Summary. Long-term use of glucocorticoids (GC) causes rapid bone loss and increases the risk of osteoporotic fractures. Matrix metalloproteinase (MMPs), the most prominent kind of proteases implicated in the proteolytic degradation of the extracellular matrix (ECM), have been reported to be involved in pathological process of GC induced osteoporosis. However, the underlining mechanisms are still unclear. The aim of this study was to investigate the spatial expression and the potential function of MMP 2, 9 and 13 in osteoporosis induced by prednisolone in the tibiae of mice. In this experiment, mice were given prednisolone (15 mg/kg body weight) in PBS intragastrically every other day, or only PBS as control. Two weeks later, mice were fixed with transcardial perfusion of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4), and tibiae were extracted for histochemical analysis. Compared with control group, the number of TRAP-positive osteoclasts and the immunoreactivity of MMP 2, 9 and 13 were significantly increased in the trabecular bone of mice administered with prednisolone, leading to the decrease of trabecular bone volume. On the other hand, lighter eosin staining areas containing numerous empty lacunae of osteocytes and crevices were seen in the narrowing cortical bone. Furthermore, intense immunoreaction of MMP 2 and MMP 13 were found in the enlarged lacunae and the crevices, respectively. Taken together, we concluded that prednisolone administration induced the increase of MMP 2, 9 and 13 expressions, while MMP 2 and MMP 13 played essential roles in the osteocytic osteolysis and the early impaired areas in the cortical bone. Therefore, MMPs might be new potential therapeutic targets for prevention and treatment of glucocorticoid induced osteoporosis, especially osteocytic osteolysis.

Key words: Prednisolone, MMPs, Osteoclast, Osteocytic osteolysis, Osteoporosis

Introduction

Glucocorticoids (GCs) are widely used for the treatment of numerous immunologic, rheumatologic, neoplastic and allergic diseases. However, long term exposure to GCs is responsible for many adverse events such as osteoporosis, diabetes mellitus or infection (Fardet et al., 2011; Morin and Fardet, 2015). As the most common cause of secondary osteoporosis, glucocorticoid-induced osteoporosis (GIO) is characterized by the dynamic balance disturbances of bone remodeling leading to increased fracture risk (Carbonare et al., 2001; Roux et al., 2012). GCs increase bone resorption by stimulating osteoclastogenesis by increasing the expression of receptor activator of nuclear factor kB ligand (RANK-L) (Pichler et al., 2013; Shi et al., 2014) and decreasing the expression of its soluble decoy receptor osteoprotegerin (OPG) in stromal and osteoblastic cells (Sasaki et al., 2001, 2005). GCs also enhance the expression of macrophage colony-
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Aims and methods

Materials and methods

Histological examination and image analysis

Prednisolone induces osteoclastic bone resorption and trabecular bone loss through a direct osteoclast action and an indirect effect on osteoblast activity. The aim of this study is to investigate the effects of prednisolone on bone remodeling and to determine the role of MMPs in the process of bone resorption.

Materials and methods

All animal experiments in this study were conducted according to the Guidelines for Animal Experimentation of Shandong University. Twenty 6-week-old Kunming mice were obtained from Laboratory Animal Centre of Shandong University (Jinan, China) and kept in plastic cages (3-5 mice per cage) under standard laboratory conditions with a 12-h dark,12-h light cycle and a constant temperature of 20°C and humidity of 48%. All mice were fed a standard rodent diet ad libitum.

After 1 week acclimation, the mice were randomly divided into two groups (ten mice per group) and were given prednisolone (15 mg/kg body weight) in PBS intragastrically every other day, or only PBS as control. Two weeks later, the mice were anesthetized with an intraperitoneal injection of 10% chloral hydrate (400 mg/100 g body weight) and fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) by transcardial perfusion. After fixation, tibiae were removed and immersed in the same fixative for additional 24 h. Following that, samples were decalcified with 10% EDTA-2Na solution for 3 weeks at 4°C. Then the specimens were dehydrated through an ascending ethanol series and then embedded in paraffin using standard procedures. Serial longitudinal 5-μm-thick sections were prepared for following histological analysis using rotary microtome (LEICA SM 2010R, German).

Histological examination and image analysis

Hematoxylin and eosin (H&E) was performed to investigate the morphology of metaphysis in both groups. After being dewaxed and hydrated, the prepared sections were immersed in Ehrlich’s haematoxylin for 15 minutes. Then the sections were washed with distilled water and counterstained with eosin.
water and differentiated in 1% HCl in 70% alcohol for 1 minute and washed again for 2 minutes. After that, the sections were stained with 1% eosin for 10 minutes and washed with distilled water. Finally, all sections were dehydrated and mounted. The stained sections were observed and then digital images were taken with a light microscope (Olympus BX-53, Japan). With the aid of Image Pro Plus 6.2 software (Media Cybernetics, Silver Spring, MD), trabecular bone volume (BV/TV, trabecular bone volume/ tissue volume × 100%) was measured. Specifically, 10 slices of each sample were used for quantitative histomorphometric analysis to get the mean value.

**Histochemistry (TRAP staining) for osteoclasts**

To evaluate the osteoclast, tartrate-resistant acid phosphatase (TRAP) staining was performed as previously showed (Li et al., 2013). In brief, dewaxed paraffin sections were submerged in a mixture of 3.0 mg of naphthol AS-BI phosphate, 18 mg of red violet LB salt, and 100 mM L(+)-tartaric acid (0.36 g) diluted in 30 ml of 0.1 M sodium acetate buffer (pH 5.0) for 15 min at 37°C. Counter staining was performed with methyl green.

**Immunohistochemistry for MMP 2, 9 and 13**

Prepared 5 μm thick paraffin sections were used for MMP 2, 9 and 13 immunolabeling. Briefly, after xylene treatment, dewaxed paraffin sections were pretreated with 0.3 % hydrogen peroxide for 30 minutes, and then with 1% bovine serum albumin (BSA; seologicals proteins Inc. Kankakee, IL, USA) in PBS (1% BSA-PBS) for 20 min to reduce non-specific staining. The treated sections were incubated with the primary...
antibodies of mouse antibody against MMP 2 (Millipore, USA) at a dilution of 1:50, goat antibody against MMP 9 (R & D Systems, Inc., Minneapolis, USA) at a dilution of 1:100 and goat antibody against MMP 13 (Millipore, USA) at a dilution of 1:50 with 1% BSA-PBS for 2 h at room temperature, respectively. After rinsing with PBS, they were immersed in horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG for MMP 2, rabbit anti-goat IgG for MMP 9 and rabbit anti-goat IgG for MMP 13 (Jackson Immunoresearch laboratories, Inc., Baltimore, USA; Abcam, Hong Kong) respectively, at a dilution of 1:100 for 1 h at room temperature. The immunoreaction was visualized with diaminobenzidine (DAB) (Sigma-Aldrich, St. Louis, MO, USA). All sections were counterstained faintly with methyl green and observed under a light microscope (BX53, Olympus Corp., Japan). Specimens were scored according to the intensity of the dye color and the number of positive cells as previously described (Ma et al., 2010). The intensity of the dye color was graded as 0 (no color), 1 (light yellow), 2 (light brown), or 3 (brown), and the number of positive cells was graded as 0 (<5%), 1 (5-25%), 2 (25-50%), 3 (51-75%), or 4 (>75%). The two grades were added together and specimens were assigned to one of 4 levels: 0-1 score (Negative), 1-2 scores (Weak), 3-4 scores (Moderate), more than 5 scores (Intense). The positive expression rate was expressed as the percent of the addition of (Weak), (Moderate) and (Intense) to the total number.

Parameters measurement and statistical analysis

Image Pro Plus 6.2 (IPP 6.2) software (Media Cybernetics, Silver Spring, MD) was used for counting...
the trabecular bone volume, thickness of growth plate, number of TRAP-positive osteoclasts in the trabecular bone and the cortical bone width, the number of vital osteocytes and empty lacunae in the cortical bone of the prednisolone-treated group and control. Because of the metabolic stability, diaphysis was chosen as the measurement area for the cortical bone width and the number of osteocytes. Immunostaining intensities (optical density, OD) also were analyzed using the same software. Positive reaction areas of MMP 2, 9 and 13 were manually selected in a color cube based manner. At least 10 sections from each sample were analyzed. All values are presented as mean ± standard deviation (SD).

The differences between prednisolone administered group and control were assessed by student’s t-test, and considered statistically significant at p<0.05 and p<0.01.

Results

Histological alterations and TRAP staining for osteoclasts

Two weeks after prednisolone administration, the tibiae of prednisolone administration group showed obvious histological manifestation of osteoporosis, including narrowed growth plate, decreased trabecular

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![Fig. 3. TRAP staining for osteoclasts and immunolocalization for MMP 2, 9, 13 in the trabecular bone at higher magnification in prednisolone-administered group.](image)

A. A large number of TRAP-positive osteoclasts were seen on the surface of trabecular bone. B. Moderate expression of MMP 13 was found in the bone matrix indicated by black arrows. C. Intense expression of MMP 9 was seen on the surface of trabecular bone and adjacent to the growth plate indicated by black arrows. D. Moderate expression of MMP 2 was mainly seen on the surface of trabecular bone shown by black arrows. TB: trabecular bone; OC: osteoclast. x 400
bone volume and thinned cortical bone (HE staining, Fig. 1A,B). Statistical analysis revealed several significant differences between prednisolone-administered group and control with regard to BV/TV (29.710±1.240% in the prednisolone administration group vs 82.100±1.620% in control, p<0.01, Fig.1C), thickness of growth plate (160.000±5.770 μm in the experiment group vs 336.700±12.020 μm in control, p<0.01, Fig.1D) and width of cortical bone (148.700±5.930 μm in the prednisolone-administered group vs 221.700±4.410 μm in control, p<0.01, Fig. 1E).

In particular, lighter eosin staining areas can be distinguished easily in the cortical bone of prednisolone-administered group (Fig. 4B). More interestingly, a number of empty or enlarged lacunae were seen in the impaired areas induced by prednisolone. Statistical analysis revealed significant differences between prednisolone-administered group and control with regard to the number of osteocytes (396.700±12.020 cells/mm² in the prednisolone administration group vs 800.000±11.550 cells /mm² in control, p<0.01, Fig. 4C) and the number of empty lacunae (220.000±3.000 empty lacunae/mm² in the prednisolone administration group vs 80.000±2.500 empty lacunae/mm² in control, p<0.01, Fig. 4D).

Compared with control group, the number of TRAP-positive osteoclasts was significantly increased on the surface of trabecular bone in mice administered with prednisolone (Figs. 2A,B, 3A). Statistical analysis found a significant difference in osteoclastic numbers between

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**Fig. 4.** Histological alterations in the cortical bone and statistical analysis. **A, B.** Histological imaging of cortical bone of control (HE staining) (A) and prednisolone treatment group (B). **A.** Control group showed uniform eosin staining and osteocytes were seen within the lacunae indicated by white arrows in the bottom right panel. **B.** Lighter eosin staining areas could be found in the cortical bone of experimental group indicated by white asterisk. Furthermore, a lot of empty lacunae and atrophic osteocytes were seen in the impaired cortical bone areas indicated by black arrows. **C, D.** Number of osteocytes and empty lacunae were counted in the cortical bone for both groups (n=10; **p<0.01). All corresponding numerical information is found in the results section. Error bars indicate ± SD. SD: Standard deviation; CB: cortical bone; ocy: osteocyte; IA: impaired area; NA: normal area. A, B, x 100; inserts, x 400.
prednisolone-administered group and control (85.400±2.540 cells/mm² in the prednisolone administration group vs 60.130±0.590 cells/mm² in control, p<0.01, Fig. 2I).

**Immunolocalization of MMP 2, 9 and 13**

In the prednisolone-administered group, trabecular bone of metaphysis showed the intense expression of MMP 9 and moderate expression of MMP 2 and MMP 13 (Figs. 2C-H, 3B-D). Statistical analysis revealed significant differences in MMP 2-, MMP 9-, and MMP 13-reactivity between prednisolone-administered group and control (MMP 2: 0.180±0.005 in the prednisolone administration group vs 0.140±0.002 in control, p<0.01; MMP 9: 0.130±0.004 in the prednisolone-treated group vs 0.080±0.002 in control, p<0.01; MMP 13: 0.080±0.003 in the prednisolone-administered group vs 0.050±0.002 in control, p<0.01, Fig. 2J). On the other hand, there is no significant difference between control and prednisolone administration group for MMP 9 expression (Fig. 5C,D,H), but intense expression of MMP 2 and moderate expression of MMP 13 can be seen in the cortical bone in prednisolone administration.
group (Fig. 5A,B,G and Fig. E,F,I). Furthermore, MMP 2 expression mainly concentrated in the lacunae (Fig. 5F), while the expression of MMP 13 was found in the crevices and periosteum (Fig 5B). Statistical analysis revealed significant differences in MMP 13- and MMP 2-expression between prednisolone-administered group and control (MMP 13: 0.050±0.002 in the prednisolone administration group vs 0.010±0.002 in control, p<0.05, Fig. 5G; MMP 2: 0.110±0.002 in the prednisolone administration group vs 0.010±0.001 in control, p<0.01, Fig. 5I).

Discussion

In this study, we investigated the expression of MMP 2, 9 and 13 in prednisolone induced osteoporosis in mice. As shown in Fig.1, tibiae of prednisolone administered mice showed the obvious features of osteoporosis including decreased trabecular bone volume, narrowed growth plate and thinner cortical bone with empty lacunae and crevices. These findings are consistent with previous studies, which reported that GC administration reduced bone formation, increased bone resorption and increased apoptosis of osteocytes and osteoblasts both in vivo and in vitro (Lo Cascio et al., 1995; Weinstein et al., 1998; O’Brien et al., 2004). GCs affect bone cells by binding to glucocorticoid receptor (GR) to form ligand-receptor complex, regulating the expression of a wide array of target genes (Surjit et al., 2011; Karmakar et al., 2013). Our results showed that intense expression of MMP 2, 9 and 13 were seen in the trabecular bone of metaphysis. Furthermore, microscopic observation at higher magnification further verified the existing viewpoints in which MMP 2 and MMP 13 are produced by osteoblasts and osteocytes, while MMP 9 is mainly expressed by osteoclasts (Reponen et al., 1994; Vu et al., 1998; Nakamura et al., 2004; Inoue et al., 2006). Schema graph of prednisolone induces osteoporosis through affecting the expression of MMP 2, 9, 13 was shown in Fig. 6.

Recently, many researchers considered that the loss of trabecular mass, trabecular architecture, and deposition bone mass does not explain the increase in fracture risk from GCs, and the adverse effects of GCs on cortical bone quality may be an independent factor worth further investigation (Black et al., 2010; Jia et al., 2011). As a meaningful finding of this study, we observed two parallel belt-like lighter eosin staining areas containing abundant tiny strip-like crevices, increased number of empty or enlarged lacunae with nucleus condensed osteocytes in the cortical bone of prednisolone administered group. The finding may be new and valuable histological evidence for the adverse effects of GCs on cortical bone quality. In addition, previous studies demonstrated that MMP 13 was synthesized and secreted by osteoblastic cells and translocated to the resorption lacunae. Furthermore, MMP 13 was also shown to be expressed in cement lines (Dew et al., 2000; Stickens et al., 2004; Tang et al., 2012). We therefore also speculate that the increased expression of MMP 13 in the crevices of cortical bone may be related to the formation of the belt-like lighter eosin staining areas in the cortical bone in prednisolone-treated mice.

GC induced osteocytic osteolysis has been paid more and more attention because of its devastating side effects. Up to now, however, most research has focused on the description of this phenomenon; the exact mechanism has not been elucidated. MMP 2 is capable of cleaving native type I collagens and over expression of MMP 2 was reported to be related to osteolysis and arthritis (Aimes and Quigley, 1995; Martignetti et al., 2001). Furthermore, MMP 2−/− mice exhibited opposing bone phenotypes featured by moderate disruption of the osteocytic networks and reduced bone density due to an impaired osteocytic canalicular network (Inoue et al., 2006). In the present study, intense immunoreactivity for MMP 2 was exhibited in the enlarged lacunae of prednisolone-treated mice.
ostecocytes with condensed nucleus in cortical bone of diaphysis in prednisolone treated mice. The finding suggested that prednisolone administration induced high expression of MMP 2 in lacunae and demonstrated that MMP 2 may play a crucial role in osteocytic osteolysis. However, histological examination was not sufficient and further research might be necessary.

In conclusion, our data suggested that the expressions of MMP 2, 9, 13 were obviously increased in prednisolone induced osteoporosis, especially MMP 2 and MMP 13, which might play key roles in osteocytic osteolysis and the formation of lighter eosin staining impaired areas in the cortical bone (Figure 6). Concurrently, we considered that MMPs might be new potential therapeutic targets for glucocorticoid induced osteoporosis, especially osteocytic osteolysis.

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