Short Communication

Risedronate Has No Adverse Effects on Mouse Haematopoiesis

(risedronate / haematopoiesis / stem cells)

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Abstract. Bisphosphonates are commonly used for treatment of osteoporosis. They inhibit osteoclast activity and thus bone resorption. It was shown that they also affect some other cell types including tumour and endothelial cells. The effects of risedronate on bone marrow microenvironment were not studied yet. As endothelial cells are integral part of bone marrow microenvironment, it is important to know whether prolonged administration of bisphosphonates does not affect haematopoietic stem cells and bone marrow haematopoiesis. We fed mice two weeks with risedronate. We found no effect of risedronate treatment on bone marrow stem cells using the method of congenic bone marrow repopulation. Risedronate administration in the dose which is considered to be comparable to a dose of risedronate used for treatment of osteoporosis in women seems to be safe in terms of effects on mouse haematopoiesis.

Introduction

Bisphosphonates (originally called disphosphonates) reduce the survival and function of osteoclast, the bone resorbing cell (Rogers et al., 2000). Like pyrophosphate, bisphoshonates have high affinity for bone mineral. The molecular mechanism by which bisphosphonates inhibit osteoclast activity can be classified into two groups. Bisphosphonates that lack nitrogen in the chemical structure of the R2 chain (such as etidronate, clodronate and tiludronate) are metabolically incorporated into nonhydrolyzable analogues of ATP that inhibit ATP-dependent intracellular enzymes (Schmidt et al., 1996). In contrast, nitrogen-containing bisphosphonates (such as pamidronate, risedronate, alendronate, ibandronate and zoledronic acid) inhibit the activity of farnesyl diphosphonate synthase, a key enzyme in the mevalonate pathway, thereby preventing the prenylation of small GTPases that are essential for osteoclast function (Reszka et al., 1999). However, it is now clear that bisphosphonates not only act on osteoclasts, but also on other cell types. Bisphosphonates inhibit *in vitro* growth of human myeloma, melanoma, breast and prostate carcinoma cell lines (Fournier et al., 2002). It is possible that the mechanism of growth inhibition induced by bisphonates is cell type-specific. Beside the fact that bishosphonates affect various biological properties of tumour cells and osteoclasts that contribute to development of osteolytic lesions, they can also act on bone marrow stromal cells. The treatment of endothelial cells with bisphosphonates (clodronate, risedronate, ibandronate, zoledronic acid) reduces proliferation, induces apoptosis and decreases capillary-like tube formation *in vitro* (Daubine et al., 2007). This could initiate adverse effects on haematopoiesis *in vivo*. In order to test the effect of risedronate on bone marrow haematopoietic stem cells, we used the murine model of competitive repopulation (Chang et al., 2005).

Material and Methods

Adult male strain C57Bl/6 (Ly5.2) and congenic Ly5.1 mice weighing approximately 22 grams were used for the experiment. They were maintained on standard laboratory diet containing 23% protein, 1.2% calcium and 0.6% phosphorus with water *ad libitum*, and were kept in an indirectly illuminated room with controlled temperature at 22 ± 2° C. After acclimation to the new environment, one group of five mice (Ly.5,1) was fed risedronate – Actonel. A second group of five mice (Ly5.1) served as the control group. The drug was incorporated into the animal diet. Each animal received 5.0 g of food per day and treatment continued for two weeks. Because pharmacokinetical data are not available for the administration of risedronate in mice, we used the dose 70 μg/kg/day, which is considered to be comparable to a dose of risedronate used for treatment of osteoporosis in women. The food consumption by both groups was controlled daily, for a period through

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which the intake of the drug in a dose shown above was supported.

After two weeks, the animals were killed and both femurs and also the seminal vesicles from individual donors were removed. The weight of seminal vesicles was determined. Blood was withdrawn from the heart. Calcium was measured by the method of Gitelman (1967) and phosphorus according to Kraml (1966).

The method of bone marrow repopulation after transplantation into a congenic recipient was used to monitor the effects of risedronate on haematopoietic stem cells. This test allows evaluation of both short-term repopulation activity caused by haematopoietic progenitor cells and long-term repopulating activity provided by haematopoietic stem cells, thus giving an advantage over *in vitro* clonal assays related to progenitor/stem cells with limited repopulation ability only.

Bone marrow was flushed out into cold PBS (0.5% BSA), nucleated cell counts were performed, and 4 million cells from each donor were transplanted into three recipient Ly5.2 mice irradiated sublethally with 6 Gy TBI (^{60}Co). Donor stem cells had to compete with surviving recipient stem cells to form blood cells. The chimerism (percentage of nucleated blood cells originated from donor cells – Ly5.1) was measured in peripheral blood two, four and eight weeks after transplantation. Briefly, blood was lysed by ammonium chloride solution and stained with fluorochrome-conjugated antibodies against Ly5.1 (donor cells) and Ly5.2 (recipient cells). The actual chimerism was measured by flow cytometry (FACSCalibur, Becton Dickinson, San Jose, CA) (Chang et al., 2005).

The chimerism after two and four weeks after transplantation reflects the action of short-term repopulating cells (STRC), while the numbers obtained after eight weeks or more are derived from long-term repopulating cells (LTRC).

Statistical analysis

The results are expressed as means ± SEM. Student's t-test was used for comparison of two variables. $P > 0.05$ was taken as a not significant difference.

Results

The results are summarized in Table 1. There were no clinical signs of morbidity in any of the two groups studied. We did not observe decreased food intake in risedronate-treated animals. Administration of risedronate did not significantly affect plasma calcium and phosphate. We did not observe significant changes in the weight of the seminal vesicles, a highly androgen-dependent tissue.

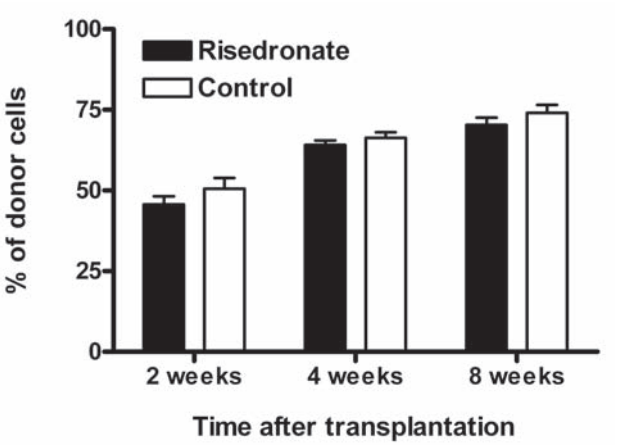

Fig. 1. White blood cell chimerism in transplanted recipient mice after bone marrow transplantation.

There were no differences in bone marrow cellularity in treated and control mice at the time of bone marrow collection – the number of cells in one femur did not statistically differ between risedronate-treated mice and control mice.

Both early (after two and four weeks) and long-term (after eight weeks) engraftment did not differ between risedronate-treated and control mice (Fig. 1). Risedronate had no effects on transplantability of haematopoietic stem cells in the congenic repopulation assay.

Discussion

Risedronate (ethylidene bisphosphonic acid monosodium salt) is a pyridinyl bisphosphonate powerful inhibitor of osteoclast-mediated bone resorption. Risedronate binds to a bone and is taken up by osteoclasts. The intracellular action of risedronate leads to loss of cytoskeletal structure and disappearance of the ruffled border, and ultimately apoptosis. However, bisphosphonates do not only act on osteoclasts. There is now extensive *in vitro* evidence that bisphosphonates can act on tumour cells (Daubine et al., 2007). Bisphosphonates

Table 1. Bone marrow cellularity, seminal vesicles weight, and plasmatic calcium/phosphates of risedronate-treated and control mice at the time of bone marrow collection

	Controls (5)	Controls + Risedronate (5)
Cells/femur (millions)	34.5 ± 0.8	35.2 ± 1.7
Seminal vesicles (mg/100 g b.w)	450 ± 40	447 ± 48
Pl. calcium (mmol/l)	2.2 ± 0.1	2.1 ± 0.1
Pl. phosphate (mmol/l)	4.2 ± 0.1	4.1 ± 0.1

inhibit experimental angiogenesis *in vitro* and *in vivo* (Wood et al., 2002). Zolendronic acid, a new-generation bisphosphonate with a heterocyclic imidazole substituent, is also a potent inhibitor of angiogenesis. *In vitro* zolederonic acid inhibits proliferation of human endothelial cells stimulated with foetal calf serum, basic fibroblast growth factor and vascular endothelial growth factor.

Bone marrow stromal cells are derived from mesenchymal stem cells, including fibroblasts, adipocytes, endothelial cells and osteoblasts (Kassem, 2004). Previous studies of the roles of stromal cells in supporting haematopoietic stem cells have been based mainly on *in vitro* culture and each of these stromal cells has been suggested to be capable of supporting haematopoietic stem/progenitor cells *in vitro* (Charbord, 2001). As endothelial cells are integral part of bone marrow microenvironment, it is important to know whether prolonged administration of bisphosphonates does not affect haematopoietic stem cells and bone marrow haematopoiesis. We investigated the *in vivo* effect of risedronate on haematopoietic stem cells, on their ability to repopulate the host microenvironment after autologous (congenic) transplantation.

We fed mice with 70 μg/kg/day risedronate for 14 consecutive days. This dose is comparable with the dose used for osteoporosis treatment in women. Since data on risedronate pharmacokinetics in mice is insufficient, the dose might not be pharmacodynamically equivalent to humans, and also the follow-up period might not have been sufficient. Possible limitations of the study should be taken into account.

Risedronate in our experiment did not induce changes in the weights of seminal vesicles of mouse tissue, which is extraordinarily sensitive to androgens. Testosterone treatment rapidly induces vasodilatation of blood vessels in the ventral prostate of castrated rats (Frank-Lissbrant et al., 1998). Ibandronate or zoledronic acid induced a 50% reduction of the revascularization of the prostate gland (Fournier et al. 2002). Based on our experiment, we can exclude the influence of indirect effects on stem cells caused by changes in calcaemia, phosphataemia and testosterone.

Our results showed no significant change in bone marrow nucleated cell counts or in the content and/or quality of stem cells. There was no difference in both short-term (STRC) and long-term bone marrow repopulating cells (LTRC) between risedronate-treated and control mice. Risedronate thus does not affect haematopoiesis in the mouse. Competitive repopulation was measured by the ability of grafted stem cells to produce nucleated blood cells. Nevertheless, blood cell counts including red blood cells and platelets were not affected (data not shown). Risedronate seems to be safe in regard to the effects on haematopoietic tissue in the mouse.

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