ORIGINAL RESEARCH

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Zoledronic acid, an amino-bisphosphonate, prolongs survival of skin allografts

Abstract

Purpose: Zoledronic acid (ZOL), an effective nitrogen-containing bisphosphonate used to prevent excessive bone loss in clinical practice, has been shown to affect the development of dendritic cells by redirecting differentiation toward a state of atypical maturation. The study was aimed to examine whether ZOL can reduce acute rejection of skin allografts.

Methods: A skin transplantation model using C57BL/6 to BALB/c mice was used. ZOL was injected intraperitoneally into transplant recipients post-surgically. Graft survival, body weight, leukocyte count, hepatic and renal functions were assessed.

Results: ZOL treatment significantly prolonged skin allograft survival in mice. In terms of toxicity, there were no significant differences in body weight, leukocyte count, plasma alanine aminotransferase or creatinine levels between the ZOL-treated and control groups. Histopathology showed that the loss of skin integrity seen in control group was prevented by ZOL treatment. In draining lymph nodes and spleen, the number and clustering extent of mononuclear cells were markedly declined by ZOL treatment. The plasma IL-6 levels were reduced by treatment of ZOL.

Conclusion: ZOL can prolong skin allograft survival without major toxicity.

Manuscript submitted 9th July, 2011

Manuscript accepted 18th June, 2012

Clin Invest Med 2012; 35 (4): E165-E172.

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Graft transplantation has been in development for decades as a method of restoring function in end-stage organs. The most critical issue in graft survival remains the success of immunosuppressive agents. Currently used immunosuppressants act through inhibition of calcineurin, mammalian target-of-rapamycin (mTOR) and the other biochemical pathways/mediators. The complications resulting from their long-term administration, such as renal and cardiovascular toxicity, hyperlipidemia, myelosuppression and impaired wound healing, [1-2] limit the clinical outcome of graft transplantation; clearly, it is of critical importance to develop novel immunosuppressive drugs.

Dendritic cells (DCs) are the most potent antigen-presenting cells (APCs) - inducing both innate and adaptive immune responses. [3] DCs can be induced to differentiate into a less mature stage or to acquire a tolerogenic phenotype. [4-6] This induced differentiation suggests that modulation of DC development could have therapeutic potential in the treatment or prevention of diseases involving unwanted immune response, such as rejection of transplanted grafts. [7]

Bisphosphonates have been widely used for treatment of excessive bone loss arising from increased number and activity of osteoclasts, such as that seen in osteoporosis from the aging process and bone metastasis from cancers. [8-9] Among various kinds of bisphosphonates, the nitrogen-containing bisphosphonates have been shown to inhibit farnesyl diphosphate synthase [10] and squalene synthase [11] of the mevalonate pathway, which interferes with protein prenylation of small GTPase to induce osteoclast apoptosis. [12] Zoledronic acid (ZOL) is a third-generation nitrogen-containing bisphosphonate with much more potent apoptosis-inducing activity in osteoclasts than the other bisphosphonates. [13] In our previous investigation, ZOL was seen to redirect DC differentiation toward a state of atypical maturation through prevention of Rap1A prenylation. [14]

Taken together, these data suggest that ZOL might increase transplant graft survival due to an immunosuppressive activity via modulation of DC development. In this study, a skin transplantation model was used to evaluate the effect of ZOL on skin allograft survival and to assess toxicity in vivo.

Materials and Methods

Animals

Male C57BL/6 (H-2^b) and BALB/c (H-2^d) mice aged 6 – 8 weeks old were obtained from the Animal Resource Center of the National Science Council of Taiwan (Taipei, Taiwan). Mice were housed in a pathogen-free environment. Experi-

ments were approved by the animal safety committee of Mackay Memorial Hospital, Taipei, Taiwan. The animal study was performed in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals, Taiwan.

Skin transplantation

Transplantation operations were performed under anesthesia with a single dose of intraperitoneal (i.p.) ketamine hydrochloride (500 mg/kg body weight; Sigma, St. Louis, MO) and xylazine 6 mg/kg. Skin transplantation was conducted by a procedure modified from that described previously [15]. In brief, a 2 x 1 cm full-thickness skin was removed from the flank of a euthanized C57BL/6 donor mouse, and the fat and muscle at underside were removed by gently scraping with a scalpel. Before transplantation, the right flank of anesthetized BALB/c recipient mouse was shaved and washed with 70% ethanol. A graft bed was prepared by using fine scissors to remove epidermis and dermis down to the depth of intrinsic muscle over a graft-compatible area. The graft was fixed to the recipient graft bed with ten interrupted sutures of 5-0 monofilament nylon thread (Dermalon, Davis & Geck, St. Louis, MO). This technique was developed in a preliminary study: skin graft survival was evaluated three times a week by visual and tactile examination by a single observer and verified by a surgeon. The percentage of viable graft was assessed using a transparent grid-template overlay. Rejection was defined as necrosis over greater than 80% of the epidermal surface of the graft.

Administration of ZOL

ZOL (synthesized and provided by Novartis (East Hanover, NJ, USA)) was stored at 4°C and dilution was prepared with 5% glucose water immediately before i.p. injection. The 32 recipient mice were randomly assigned to four groups and treated according to one of the following regimens: 5% glucose water as a vehicle control, 5 µg/kg ZOL i.p. twice a day, 25 µg/kg ZOL i.p. twice a day, or 125 µg/kg ZOL i.p. twice a day. All treatments were started on the day of transplantation and continued for 14 days.

Evaluation of leukocyte count, hepatic and renal functions

The weight of each mouse was determined every other day by a single observer. Blood samples were obtained via retroorbital plexus to analyze the white blood cell counts on an automatic Coulter counter (Model Z1, Beckman Coulter Electronics, Fullerton, CA). Plasma levels of alanine aminotransferase (ALT) and creatinine were measured by a standard colorimet-

TABLE 1. Plasma levels of alanine aminotransferase and creatinine in recipient mice.

	Day 0		Day 7		Day 15	
	ALT	Cr	ALT	Cr	ALT	Cr
Control	25.2±2.1	0.28±0.05	36.1±2.6	0.32±0.06	46.3±3.9	0.30±0.11
ZOL 5 µg/kg	26.1±1.9	0.26±0.09	33.2±4.1	0.35±0.08	48.8±5.3	0.29±0.09
ZOL 25 µg/kg	25.9±3.3	0.31±0.10	37.4±4.5	0.33±0.11	48.2±4.7	0.34±0.12
ZOL 125 µg/kg	25.5±2.9	0.29±0.06	34.9±3.7	0.32±0.07	46.5±4.1	0.33±0.08

ALT: alanine aminotransferase ; Cr: creatinine; Unit: IU/L for ALT, mg/dL for Cr. There are no significant differences in either variable among the four groups during the duration of the experimental protocol.

FIGURE 1. The effect of ZOL on the survival of skin grafts. BALB/c mice, transplanted with skin grafts from C57BL/6 donors, were treated daily with ZOL 5, 25 or 125 µg/kg starting on the day of transplantation via intraperitoneal route. A control group was grafted with vehicle. Eight mice were tested in each group. One-way analysis of variance (ANOVA) with repeated measurement followed by Tukey test was used to compare skin graft survival among groups. There were significant differences in graft survival among ZOL-treated groups and control group ($p < 0.05$).

(A) Day 0 of skin allograft transplantation

(B) Day 7 of skin allograft transplantations

FIGURE 2. Comparison of gross appearance of skin allografts between the ZOL-treated and control mice. Representative photographs of mouse skin allografts. Skin grafts were evaluated by visual and tactile inspection until necrosis.

Control 5 µg/kg 25 µg/kg 125 µg/kg

(A) skin

(B) draining lymph node

(C) spleen

FIGURE 3. The histopathological features of transplanted skin, recipient draining lymph node and spleen. (A) skin; (B) draining lymph node; (C) spleen. The specimen was harvested on day five, fixed in formalin and embedded in paraffin. Five µm sections were made, deparaffinized, rehydrated and stained with hematoxylin and eosin. Magnification 1000x.

FIGURE 4. The plasma cytokine levels in mice. (A) IL-6; (B) IL-10; (C) IL-12; (D) TNF- α ; (E) TGF- β 1. Comparison of cytokine levels was performed using the Kruskal Wallis test and pairwise comparison was performed by Mann-Whitney U test. A p value of < 0.05 was considered as statistically significant.

FIGURE 5. Body weight and leukocyte count in recipient mice. Body weight (A) and leukocyte count (B) of ZOL-treated mice compared with controls after skin transplantation. There were no significant differences in either variable among the four groups.

ric method using a Synchron LX20 spectrophotometer (Beckman Coulter).

Histopathological evaluation

For histological assessment, skin grafts were fixed in formalin and embedded in paraffin. Five µm sections were made, deparaffinized, rehydrated and stained with hematoxylin and eosin (HE).

Measurement of plasma cytokine levels

The plasma was collected on day 5 for measurement of cytokine levels. The levels of interleukin (IL-6), IL-12 (p70), IL-10, tumor necrosis factor-α (TNF-α) and transforming growth factor-β1 (TGF-β1) in the mice plasma were measured using enzyme-linked immunosorbent assay (ELISA) (R&D Systems) according to the manufacturer's instructions.

Statistics

Comparison of cytokine levels was performed using the Kruskal Wallis test and pairwise comparison was performed by Mann-Whitney U test. One-way analysis of variance (ANOVA) with repeated measurement followed by Tukey test

was used to compare skin graft survival among groups. A *p* value of < 0.05 was considered as statistically significant.

Results

Effects of ZOL on graft survival

By using Kaplan-Meier method and log-rank analysis, a significant difference was observed between each drug level and control group (*p* < 0.05) [Figure 1]; however, no difference was noted among groups treated with 5, 25 and 125 µg/kg ZOL. A representative set of photographs showing the effect of ZOL on the integrity of skin allograft at day 7 is shown in Figure 2.

Assessment for histopathology and plasma cytokine levels

To elucidate the possible mechanism of action of ZOL, the plasma concentrations of various cytokines were measured and the histopathological features of transplanted skin, recipient draining lymph node and spleen were evaluated. In the tissue sections with HE stain, the loss of skin integrity in the control group due to rejection was prevented by ZOL treatment. In draining lymph node and spleen, the number and clustering extent of mononuclear cells were markedly decreased by ZOL treatment [Figure 3]. The plasma IL-6 levels were reduced by

treatment with ZOL ($p = 0.024$). The other cytokines, included IL-10, IL-12, TGF- β 1 and TNF- α , were not significantly changed from control values [Figure 4].

Changes in body weight and leukocyte count

There were no significant changes in body weight in all groups during the experimental period [Figure 5A]. The white blood cell count decreased after skin transplantation and then rose gradually in all groups. There were no significant differences in white blood cell counts among groups at each time point [Figure 5B].

Evaluation of hepatotoxicity and nephrotoxicity

After skin transplantation, plasma ALT levels, but not creatinine levels, in control and ZOL-treated mice increased slowly up to approximately twice baseline levels by day 15 [Table 1]. No significant differences between the control and ZOL-treated mice in either ALT or creatinine levels were noted.

Discussion

In this study, the major finding is that ZOL treatment prolongs survival of skin allografts in major histocompatibility complex-incompatible mice. No significant toxicity to bone marrow, liver or kidney was noted. This is the first study showing the therapeutic potential of ZOL in preventing transplantation rejection.

It has been previously reported that ZOL can modulate DC development toward a tolerogenic phenotype. [14] Given that DC play a critical role in the reject reaction of grafts, the skin allograft survival benefit due to treatment with ZOL may be attributed to this effect on DC development. This postulated mechanism of action needs further validation; perhaps by examining the DC distribution and phenotype in the draining lymph nodes of recipient mice.

Interferon-gamma (IFN- γ) is a cytokine classified as type II interferon. IFN- γ possesses anti-viral, anti-tumor and immunomodulatory activity [16] and can activate macrophage to perform anti-bacterial activity. [17] In previous investigations, ZOL was shown to stimulate the production of IFN- γ from a T cell subset, gammadelta (V γ 9V δ 2) T cells, both in vitro and in vivo in humans. [14, 18] Given that ZOL is a water-soluble infusional drug, widely accepted for the treatment of bone metastasis in cancer patients, the safety, administration and adverse effect profile have been well established.

The major safety consideration is the jaw osteonecrosis caused by ZOL [19]: this may not compromise its use in skin transplantation because the majority of cases of osteonecrosis

had previous invasive dental procedure, which could be avoided or screened for. The absence of significant effects of ZOL on leukocyte counts makes this agent distinct from many currently used immunosuppressants, which are well known to reduce leukocyte count. Another safe immunosuppressant for rejection prevention is FTY720. FTY720 has been reported to be capable of preventing rejection without causing toxicity, but it does affect leukocyte numbers. [20, 21] Taken together, these data suggest that ZOL may have the potential to be developed as a new category of immunosuppressants possessing a better pharmacological profile and therapeutic window than those currently in use.

In conclusion, ZOL may have unique benefits in transplant medicine. Further investigation is needed to evaluate whether, when used during transplantation, ZOL maintains the property of modulation of DCs. Clearly this promising agent is worthy of further clinical investigation.

Acknowledgments

This work was supported by a grant (Grant #MMH-9924) from Mackay Memorial Hospital, Taipei, Taiwan.

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