissolved in 5% minimum essential medium, and unmodified medium was used as a vehicle control. PTH has different effects depending on the method of administration—continuous or pulsed dosing [18–20]. Therefore, teriparatide was added every 2 days (several times administration).

2.4. Cell number and morphology in groups treated with pulsed vancomycin plus 100 ng/ml teriparatide

Osteoblasts were placed in a 24-well plate at a density of 1.0 × 10^4 cells/mL until they reached 70% confluency in approximately 3 days. To examine the cytotoxic effects of pulse exposure to high-dose VCM and teriparatide, osteoblasts were cultured in the presence of VCM and teriparatide for 24 h after which 100 ng/mL of teriparatide was added every 2 days (several times administration) [21]. Cell numbers were compared between the VCM and VCM + PTH groups on days 1, 3, and 7. For counting, cells were detached using 0.25% trypsin–EDTA. The number of viable cells was determined under a microscope using the Trypan blue dye exclusion test. The proliferation of these cells and morphological changes were examined using an Olympus IX70 microscope (Olympus Corp, Tokyo, Japan).

2.5. Assessment of alkaline phosphatase staining and activity

2.5.1. Alkaline phosphatase staining

The treated osteoblasts were stained for ALP using an alkaline phosphatase staining kit (Cosmo Bio Ltd, Tokyo, Japan). The experiments were conducted using VCM concentrations yielding the most effective combined effect. To evaluate the protective effect of teriparatide on osteoblasts treated with VCM, ALP activity was measured on day 7 using the VCM concentration with the most pronounced detrimental effect (7500 µg/mL). Pre-confluent cultures were stratified into the following three groups: 7500 µg/mL VCM, 7500 µg/mL VCM + 100 ng/mL teriparatide, and control. For each group, we performed ALP staining and evaluated the ALP activity. ImageJ was used to quantitatively evaluate cell staining under a microscope.

2.5.2. Alkaline phosphatase activity assay

ALP assay was performed using an alkaline phosphatase kit (Wako Pure Chemical Industries). MC3T3-E1 cells were briefly added to the wells of a 96-well plate at a density of 1.0 × 10^4 cells/mL and divided into the same treatment groups as used for ALP staining. ALP activity was measured after incubation for 7 days using p-nitrophenyl phosphate as the ALP substrate. For the assay, 100 µL of the substrate was added to each well, and 20 µL of cell supernatant that was obtained from the control, VCM, and VCM + PTH groups was added to each well. After agitating the cells for 1 min using a plate mixer, we incubated the cells at 37°C for 15 min. The reaction was then terminated by adding 80 µL of stop solution to each well and agitating for 1 min using a plate mixer. Absorbances were measured at 405 nm using a microplate reader Model 680 (Bio-Rad Laboratories, Inc., Tokyo, Japan).

2.6. Alizarin red staining

To evaluate the mineralization of osteoblasts, we performed alizarin red S staining using paraformaldehyde and alizarin red S solutions purchased from Sigma–Aldrich (St. Louis, MO, USA). Specifically, the differences in the calcified nodules produced in response to treatment with 7500 µg/mL VCM and 7500 µg/mL VCM + 100 ng/mL teriparatide were analyzed. After cells were cultured for 28 days, they were stained with 2% alizarin red S (pH 4.2) for 10 min at 20–25°C with minimal exposure to light. Microscopic observations were performed using a light microscope. ImageJ was used to quantitatively evaluate the staining of cells under a microscope.

2.7. RNA isolation and gene expression analysis

Total RNA was isolated from osteoblasts using TRIzol Reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instruction. For the quantitative reverse transcription-polymerase chain reaction analysis, total RNA was reverse transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Data analysis using the SYBR Green real-time reverse transcription polymerase chain reaction technique was performed using a 7500 Fast Real-Time Polymerase Chain Reaction System (Applied Biosystems). We used the following polymerase chain reaction primers: collagen type I 5′-CTGCTTCTTGGCGCC-3′ (forward) and 5′-ACCTTTAACCAGTACACCCGCT-3′ (reverse); alkaline phosphatase, 5′-GGATACGCGATGAGAAGGGC-3′ (forward) and 5′-CGTTCAGTGGTTCCAGACATAG-3′ (reverse); and 18S rRNA, 5′-ACCTTTAACCAGTACACCCGCT-3′ (forward) and 5′-ACCTTTAACCAGTACACCCGCT-3′ (reverse).
Expression of collagen type I (vancomycin alone: 0.4095 ± 0.02723 [95% CI 0.38710 to 0.43191]; p = 0.0657) and alkaline phosphatase (vancomycin alone: 0.5629 ± 0.07088, vancomycin + PTH: 0.6829 ± 0.01485; mean difference: 0.0657) and alkaline phosphatase gene expression in VCM-induced cytotoxic osteoblasts.

2.8. Statistical analysis

Statistical analyses were performed using Graph Pad Prism version 7.0 (San Diego, CA, USA). Student’s t-test was used for comparisons between the two groups, and ANOVA, followed by Tukey’s multiple-comparison test, was used to make comparisons between multiple groups. The statistical significance was set at a threshold of p < 0.05. Significance was determined as *: p < 0.05, **: p < 0.01 and ***: p < 0.001. All error bars represent standard deviation.

3. Results

3.1. Effect of teriparatide in preventing VCM-induced cytotoxicity in osteoblasts

When osteoblasts were treated with pulsed VCM for the first 24 h despite the presence of teriparatide, an increased number of cells were observed at all examined time points (Fig. 1A–C). Teriparatide had a protective effect when VCM (2500 µg/mL) was administered only for 24 h (cell number with 2500 µg/mL, day 7: VCM alone, 16.4 ± 10^4 ± 0.743 × 10^4 cells/mL, VCM + PTH, 21.8 ± 10^4 ± 0.75 × 10^4 cells/mL, mean difference 5.33 ± 1.056 cells/mL, [95% CI 3.14–7.52]; p < 0.001). Teriparatide had a recovery effect when 5000 µg/mL VCM was administered only for 24 h (cell number with 5000 µg/mL, day 7: VCM alone 11.5 ± 10^4 ± 0.5 × 10^4 cells/mL, VCM + PTH, 15.33 ± 10^4 ± 1.196 × 10^4 cells/mL, mean difference 3.83 ± 10^4 ± 1.296 × 10^4 cells/mL [95% CI 1.146–6.521]; p < 0.01). It also had a recovery effect when 7500 µg/mL VCM was administered only for 24 h (cell number: 7500 µg/mL; day 7: VCM alone 8.417 ± 10^4 ± 0.6211 × 10^4 cells/mL, VCM + PTH, 10.25 × 10^4 ± 0.617 × 10^4 cells/mL, mean difference 1.833 ± 10^4 ± 0.8755 × 10^4 cells/mL [95% CI 0.0176–3.649]; p < 0.001). Compared with cells in the 7500 µg/mL VCM group, those in the 7500 µg/mL VCM + 100 ng/mL PTH group tended to develop a spherical morphology, and the number of cells increased in a time-dependent manner. The results show that there was no difference between the control and the VCM 2500 + PTH or VCM 5000 + PTH over time.

Microscopic examination indicated the absence of cytotoxicity in osteoblasts exposed to teriparatide and 7500 µg/mL VCM (Fig. 2A–D). The observed morphological changes indicated that teriparatide also prevented cell damage in response to treatment with a higher VCM concentration.

3.2. Teriparatide facilitates normal differentiation, calcification and gene expression in osteoblasts treated with vancomycin

ALP staining of untreated cells was observed under the microscope; very little staining was observed in osteoblasts after exposure to a high VCM concentration (7500 µg/mL). However, we also observed the recovery of ALP staining after adding teriparatide (Fig. 3A–B). Similar differences were observed using an ALP activity kit (Fig. 3C). ALP staining was recovered upon the addition of teriparatide, which is evidence that teriparatide helps restore the differentiation of osteoblasts. Calcification of the untreated osteoblasts was examined both macroscopically and microscopically; very little staining was observed in osteoblasts after exposure to VCM (Fig. 4A, B). However, recovery of staining, indicating calcification, was observed after the addition of teriparatide (Fig. 4A, B). We observed no significant difference in the gene expression of collagen type I (vancomycin alone: 0.4095 ± 0.01431, vancomycin + PTH: 0.478 ± 0.02316; mean difference: 0.06489 ± 0.02723 [95% CI −0.07104 to 0.1441]; p = 0.0657) and alkaline phosphatase (vancomycin alone: 0.5629 ± 0.07088, vancomycin + PTH: 0.6829 ± 0.01485; mean difference: 0.12 ± 0.07242 [95% CI −0.08111 to 0.321]; p = 0.1730) (Fig. 5).

4. Discussion

In this study, teriparatide showed a recovery effect when VCM was administered only for 24 h. The number of cells increased in a time-dependent manner. Addition of teriparatide led to the recovery of ALP and alizarin red staining and gene expression. We found that teriparatide could be a candidate concomitant drug that helps ameliorate VCM-induced cytotoxicity.

Some studies have reported that high local VCM concentrations may have cytotoxic effects on osteoblasts [22,23]. Tsuji et al.
Fig. 2. Protective effects of PTH on the morphology of osteoblasts after vancomycin administration for 24 h. The cells were exposed to (A) 0, (B) 2500, (C) 5000, (D) 7500 μg/mL of vancomycin. Scale bar indicates 50 μm.
investigated VCM-induced cytotoxicity in osteoblasts in vitro [9]. VCM was cytotoxic despite its pulsed administration for a short time of 24 h (Fig. 1).

To our knowledge, there are no reports on the mechanism underlying the cytotoxic effects of VCM on osteoblasts. High local VCM concentrations used routinely are fraught with cytotoxicity in osteoblasts, contributing to non-union after spinal surgery [2]. Therefore, concomitantly administered drugs promoting osteogenesis may ameliorate these cytotoxic effects in osteoblasts. Although vitamin D3 is a candidate drug that helps ameliorate VCM-induced cytotoxicity, it is necessary to examine other candidate concomitant drugs that promote osteogenesis. In addition to vitamin D3, various osteoporosis drugs are used clinically. Similar to vitamin D3, teriparatide is a bone anabolic agent that induces osteoblast activation, increases bone formation and BMD, and prevents vertebral fracture [11,24]. Drugs for treating osteoporosis commonly include bisphosphonates, which inhibit bone inhalation. The best drug for treating osteoporosis would be a bone anabolic agent that increases bone mass by remodeling the bone. Teriparatide, a bone anabolic agent, is used worldwide to treat osteoporosis. It is believed that the effect of increasing BMD is higher than that of bisphosphonates. Teriparatide has a 34-amino acid peptide derived from the N-terminus of the PTH, an endocrine factor secreted by the parathyroid glands and is the most important hormone for in vivo regulation [19]. PTH experts have demonstrated both anabolic and catabolic influences on bone, depending on the exposure time [24].

Osteoblasts and osteoclasts are involved in bone remodeling. PTH stimulates the proliferation and differentiation of osteoblast progenitor cells and pre-osteoblasts, suppresses osteoblast apoptosis, and increases cell number [25]. In contrast, osteoblasts induce RANKL expression, promote M-CSF expression, and suppress OPG expression, indirectly activating osteoclasts and promoting bone resorption. Moreover, PTH has not been reported to promote bone resorption through osteoclasts alone [26]. Therefore, we conducted an in vitro study using osteoblasts alone.

Functional PTH receptors are present on cells of the osteoblast lineage, ranging from early skeletal stem cells to matrix-embedded osteocytes [27]. PTH may have effects on osteoblasts in several aspects including cell proliferation, differentiation, and apoptosis. Although intermittent treatment with PTH analogs regulates bone formation by suppression of osteoblast apoptosis, activation of bone lining cells, and differentiation of precursor cells [28], the mechanisms underlying the anabolic effects are still unknown. During osteoblast differentiation, cell morphological changes are often detected, and collagen type I and alkaline phosphatase are commonly used as indicators. Collagen type I promotes cell attachment and stimulates cell differentiation. Alkaline phosphatase increases the local content of the phosphoric marker to assess osteoblast activity and tissue calcification capability [29]. These genes (Col-I and Alp) are of fundamental importance in the regulation of osteoblast differentiation and function. Since PTH administration increases Col-I and Alp gene expression in osteoblasts [16], the expression of both genes was measured in this
study. We found that VCM cytotoxicity reduced Col-I and Alp gene expression, but PTH administration slightly increased both gene expressions, although not significantly. We hypothesized that PTH administration acts to increase the activity of osteoblasts under toxic conditions of VCM and also to restore gene expression of Col-I and Alp. Although mitochondrial disruption is involved in the cytotoxicity of rifampin and gentamicin on osteoblasts, the mechanism of VCM cytotoxicity on osteoblasts remains unclear [30]. This remains a subject of further study.

Further, our results show that PTH has a higher ability to alleviate bone formation inhibition than vitamin D3 does (Fig. S1). It has been reported that PTH has different effects depending on the administration method, such as one time or several times administration [18–20]. Bone resorption exceeded osteogenesis with one time administration, while osteogenesis exceeded bone resorption with several times administration. Although there is a difference in PTH action depending on the administration method (one time or several times administration), clinically, several times administration of PTH is more common for treatment of osteoporosis [19,20].

In clinical practice, several times administration of PTH is done daily or once a week [19]. In clinical practice, pulsed administration of PTH is done daily (20 μg) or once a week (56.5 μg). The once-weekly administration resulted in osteogenesis after approximately three weeks [11]. Both the cancellous bone and cortical bone increased, and the treated vertebral body was stabilized after

---

**Fig. 4.** Analysis of calcified nodules in the VCM with or without PTH. Microscopy images (A). Quantitative evaluation (B). Scale bar indicates 50 μm.
6 weeks of treatment [11]. Even with several times administration, the effects differ between daily administration and administration once a week. It has been reported that the expression of osteogenic markers increases only slightly for 1 week, while the bone resorption marker decreases for 1 week. In this study, we investigated the topical administration of teriparatide. In an in vitro study that examined the promotion of osteogenesis via osteoblasts by teriparatide, Wang et al. reported that several times administration of teriparatide at a concentration of 100 ng/mL effectively promoted bone differentiation [16]. In our study, when the vancomycin was introduced to osteoblasts with teriparatide for the first 24 h, we observed an increased number of cells at all examined time points. Similarly, ALP activity and mineralization of osteoblasts were increased. These results indicate that teriparatide could ameliorate vancomycin-induced cytotoxicity in osteoblasts. Even when one time administration of teriparatide was performed in advance, long-term administration reduced cell proliferation and had no positive effects on ALP production or calcification (data not shown) [18–20]. To date, no studies have examined the combined effects of vancomycin and teriparatide. It has been shown that this candidate drug may suppress vancomycin-induced cytotoxicity.

In this study, the combination of PTH with VCM was effective in alleviating cytotoxicity. In clinical practice, local administration of PTH in combination with VCM at the time of wound closure is thought to be effective in preventing infection and in alleviating toxicity and may prevent postoperative pseudoarthrosis. However, because the osteogenesis-promoting effect is not observed unless the drug is administered several times, determining how to administer the drug intermittently is an issue that needs to be investigated in a future study.

There are a few limitations to this study. First, we only investigated the effects of a single dose of 100 ng/mL teriparatide based on a previous study [16]. Studies on the most effective concentration of teriparatide in this context are currently ongoing. Second, the results need to be confirmed in vivo in a future study.

5. Conclusion

We found that teriparatide can reduce the cytotoxicity caused by the high local VCM concentration for 24 h. Thus, teriparatide, which is frequently used to treat osteoporosis, may be effective as a concomitantly administered drug, inducing the proliferation of osteoblasts. These results indicate that the combination therapy of VCM and teriparatide may prevent adverse events, such as osteoblast cytotoxicity. However, further studies are necessary to determine whether these findings can be applied in vivo and in clinical practice.

Declaration of competing interest

None.

Acknowledgment

This work was supported in part by Grants-in-Aid for Toho University Grant for Research Initiative Program (TUGRIP) from Toho University (to KTa), and The Project Research Grant (No. 18-23) from Toho University (to KTu, SK).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jos.2022.09.018.

References


Lu X, Ding Y, Niu Q, Xuan S, Yang Y, Jin Y, et al. ClC-3 chloride channel me-

Matsumoto T, Ito M, Hayashi Y, Hirota T, Tanigawara Y, Sone T, et al. A new active vitamin D3 analog, edecacitol, prevents the risk of osteoporotic frac-

effects of vancomycin on osteoblasts? Clin Orthop Relat Res 2020
Feb;478(2):420–33.


Nakamura T, Sugimoto T, Nakano T, Kishimoto H, Ito M, Fukunaga M, et al. Randomized Teriparatide [human parathyroid hormone (PTH) 1-34] Once-

Czekanska EM, Stoddart MJ, Ralphs JR, Richards RG, Hayes JS. A phenotypic


Lu X, Ding Y, Niu Q, Xuan S, Yang Y, Jin Y, et al. CIC-3 chloride channel med-

Duewelhenke N, Krut O, Einhorn TA. The role of growth factors in the repair


