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Alleviation of Radiotherapy-Induced Local Trabecular Bone Loss by PTH(1-34) is Associated with Improved DNA Repair and Cell Survival in Osteoblasts

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Introduction

Radiotherapy is a common therapeutic for cancers, but it has inevitable adverse effects on bones within irradiated region and causes problems, such as osteoporosis, osteoradionecrosis, and fractures. To date, there is no preventive or curative treatment for radiation-induced bone damage. We have previously reported that parathyroid hormone (PTH, 1-34), an anabolic treatment for osteoporosis, prevents the adverse effects of radiation generated from multiple longitudinal micro-computed tomography (μ CT) scans on the trabecular bone architecture in young rats.¹ However, μ CT scan does not mimic clinical radiator due to its low and fixed dosage. Furthermore, the mechanism by which PTH reverses radiation-induced bone loss has not been elucidated. In this study, we investigated the effectiveness of PTH in a clinically relevant radiation model using skeletally mature rats. The mechanisms of PTH's rescue effects on bone loss were studied at structural, cellular, and molecular levels.

Methods

Animal protocols- 3-month-old female Sprague Dawley rats (n=5/group) were radiated by SARRP at their right tibial metaphyseal region at a dose of 8 Gy twice (day 1 and 3). This radiation protocol mimics the dose to the hip during hypofractionated radiotherapy for prostate cancer. The contralateral non-radiated tibiae served as paired controls. Rats were then separated into two groups receiving either vehicle or rhPTH(1-34, 60 μ g/kg/day) treatment for 4 weeks. **In vivo μ CT and analysis-** To avoid the bone growth issues in rats, we developed a unique 3D μ CT registration method to identify the same trabecular structure at day 28 that matches the metaphyseal area at 2.5-4 mm below the growth plate at day 0 in the same tibia. The structural parameters and mechanical competence of the same block of trabecular bone before and after radiation were then calculated based on standard image analysis and finite element analysis (FEA). **Histology-** Tibiae were harvested at 4 weeks for measuring osteoblast numbers using H&E staining or at 2 weeks for paraffin sections with TUNEL assay for detecting

apoptotic osteoblasts. Apoptosis assay of UMR-106 cells after radiation and PTH treatment- UMR cells were radiated at 8 Gy followed by 10 nM PTH treatment in presence or absence of a PKA inhibitor (H89) or WNT-inhibitor (IWR). Ethidium Bromide (EB)/Acridine Orange (AO) staining was performed 2 days later to count apoptotic cells. Single cell gel electrophoresis- Comet assay was performed under alkaline conditions to measure the extent of DNA damage at a single cell level. **Immunocytochemistry-** Cells were immunostaining with antibodies against γ -H2AX, caspase 3, or β -catenin to detect their cellular localization.

Results

3D registration of the same trabecular bone before and after radiation demonstrated that trabecular bone volume fraction (BV/TV) in non-radiated tibiae of vehicle-treated rats at day 28 increased significantly (69%) with increased trabecular thickness (Tb.Th, 36%) and unchanged trabecular number (Tb.N) compared to its own at day 0. However, BV/TV in radiated tibiae of vehicle-treated rats only increased slightly (36%) compared to its own at day 0 but it was 19% significantly less than that in non-radiated contralateral tibiae at day 28 (Figure 1A). This was mainly due to an 18% decrease in Tb.N and a 26% decrease in connective density (Conn.D). Carefully examining the reconstructed 3D μ CT images reveals that, while almost all trabecular elements were preserved in non-radiated samples during 4 weeks of growth, some small trabecular elements were apparently lost after radiation (red circles in Figure 1B), implicating that radiation shifts the balance of bone remodeling toward more resorption. FEA revealed a 57% decrease in trabecular bone stiffness in radiated bones compared to non-radiated controls (Figure 1C), suggesting that the mechanical strength is severely impaired after radiation. Interestingly, PTH(1-34) treatment had a great anabolic effect on trabecular bone and remarkably stimulated bone mass in both non-radiated and radiated tibiae to a similar level (Figure 1A). A close look at the 3D registered μ CT images uncovered that PTH not only thickened the trabecular elements but also preserved them

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from radiation-induced loss (Fig. 1B). Trabecular bone strength was also maintained in radiated bones by PTH treatment (Figure 1C).

Further histological analysis indicated that radiation caused apoptosis in osteoblasts, leading to few viable and functional osteoblasts (30% of non-radiated control) on the trabecular bone surface (Figure 2A-C), while PTH injections increased their number and protected them from cell death (Figure 2C). To confirm and expand these *in vivo* findings, we radiated osteoblast-like UMR106-01 cells followed by vehicle or PTH (1-34) treatment. Radiation greatly stimulated the percentage of apoptotic cells from 1.0% to 12.6% and 10 nM PTH diminished this increase to 2.6% (Figure 2D). Inhibitor assays revealed that the survival action of PTH is mediated by PKA and β -catenin. Indeed, PTH enhanced β -catenin amount and its nuclear translocation in osteoblasts after radiation via PKA pathway (Figure 2E and data not shown). Radiation induces highly lethal DNA damage, among which double-strand breaks (DSBs) is the major factor responsible for cell death. A sensitive method to detect DSBs is the immunofluorescence staining of γ -H2AX. We found that PTH

reduced the radiation-induced γ -H2AX foci number as early as 2 h (Figure 3A). Comet assay further confirms that PTH treatment blocks radiation-induced DNA damage (represented by comet tails) in a PKA- and β -catenin-dependent manner (Figure 3B). The occurrence of DSBs invokes the repair pathway. Interestingly, we found that 30 min of PTH treatment significantly increased the amount of Ku70, a critical component of repair complex, regardless of radiation (Figure 3C). If DNA lesions induced by radiation cannot be resolved in a timely fashion, cells will begin the process of programmed cell death. Western blot showed that radiation reciprocally regulated anti-apoptotic Mcl1 and pro-apoptotic Bim for cell death and that PTH had opposite effects (Figure 3D). At last, radiation activated the downstream protein of apoptotic pathway, caspase 3 while PTH suppressed it (data not shown).

Discussion

Our ability to accurately trace the same bone before and after radiation provides strong and direct evidence that PTH treatment is able to alleviate radiotherapy-induced local loss

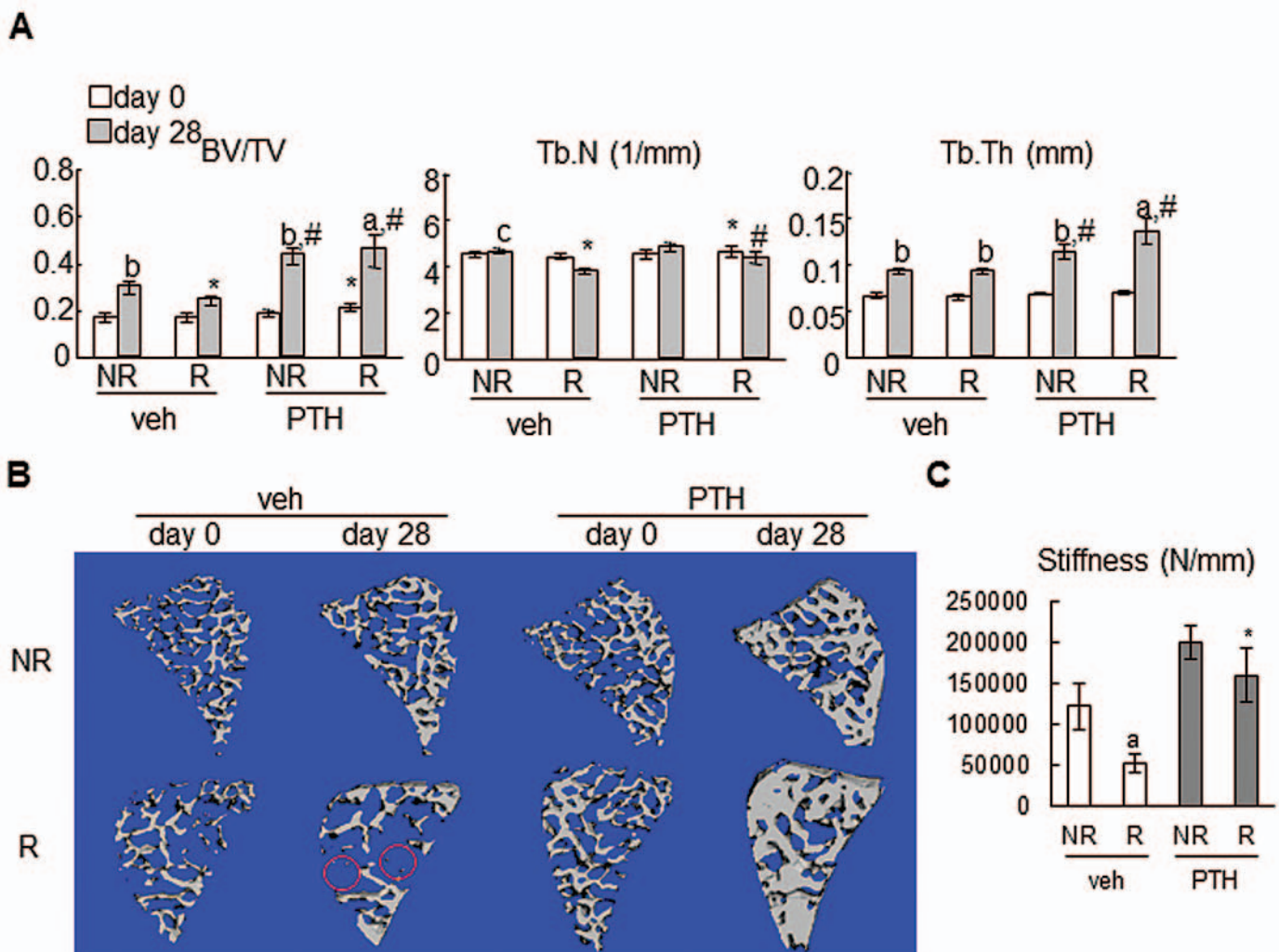


Figure 1. (A) PTH reverses SARRP radiation-induced bone loss and damage. (B) μ CT images of the rat proximal metaphyseal region of radiated (R) and non-radiated (NR) tibiae of vehicle or PTH treated animals. (C) Measure of bone strength by finite element analysis. a<0.05; b: p<0.01; c: p<0.001, d 28 vs. d 0. *: p<0.05, R vs. NR. #: p<0.05, PTH vs. veh.

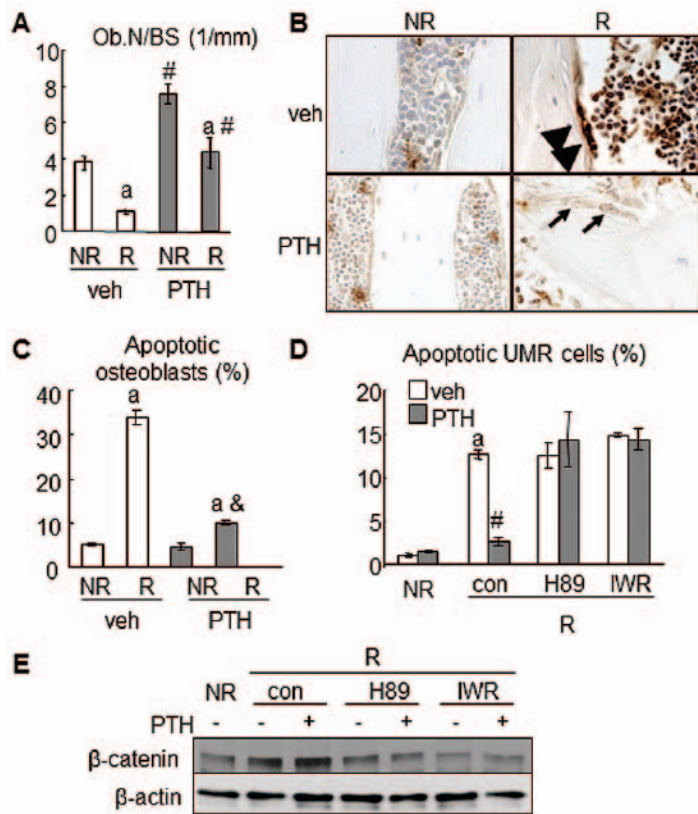


Figure 2. PTH alleviates radiation-induced apoptosis in osteoblasts. (A) Bone histomorphometry to measure osteoblast number and (B,C) TUNEL staining (arrowheads indicate apoptotic cells and arrows indicate healthy osteoblasts of tibiae sections and its quantification (D) Apoptotic assay in UMR cells after radiation and PTH treatment (E) β-catenin immunoblots after radiation and PTH treatment. a: $p < 0.01$, R vs. NR, #: $p < 0.01$, PTH vs. veh.

of trabecular elements and deterioration in bone strength. Using in vivo and in vitro approaches, we report that PTH achieves its therapeutic effect by accelerating DNA repair and stimulating anti-apoptotic signals via a PKA/β-catenin pathway in mature osteoblasts, which leads to their preservation against radiation-induced cell death.

Significance

The improved survivorship rate and the increased age of cancer patients receiving radiotherapy emphasize the

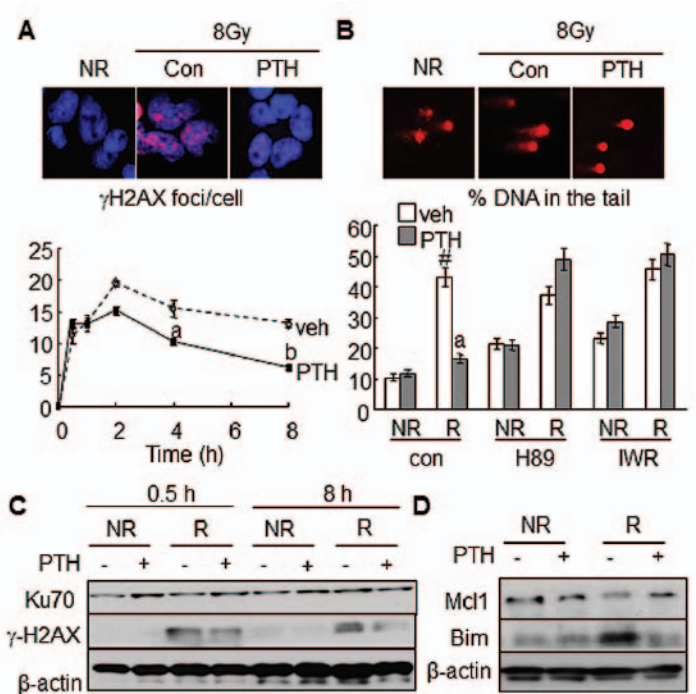


Figure 3. PTH promotes DNA repair and subsequently survival in osteoblasts (A) immunofluorescence assay detecting γ-H2AX post-radiation and PTH treatment. (B) Comet assay to determine DNA damage. (C) Immunoblot for Ku70 and γ-H2AX(D) Immunoblot of Mcl1 and Bim. a: $p < 0.01$; b: $p < 0.001$, PTH vs veh, #: $p < 0.01$, R vs NR.

importance of understanding the mechanism of radiation-induced osteoporosis and identifying a treatment for this disease. Our studies showing that PTH(1-34) can effectively prevent radiation-induced bone damage in rats is a major and unique step ahead, promising to pave the way toward effective treatments to reduce radiation damage on bone and thereby maximize the therapeutic index for radiotherapy.

Acknowledgments

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References

1. Chandra A, et al. PTH prevents the adverse effects of focal radiation on bone architecture in young rats. *Bone* 55, 449–457 (2013).