Testosterone inhibits osteoclast formation stimulated by parathyroid hormone through androgen receptor

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Abstract Androgens play an important role in the regulation of bone metabolism in animals and humans. The present study was performed to investigate whether androgens would affect osteoclast formation stimulated by parathyroid hormone (PTH) in mouse bone cell cultures and its mechanism. Testosterone as well as α-dihydrotestosterone (DHT) concentration-dependently inhibited osteoclast formation induced by PTH-(1-34). 10^{-8} M ICI 182780, an estrogen receptor inhibitor, did not affect PTH-induced osteoclast formation antagonized by 10^{-8} M testosterone, although it completely antagonized the effects of 10^{-8} M 17β-estradiol. Moreover, 3 μM 4-androsten-4-ol-3,17-dione, an aromatase inhibitor, did not affect PTH-induced osteoclast formation antagonized by testosterone. Hydroxyflutamide, an androgen receptor antagonist, concentration-dependently antagonized the inhibitory effects of testosterone as well as DHT on PTH-stimulated osteoclast formation. In conclusion, the present study first demonstrated that testosterone inhibited osteoclast formation stimulated by PTH through the androgen receptor, but not through the production of intrinsic estrogen in mouse bone cell cultures. **© 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Androgen; Testosterone; Osteoclast; Osteoclast formation; Parathyroid hormone; Androgen receptor

1. Introduction

Androgens play an important role in the regulation of bone metabolism in animals and humans [1–4]. Osteoporosis in men is a serious health problem in an aging population [5], and decreased androgen levels have been linked to lower bone mineral density in men [6]. Moreover, there is a strong correlation between hypogonadism in elderly men and spinal osteoporosis [5,6], and androgen treatment of osteoporotic men and women is effective in increasing bone mineral density [7].

Gonadal androgens including testosterone act directly on osteoblasts, stimulating growth and differentiation of osteoblastic cells in vitro by binding to an androgen receptor [8,9]. As for bone resorption, Mizuno et al. [10] reported the existence of androgen receptors in mouse osteoclast-like cells and the recent study [11] revealed that androgens regulated bone-resorbing activity of isolated chicken osteoclasts. Osteoclastic bone resorption is accelerated by the development of new osteoclasts as well as the activation of quiescent osteoclasts. However, no reports have been available about whether androgens would affect osteoclast formation or not.

Parathyroid hormone (PTH) stimulates bone resorption by accelerating osteoclast formation and bone-resorbing activity of mature osteoclasts [12,13]. Our previous study [14] revealed that estrogen blocked PTH-stimulated osteoclast formation by selectively affecting PTH-responsive cAMP pathway in mouse bone cell cultures. Several studies [15–18] suggested that androgens act on bone cells mainly through the conversion of testosterone to estrogen. The present study was, therefore, performed to investigate whether androgens such as testosterone and α-dihydrotestosterone (DHT), an active form of testosterone, would affect osteoclast formation induced by PTH in mouse bone cell cultures and to clarify its mechanism.

2. Materials and methods

2.1. Materials

ICR mice were obtained from Japan Clea Co. (Tokyo, Japan). 17β-Estradiol (17β-E_2), testosterone, DHT and 4-androsten-4-ol-3,17-dione were purchased from Sigma Chemical Co. (St. Louis, MO, USA), human (h) PTH-(1-34) from Peptide Institute (Osaka, Japan), ICI 182780 was from Tocris (Ballwin, MO, USA). 1,25-Dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) and hydroxyflutamide were kindly provided by Chugai Pharma Co. Ltd. (Shizuoka, Japan) and Nippon Kayaku Co. Ltd. (Tokyo, Japan), respectively. All other chemicals used were of analytical grade.

2.2. Mouse bone cell culture and osteoclast formation

Osteoclast formation from mouse unfractionated bone cells was measured, as previously described [12]. Femora and tibiae of 10–15-day-old ICR mice were aseptically removed. The bones from 10 mice were dissected free of soft tissues and mechanically minced with a scalpel blade in phenol red-free Eagle's essential medium (MEM) containing 5% charcoal-treated fetal calf serum (FCS). After removal of bone fragments by sedimentation under normal gravity, unfractionated bone cell suspensions were collected from the supernatant. These unfractionated bone cells included tartrate-resistant acid phosphatase (TRAP)-positive multinucleated cells (MNCs), alkaline phosphatase-positive mononuclear cells (probably osteoblasts) and other bone marrow cells. For cell spotting, the number of TRAP-positive cells containing three or more nuclei was counted as TRAP-positive MNCs in these bone cell suspensions. To examine the effect of each chemical on osteoclast formation using a population of preexistent osteoclast-free bone cells, we spotted these mouse bone cells in 96-well plates at 160 TRAP-positive MNCs/well (1×10^5 cells/well). Unfractionated bone cells were cultured in MEM containing 5% FCS at 37°C in a humidified 10% CO_2–90% air atmosphere for 5 days, by which time preexistent osteoclasts (mature osteoclasts existing at the beginning of culture) had degenerated and few TRAP-positive MNCs were observed after 5 days of culture, as previously described in detail [12]. Thus, we could test the effects of osteotropic factors on the sequential process leading to osteoclast formation by adding those factors to the cultures after degeneration of preexistent osteoclasts.

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That is, this method allows us to analyze osteoclast formation in the presence of osteoblasts. Each chemical was added to these osteoclast-free bone cell cultures. After 7 days of culture, cells adherent to the plates were washed with phosphate-buffered saline, dried, and promptly stained for TRAP. Cells were viewed under an inverted phase-contrast microscope and the number of TRAP-positive MNCs was counted. These newly formed TRAP-positive MNCs had various characteristics of osteoclasts, including responsiveness to calcitonin and bone-resorbing activity, as previously described [12].

2.3. Statistical analysis

Statistical analysis was performed by Student's *t* test or Duncan's multiple range test. Results of figures are representative of at least three separate cell preparations. Similar results were obtained from other cell preparations.

3. Results and discussion

We first examined whether androgens would affect osteoclast formation stimulated by PTH and 1,25-$(OH)_2D_3$ in mouse bone cell cultures. As shown in Fig. 1, 10^{-8} M PTH-(1-34) stimulated MNC formation and testosterone concentration-dependently inhibited MNC formation induced by PTH. However, testosterone did not affect MNC formation induced by 10^{-8} M 1,25-$(OH)_2D_3$. This is the first evidence that testosterone inhibits osteoclast formation. In our previous study [14], estrogen inhibited osteoclast formation induced by PTH, although it did not affect osteoclast formation induced by 1,25-$(OH)_2D_3$ in mouse bone cell cultures. The effects of testosterone in the present study are analogous to those of estrogen in our previous study. DHT has a greater affinity for androgen receptors and cannot be aromatized. Human bone is capable of metabolizing testosterone to DHT [19]. We, therefore, examined whether DHT would affect osteoclast formation stimulated by PTH in mouse bone cell cultures. As shown in Fig. 1, DHT concentration-dependently inhibited MNC formation stimulated by PTH and its potency seemed to be somewhat stronger, compared to that of testosterone.

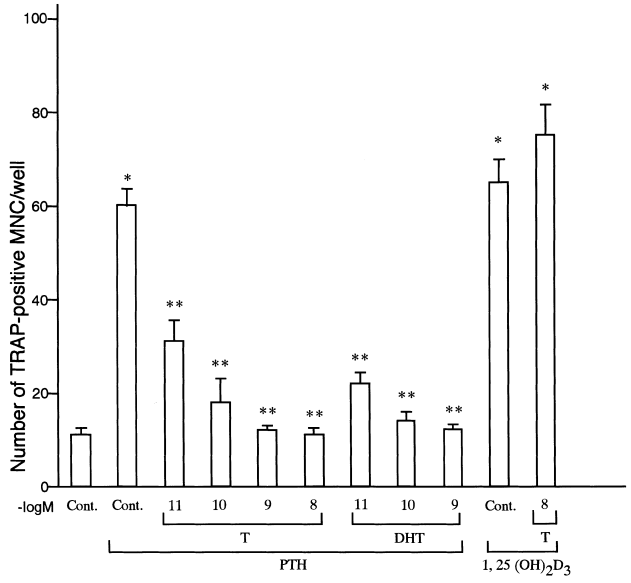

Fig. 1. Effect of androgens on osteoclast formation stimulated by PTH or 1,25-$(OH)_2D_3$ in mouse bone cell cultures. After degeneration of preexistent osteoclasts, 10^{-8} M hPTH-(1-34) (PTH) and 10^{-8} M 1,25-$(OH)_2D_3$ were added with or without the indicated concentrations of testosterone (T) or DHT, followed by 7 days of culture. Then, the number of TRAP-positive MNCs was counted, as described in Section 2. Data are expressed as the mean ± S.E.M. of four determinations. $*P<0.01$, compared to control. $**P<0.01$, compared to PTH-treated group.

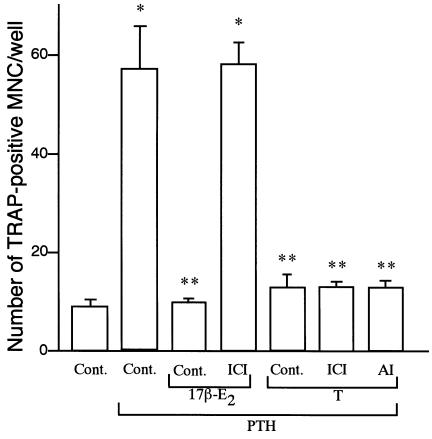

Fig. 2. Role of estrogen receptor and aromatase in the effects of testosterone on PTH-stimulated osteoclast formation. After degeneration of preexistent osteoclasts, 10^{-8} M hPTH-(1-34) (PTH), 10^{-8} M PTH and 10^{-8} M 17β-E_2, and 10^{-8} M PTH and 10^{-8} M testosterone (T) were added with or without 10^{-8} M ICI 182780 (ICI) or 3 μM 4-androsten-4-ol-3,17-dione (AI), followed by 7 days of culture. Then, the number of TRAP-positive MNCs was counted, as described in Section 2. Data are expressed as the mean ± S.E.M. of four determinations. $*P<0.01$, compared to control. $**P<0.01$, compared to PTH-treated group.

Several studies [15,16] revealed that bone mineral density of the lumbar spine and hip was positively related to serum concentration of estradiol in men, supporting the concept that the actions of testosterone on the male skeleton may be mediated in part by aromatization to estrogen [17]. Moreover, in a recent case report of osteoporosis in a 28-year-old man with a mutation of the aromatase gene, treatment with intramuscular testosterone produced no benefit but transdermal estradiol resulted in skeletal maturation, a rapid increase in lumbar spine bone density and closure of the epiphyses [18]. On the other hand, many studies have confirmed the presence of androgen receptors in bone cells and the ability of these cells to respond to androgens [20]. These findings have raised the following possibilities: first, testosterone might inhibit osteoclast formation induced by PTH through the conversion to estrogen by aromatase in bone cells. Second, testosterone might inhibit osteoclast formation induced by PTH through the androgen receptors in a manner independent of intrinsic estrogen in bone cells. We, therefore, employed a specific estrogen receptor antagonist, ICI 182780, and an aromatase inhibitor, 4-androsten-4-ol-3,17-dione, to examine whether testosterone would inhibit osteoclast formation induced by PTH through estrogen receptor or aromatase in mouse bone cell cultures. As shown in Fig. 2, 10^{-8} M 17β-E_2 significantly inhibited PTH-induced MNC formation and 10^{-8} M ICI 182780 completely antagonized the effects of 17β-E_2. However, 10^{-8} M ICI 182780 did not affect MNC formation antagonized by 10^{-8} M testosterone. These data indicated that testosterone inhibited osteoclast formation induced by PTH by the mechanism other than estrogen receptor. Moreover, 3 μM 4-androsten-4-ol-3,17-dione did not affect PTH-induced MNC formation antagonized by testosterone in mouse bone cell cultures (Fig. 2), indicating that testosterone inhibited osteoclast formation induced by PTH in a manner indepen-

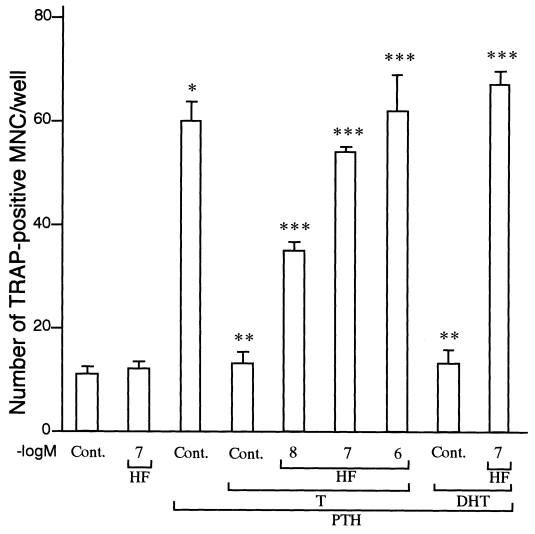

Fig. 3. Role of androgen receptor in the effects of testosterone on PTH-stimulated osteoclast formation. After degeneration of preexistent osteoclasts, 10^{-8} M hPTH-(1-34) (PTH), 10^{-8} M PTH and 10^{-8} M testosterone (T), and 10^{-8} M PTH and 10^{-9} M DHT were added with or without the indicated concentrations of hydroxyflutamide (HF), followed by 7 days of culture. Then, the number of TRAP-positive MNCs was counted, as described in Section 2. Data are expressed as the mean ± S.E.M. of four determinations. $*P < 0.01$, compared to control. $**P < 0.01$, compared to PTH-treated group. $***P < 0.01$, compared to PTH and T- or DHT-treated group.

dent of the conversion to estrogen by aromatase. The present findings indicated that testosterone inhibited osteoclast formation induced by PTH in a manner independent of the production of intrinsic estrogen in bone cells.

We, next, examined whether testosterone would inhibit osteoclast formation through androgen receptor in mouse bone cell cultures. We employed hydroxyflutamide, the androgen receptor antagonist, to clarify this issue. As shown in Fig. 3, hydroxyflutamide concentration-dependently antagonized the inhibitory effects of testosterone on PTH-induced MNC formation in mouse bone cell cultures, although hydroxyflutamide itself did not affect MNC formation. Moreover, 10^{-9} M DHT significantly inhibited MNC formation stimulated by PTH and 10^{-7} M hydroxyflutamide significantly antagonized the inhibitory effects of DHT. These findings indicated that testosterone inhibited osteoclast formation by an androgen receptor-mediated mechanism in mouse bone cell cultures. Androgen receptor exists on osteoclasts as well as on osteoblasts [8–10]. Mouse bone cell cultures employed in the present study include osteoblasts, osteoclast precursors and osteoclasts. We, therefore, could not clarify which cells are responsible for the inhibitory effects of testosterone on osteoclast formation in the present study. Further study is necessary to clarify these issues.

In conclusion, the present study first demonstrated that testosterone inhibited osteoclast formation induced by PTH through the androgen receptor, but not through the production of intrinsic estrogen in mouse bone cell cultures.

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