REVIEW

Androgen effects on bone metabolism: recent progress and controversies

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Abstract

Androgens have beneficial effects on skeletal development and maintenance in women and men. The detection and functional characterization of androgen receptors in bone cells has implicated bone tissue as a potential target tissue for androgens. Gonadal and adrenal androgens directly regulate various aspects of osteoblastic lineage cells, including proliferation, differentiation, mineralization, and gene expression. These effects may differ depending on the stage of differentiation, the number of androgen receptors, and other inherent characteristics (species, site, cell biology) of the osteoblastic cell system. In addition, recent studies have suggested that some of the anabolic and anti-resorptive effects of androgens on bone may be mediated by regulation of autocrine and paracrine factors in the bone microenvironment, including transforming growth factor- β , insulin-like growth factors (and their binding proteins), and interleukin-6. This review summarizes the recent progress made in our knowledge of androgen receptor action, local androgen metabolism in bone, and direct and indirect effects of gonadal and adrenal androgens as well as androgen receptor antagonists on bone cells.

European Journal of Endocrinology 140 271–286

Introduction

Sex steroid hormones have major beneficial effects on the development and maintenance of the skeleton (1, 2). These include control of growth plate maturation and closure during longitudinal bone growth, differential regulation of cortical and cancellous bone metabolism, stimulation of the acquisition of peak bone mass, and inhibition of bone loss (1–4). Both estrogen and androgens play important roles in skeletal metabolism. Androgen deficiency results in various abnormalities of bone metabolism, including a tall eunuchoid stature due to unfused growth plates and thinner (particularly cortical) bones as well as a lower peak bone mass and accelerated bone loss due to increased bone resorption, resulting in a higher fracture risk (1). While the clinical and biochemical effects of androgens (and androgen deficiency) on bone metabolism are well appreciated (1, 5–15), the cellular and molecular action(s) of androgens on bone cells have remained largely unclear.

The cloning of the human androgen receptor (AR) (16, 17) and its detection and characterization in various bone cells, including osteoblasts (18), bone marrow-derived stromal cells (19), osteocytes (20), hypertrophic chondrocytes (20), and osteoclasts (21), have unequivocally identified bone as a direct androgen target tissue. In addition, subsequent studies over the last decade have identified a variety of

androgen-dependent autocrine and paracrine cytokines which are produced by bone cells and mediate some of the anabolic and anti-resorptive effects of androgens.

This review summarizes the molecular and biochemical basis for androgen action on bone and the direct and indirect effects of various androgens on proliferation, differentiation, and gene expression of bone cells.

Molecular basis of androgen action – the AR

The AR, cloned a decade ago, is a member of the closely related steroid hormone–thyroid hormone–retinoic acid receptor superfamily (16, 17). Androgens, by virtue of their lipophilia, enter the plasma membrane and the nucleus of cells by diffusion and bind to the AR located in the peri- and intranuclear region of the cell. Subsequently, binding of androgen ligands to the AR induces a cascade of nuclear events, including a conformational change in the AR, dissociation from AR-associated proteins (e.g. heat-shock proteins), phosphorylation of the AR, and dimerization with either other steroid receptor monomers (heterodimerization) or AR monomers (homodimerization) (22) (Fig. 1). The activated ligand–receptor complex acts as a nuclear transcription factor which binds with its DNA-binding domain to *cis*-acting DNA sequences (androgen

Figure 1 Steroid hormone receptor: molecular mode of action. Following binding of the ligand to the ligand-binding domain of the steroid hormone receptor, heat shock proteins (HSP) detach from the receptor, and the receptor changes its conformation, undergoes phosphorylation, and forms receptor dimers. (A) Classical pathway: the activated steroid hormone receptor then binds with its DNA-binding domain to the steroid-responsive elements in the promoter region of target genes (gene A, e.g. TGF- β) to modulate their transcription. (B) Non-classical pathway: transcription factors (AP-1, NF- κ B, C/EPB β) regulate gene transcription by directly binding to the promoter region of target genes. Without binding to DNA, the activated steroid hormone receptor regulates gene expression (gene B, e.g. IL-6) by binding transcription factors to increase (in case of inhibitory factors) or decrease transcription (in case of stimulatory factors) of target genes.

response elements (ARE)) located in the promoter region of androgen-responsive genes to increase or to decrease their transcription (22) (Fig. 1A). Of note, the consensus half-site nucleotide sequence constituting the ARE (AGAACA) is identical to that of the glucocorticoid receptor, the mineralocorticoid receptor, and the progesterone receptor (23). The specificity of this consensus half-site sequence for the AR is determined by tissue-specific co-factors. This mode of action described above is referred to as the classical pathway of steroid hormone action (Fig. 1A) (24).

In addition, some studies also suggest a non-classical pathway for sex steroid hormones whereby the activated steroid hormone receptor (e.g. the estrogen receptor (ER)) regulates gene expression by interacting with transcription factors (AP-1, NF- κ B, C/EPB β) without binding to DNA (25, 26). Transcriptional interference with AP-1 or NF- κ B, which is mediated through competition for intracellular transcriptional coactivators, may serve as an integrator for AR-mediated signaling (Fig. 1B) (27).

A recent study demonstrated the existence of two forms of the AR, AR A (87 kDa) and AR B (110 kDa) (28). In most tissues, AR B is fourfold more abundant than AR A. The functional activities of AR A and B appear to be comparable, although there are some subtle differences between AR A and B, depending on the promoter and cell-specific context of target genes (28). During embryogenesis, AR are expressed in a spatially and temporally distinct fashion in a variety of reproductive and non-reproductive tissues, including external and internal genitalia, mammary gland, adrenal glands, kidneys, muscles, pituitary gland, hypothalamus, and larynx (29).

Bone as direct androgen target – detection of the AR in bone cells

The expression of AR in bone was first described by Colvard *et al.* (18) in primary human osteoblasts using a nuclear binding assay which only detects functional AR

that bind the ligand and translocate to the cell nucleus to bind DNA (30). These findings have since been confirmed by other groups using a variety of osteoblastic cell systems from various species (31–36). Functional AR were also detected in pluripotent marrow-derived stromal cells (19), which represent osteoblast precursor cells, hypertrophic chondrocytes (20), osteocytes (20) as well as osteoclasts (21). The detection of functional AR in various bone cells has implicated bone as a target tissue for androgen action and has fueled an increase in further investigations on the direct and indirect effects of androgens on bone cells *in vitro*, as well as the sequelae of clinical and experimental androgen deficiency and its correction on bone metabolism *in vivo*.

As summarized in Table 1, a variety of osteoblastic cell models has been used to assess androgen action *in vitro*. The interpretation of results derived from primary osteoblastic systems has to take into account the variability due to differences in the age of the donor (fetal, neonatal, adolescent, adult), the skeletal site (vertebral, femoral, calvarial) (37, 38), and the predominant type (cancellous, cortical) of the bone fragments (37, 39). Other variables include species and ontogenic differences of bone development and metabolism, the origin and culture technique (spontaneously transformed osteosarcoma cell lines, conditionally immortalized cell lines, primary non-transformed osteoblasts) (40), and, perhaps most importantly, the stage of differentiation of the osteoblastic system (percentage of AP-positive cells, degree of heterogeneity, contamination by non-osteoblastic cell populations) (3, 37, 39–41).

Moreover, the reported number of AR/cell varies greatly depending on the variables mentioned above (41) and the method used for detection (nuclear,

cytosol, or whole cell-binding assay) (42). Most studies (see Table 1) have been performed in osteoblastic cell systems which have 500 to 6000 AR/cell (18, 31, 33, 34, 37, 40, 43, 44), which is in the physiological range as present in other androgen target organs, including the prostate (34), while some studies report less than 500 AR/cell (19, 32, 34). Osteoblasts with less than 200 AR/cell appear to lack androgen responsiveness (43, 44). However, one study failed to detect androgen responsiveness, as assessed by expression of androgen-responsive genes and an ARE reporter construct in three osteosarcoma cell lines (SaOS-2, TE85, and U-2 OS), although these cells have 3600 to 5800 AR/cell (40).

Various steroid hormones may modulate the number of AR expressed by osteoblasts. 5α -Dihydrotestosterone (5α -DHT) has been demonstrated to up-regulate AR expression (35) and transcriptional activation in osteoblastic cells (45). In addition, dexamethasone, 1,25-dihydroxyvitamin D₃, and 17 β -estradiol have also been demonstrated to increase AR mRNA steady state levels (37).

A detailed *in situ* study localized AR to various cells within bone, including hypertrophic chondrocytes, osteoblasts, osteocytes, and bone marrow mononuclear cells (20). In the growth plate of adolescent boys and girls, AR were mainly localized to hypertrophic chondrocytes, while in adult bone, AR were mainly expressed by cells in the vicinity of active bone remodeling (20). Heterogeneous distribution of AR within the skeleton with high expression in cortical and intramembranous bone and lower expression in cancellous bone may, in part, explain regional differences in androgen action on bone, and contribute to the development of sexual

Table 1 Number of AR/cell in various osteoblastic cell systems. Data are expressed as the mean $\pm$ S.E.M. when applicable based on various binding assays (see text and references for details).

Cells	Characteristics	n (AR)/cell	Reference no.
hOB	Human primary osteoblasts; femoral head (F, M)	146 $\pm$ 19	32
hOB	Human primary osteoblasts; femoral head (F, M)	5520 $\pm$ 240	37
hOB	Human primary osteoblasts; femoral head (F, M)	821 $\pm$ 140	18
hFOB	Human fetal immortalized osteoblastic line	152 $\pm$ 73	43
hFOB/AR-6	Derived from hFOB transfected with the AR	3987 $\pm$ 823	43
TE-85	Human osteosarcoma cell line	2800	31
		3600	40
U2-OS	Human osteosarcoma cell line	1605	34
		3600	40
SaOS-2	Human osteosarcoma cell line	1277 $\pm$ 239	34
		5800	40
+/+LDA11	Murine clonal stromal cell line	336 $\pm$ 46	19
MC3T3-E1	Murine clonal calvaria preosteoblastic cell line	14 312 $\pm$ 1884	100
		1150 $\pm$ 105	33
UMR-1060	Rat osteosarcoma cell line	74 $\pm$ 10	34

hOB, primary human osteoblastic cells; hFOB, conditionally immortalized human fetal osteoblastic cell line; hFOB/AR-6, subclone of the hFOB cells transfected with the human AR gene; F, female; M, male.

dimorphism in the skeletal phenotype between women and men (37).

Local androgen and estrogen metabolism in bone cells

In men, androgens are produced by both the testes and the adrenal glands. The major gonadal androgen is testosterone, which is secreted and circulates largely bound to albumin and sex-hormone binding globulin to reach peripheral tissues, where it is converted by the enzyme, 5 α -reductase, to the more potent 5 α -DHT (Fig. 2). Dehydroepiandrosterone (DHEA) and androstenedione are the major circulating adrenal androgens in both women and men (2). DHEA is metabolized by the enzyme, steroid sulfotransferase, to DHEA sulfate (DHEA-S) in peripheral tissues. In addition, testosterone, DHEA and androstenedione, but not 5 α -DHT, can be metabolized either directly or indirectly to estrogens by the microsomal P450 enzyme aromatase (Fig. 2). 5 α -Reductase (33, 46, 47), steroid sulfotransferase (47, 48), and aromatase (33, 46, 49) as well as 17 β -hydroxysteroid dehydrogenase (47, 50, 51) and 3 β -hydroxysteroid dehydrogenase (51) have been detected in osteosarcoma cell lines and primary osteoblastic cells. The presence of these enzymes indicates the ability of the bone microenvironment to locally form biologically potent estrogens and androgens from weaker circulating sex steroid hormones (Fig. 2). Of note, 1,25-dihydroxyvitamin D₃ stimulates and dexamethasone inhibits the activity of some of these enzymes (50).

As evident from the sex steroid hormone metabolism pathways described in Fig. 2, only the non-aromatizable androgen, 5 α -DHT, exclusively activates the AR. In contrast, testosterone, DHEA, DHEA-S, and androstenedione can be metabolized to either estrogens or to 5 α -DHT,

and biological effects derived from studies with these compounds *in vitro* or *in vivo* may thus result from activation of the AR or the ER, or both. To highlight this, we will explicitly indicate which particular androgen was used in the various studies.

There is now compelling evidence that aromatization of androgens into estrogens at the local level is crucial for maintaining normal bone remodeling in males. Thus, the administration of the non-steroidal aromatase inhibitor, vorozole, to mature (52) and growing (53) male rats decreased bone density, increased biochemical markers of bone resorption, and inhibited periosteal bone formation and endosteal bone resorption similar to orchiectomy (52, 53). In addition, treatment with 17 β -estradiol, but not testosterone, improved bone mineral density (BMD) and reduced bone turnover markers in males with inherited aromatase deficiency due to homozygous mutations in the aromatase gene (54–56). Interestingly, the features of abnormal bone metabolism in aromatase-deficient males are similar to those of a male with a homozygous mutation of the ER- α gene (57) and mice with targeted ablation of the ER- α gene (58).

In vivo effects of androgens on bone metabolism

The direct effects of androgens on bone metabolism *in vivo* have been extensively assessed in animal studies (largely rodents) (59–65) and clinical or epidemiological studies (5, 7, 8, 11–15, 66–88). The endpoints of these studies were BMD (5, 7, 8, 11–13, 59, 64–66, 68–80), static or dynamic histomorphometry (7, 59–62, 64, 65, 67, 68, 87, 88), measurement of biochemical markers of bone formation and bone resorption (5, 12, 65, 66, 69, 81–88), or biomechanical properties of bone (63). The

Figure 2 Androgen metabolism in bone cells: major biochemical pathways and enzymes involved in sex steroidogenesis in osteoblastic cells (1 = 17,20-desmolase; 2 = 17 β -hydroxysteroid dehydrogenase; 3 = 3 β -hydroxysteroid dehydrogenase; 4 = steroid sulfotransferase; 5 = 5 α -reductase; 6 = P450-aromatase).

majority of studies were performed either in spontaneous or orchiectomy-induced hypogonadism with or without subsequent androgen replacement therapy, or androgen treatment of eugonadal male or female subjects.

Important issues that have to be considered are the fundamental differences of bone metabolism between humans and rodents, differences between the growing and the mature skeleton, differential effects of androgens depending on the site (metaphyseal vs epiphyseal) and the predominant type (cancellous vs cortical) of bone, and the duration of androgen treatment (2–4). In addition, as noted earlier, androgens other than 5 α -DHT or synthetic androgens may be converted to estrogens.

Effects of androgens on BMD

There are extensive data indicating that severe male hypogonadism (as found in patients with hypothalamic dysfunction, hyperprolactinemia, anorexia nervosa, Klinefelter's syndrome, and castration) is associated with a low BMD and increased risk of fracture (89). Consequently, most clinical studies examining the effects of testosterone replacement on BMD have been performed in relatively young men with overt hypogonadism (1–4, 7, 8, 11, 12, 70, 71). In these selected populations of hypogonadal men, testosterone replacement is associated with significant increases in BMD. In a study of 21 adult males with hypogonadotropic hypogonadism studied before and after restoration of gonadal status, Finkelstein *et al.* (70) found that in men with open epiphyses, cortical and trabecular BMD increased nearly 13% over 2 years, whereas in men with fused epiphyses, cortical BMD increased only by 4% and trabecular BMD did not change. Similarly, Devogelaer *et al.* (71) studied 16 male hypogonadal patients before and following testosterone replacement and found a significant increase (6%) in distal radius BMD. Consistent with this, Katznelson *et al.* (12) demonstrated that especially spinal BMD was reduced in patients with acquired hypogonadism (–10%), and correction with testosterone for 12 months increased spinal BMD (by 5%) and trabecular BMD (by 14%) respectively. Behre *et al.* (8) observed a BMD increase of 26% after long-term administration of testosterone to previously untreated hypogonadal men with the highest percentage of increase occurring within the first year of therapy. In summary, overt androgen deficiency is associated with a low BMD, which is rapidly increased by subsequent androgen replacement therapy.

However, the relationship between smaller reductions in testosterone levels and osteoporosis in aging males is less clear. Aging men have an annual vertebral bone loss of up to 2% (73–75) and an annual cortical bone loss in the order of 1% (76). However, while several studies have noted significant correlations between either total or free testosterone levels in aging men

and BMD at various sites (13, 77, 78), others have failed to confirm this relationship (79, 80). Of interest, a recent report of an open, prospective trial of intramuscular testosterone treatment of eugonadal men with osteoporosis found that spine BMD increased in these men by 5% over 6 months, although hip BMD did not change (5, 72).

Animal studies have provided important insights into the effects of androgens on BMD. Mature male rats had a decreased BMD as early as 2 weeks after orchiectomy (59). Intact osteoblastogenesis appears to be a prerequisite for orchiectomy-induced bone loss, since mice with defective osteoblastogenesis failed to lose bone following orchiectomy (64). Finally, a recent study in female rats has demonstrated that the adrenal androgen, DHEA, contributes to the maintenance of BMD in estrogen deficiency (65). While DHEA treatment of ovariectomized rats increased BMD to 8% above that of normal animals, co-treatment with flutamide (an AR antagonist) abrogated 76% to 100% of these stimulatory effects at various skeletal sites, suggesting that these effects were specifically mediated by the AR (65).

Effects of androgens on biochemical markers of turnover

Profound hypogonadism is associated with increased bone resorption (81, 82). Tenover (83) showed that 3 months of testosterone treatment of 13 healthy men (aged 57 to 76 years) with borderline serum testosterone levels (<400 ng/dl) resulted in a 28% reduction in urinary hydroxyproline excretion. In the study of eugonadal men with osteoporosis treated with 6 months of intramuscular testosterone (84), androgen supplementation was associated with a significant reduction in bone resorption markers, urinary deoxypyridinoline (–19%) and N-telopeptide of type I collagen (–39%). Similarly, correction of acquired hypogonadism with testosterone for 12 months decreased urinary deoxypyridinoline excretion by 19% (12). In addition, treatment of ovariectomized rats with DHEA decreased the urinary excretion of hydroxyproline by 50%, which was partially reversed by co-treatment with flutamide (65). Thus, these studies suggest a significant anti-resorptive effect of androgens on biochemical markers of bone turnover.

In addition to inhibiting bone resorption, testosterone treatment may also stimulate bone formation, although the data are somewhat conflicting in this regard (12, 66, 69, 83–88). Bone loss in men after age 50 is associated with decreased bone formation as assessed by the biochemical markers bone-specific alkaline phosphatase, osteocalcin, and procollagen I C-terminal peptide (69). Raisz and associates (66) found that postmenopausal women treated with estrogen plus 2.5 mg methyltestosterone had a 24% higher serum osteocalcin level after 3 months of treatment, as compared with a 40% lower osteocalcin level in the

women treated with estrogen alone. Similarly, the administration of synthetic androgens in postmenopausal women increased markers of bone formation, at least over the short term (3 to 6 months) (86), and 3 months of testosterone therapy in elderly hypogonadal men resulted in a significant increase in serum osteocalcin levels (+45%) (88). In support of this, Martel *et al.* (65) reported an increase of serum alkaline phosphatase by fourfold in ovariectomized rats following DHEA which was reversed by flutamide co-treatment. However, long-term studies with testosterone supplementation reported decreased biochemical markers of bone formation (12, 84). Thus, short-term testosterone treatment appears to have a positive effect on bone formation.

Effects of androgens on bone histomorphometrical parameters

An extensive bone histomorphometric study in 43 healthy men reported decreases in cancellous bone volume, osteoblast–osteoid interface, and double and single labeled osteoid with age, suggesting impaired osteoblast function (68). By contrast, osteoclastic parameters were normal. In a multiple regression analysis, the log free androgen index (serum-free testosterone/sex-hormone binding globulin) best predicted the age-related reduction of iliac crest cancellous bone volume in these men (68). Serial bone biopsies in a hypogonadal man before and after 6 months of testosterone treatment revealed increases in relative osteoid volume, total osteoid surface, linear extent of bone formation, and bone mineralization following therapy (7).

As is evident from animal studies, androgen deficiency may have differential effects on cortical and cancellous bone. Orchiectomy in mature rats rapidly (within 2 weeks) decreased cancellous bone volume by 13 to 19%, while osteoblast and osteoclast surfaces and the numbers of osteoclasts increased (59). Increased cancellous bone formation was due to an increase in mineralizing surfaces and mineral apposition rate. By contrast, orchiectomy was associated with a decrease in cortical bone formation rate and mineralizing surfaces (59). These data suggest that short-term androgen deficiency leads to a stimulation of cancellous and a decrease in cortical bone turnover.

Several histomorphometric studies have assessed the potential skeletal benefits of androgen treatment in estrogen deficiency (60–62, 65, 87). Bone biopsies in 29 women with postmenopausal osteoporosis before and after oxandrolone (a synthetic androgen) treatment revealed a decrease in both resorption and formation surfaces (87). In animal experiments, treatment with 5 α -DHT partially prevented suppressed cancellous bone formation in ovariectomized rats (60). Of interest, while the AR antagonist, flutamide, had no effect on histomorphometrical parameters in ovariectomized rats, it suppressed cancellous bone formation in sham-operated rats (60). In addition, the adrenal androgen,

androstenedione, dose-dependently reduced the loss of cancellous bone in ovariectomized rats, an effect that was not blocked by the co-administration of the aromatase inhibitor, arimidex, suggesting direct mediation of these effects by the AR (61). Moreover, treatment of ovariectomized rats with DHEA stimulated trabecular bone mineral content (compared with sham-operated and ovariectomized controls) at the spine and the femur, and partially inhibited decreased trabecular volume and number and increased trabecular separation. Co-administration of flutamide abrogated most of these beneficial effects of DHEA (65).

Long-term treatment (over 2 years) with supraphysiologic levels of testosterone of female monkeys confirmed the positive effects of androgens on cancellous bone while emphasizing a potential negative effect on cortical bone (62). In treated monkeys, cancellous bone of the vertebrae had an increased cancellous bone area, a decreased osteoid perimeter, a decreased eroded perimeter, and a better mineralization profile, suggesting a decreased bone turnover (62). In contrast, cortical bone of the testosterone group revealed an increased intracortical remodeling activity with increased porosity, osteonal bone, and mean wall width compared with the control group, suggesting increased cortical bone remodeling (62). Taken together, androgens may have significant positive effects on bone histomorphometry parameters in both females and males, with a combined increase in bone formation and decrease in bone resorption mainly in cancellous bone.

Effects of androgens on the biomechanical properties of bone

Testosterone treatment of adult female monkeys for 2 years improved a variety of biomechanical properties of bone (63). Cortical bone of testosterone-treated monkeys had a higher (compared with untreated controls) energy absorption capacity (+45%), maximum shear stress (+39%), torsional rigidity (+23%), and bending stiffness (+15%) at the tibiae (63). In addition, trabecular bone of treated animals had a higher elastic modulus (+107%) and a higher compressive stress value (+28%).

Extraskelatal effects of androgens on calcium metabolism

There are several lines of evidence suggesting that androgens may also have indirect effects on calcium homeostasis by regulating the intestinal absorption and renal handling of calcium. An *in vivo* study reported that duodenal active calcium transport decreased in male rats following orchiectomy which was reversed by testosterone treatment (90). In early studies, Lafferty *et al.* (91) found that calcium absorption transiently increased following short-term treatment (2 to 3 months) with testosterone. Consistent with this, a significant

increase in the hourly fractional rate of radiocalcium absorption was found in 27 women with postmenopausal osteoporosis following 3 months of therapy with the anabolic steroid, nandrolone decanoate (92). However, since androgens may affect serum levels of vitamin D-binding protein, thus altering the levels of free 1,25-dihydroxyvitamin D₃ (93), it remains unclear whether androgens might exert their effects on intestinal calcium absorption directly or indirectly through effects on the vitamin D-endocrine system.

Some studies also suggest that androgens may affect renal calcium transport. The AR gene is expressed in kidney epithelial cells (94) and androgens have been shown to modulate calcium fluxes in mouse kidney cortex preparations (95). Reifstein & Albright (96) initially noted that testosterone propionate decreased the urinary excretion of calcium. Several studies using synthetic androgens have suggested a positive effect of androgens on renal calcium handling. Therapy with the anabolic steroid, stanozolol, for 8 to 32 months in 23 women with postmenopausal osteoporosis decreased urinary calcium excretion by 32% (97). In addition, treatment with nandrolone decanoate for 3 months significantly increased renal tubular calcium reabsorption in 27 women with postmenopausal osteoporosis (92).

Direct effects of androgens on bone cells *in vitro*

Although AR have been detected in chondrocytes, stromal cells, osteoblasts, osteocytes, and osteoclasts, detailed studies on direct effects of androgens are limited to osteoblastic lineage cells (stromal cells and mature osteoblasts) (2), which are considered the effector cells for androgen action on bone remodeling and BMD (64).

Effects of androgens on osteoblast proliferation

The effects of androgens on preosteoblastic and osteoblastic systems are summarized in Table 2. Kasperk *et al.* (98) were the first to report an increase in osteoblast proliferation following treatment with 5 α -DHT as assessed by [³H]thymidine incorporation and cell number (98). Growth stimulation was similar for various osteoblastic cells (primary mouse calvaria cells, primary human osteoblasts, human osteosarcoma cell line, TE-89) and could be prevented by administration of the competitive AR antagonists, hydroxyflutamide and cyproterone acetate (98). Subsequent studies have confirmed these results in various primary and transformed osteoblastic cell lines from different species (33, 99–102) (see Table 2). Of note, one study demonstrated that DHEA and 5 α -DHT were qualitatively similar in stimulating proliferation and DNA synthesis of primary human osteoblastic cells, although DHEA was less potent, possibly due to a lower affinity for the AR (99).

However, in two of these studies (33, 100), 5 α -DHT and testosterone only marginally increased proliferation of MC3T3-E1, a murine preosteoblastic cell line, by 10%–15% despite the presence of up to 14 000 AR/cell (100), and androgens were generally less mitogenic than estrogens (33, 100). As noted earlier, these effects may be related to the immature, partially committed stage of differentiation of this cell system.

In contrast, two studies have reported growth inhibition of osteoblasts following treatment with 5 α -DHT (31, 44). In these studies, which used the human osteosarcoma cell line, TE-85 (~2800 AR/cell) (31), and hFOB/AR-6 cells, a fetal human osteoblastic cell line (~4000 AR/cell) (43), 5 α -DHT inhibited cell proliferation by 20% to 35% (31, 44) (Table 2). Growth inhibition by 5 α -DHT in the hFOB/AR-6 cells

Table 2 Effects of androgens on osteoblast proliferation. For a detailed characterization of the osteoblastic cells see Table 1.

Cells	Androgen(s)	Effect	Reference no.
Primary human osteoblasts	DHT	↑ 50%	98
Primary mouse calvaria cells	DHT	↑ 100%	98
Primary human osteoblasts	DHT	↑ 200%	99
	DHEA	↑ 88%	99
MC3T3-E1 cells	DHT	↑ 15%	100
MC3T3-E1 cells	DHT	↑ 57%	33
	T	↑ 39%	33
Primary rat diaphyseal osteoblasts	T	↑ 100%	101
Primary rat epiphyseal cells	T	↑ 57%	101
Primary rat calvarial osteoblasts	DHT	↑ 150%	102
	T	↑ 70%	102
Osteosarcoma cell line TE-85	DHT	↓ 25%	31
	T	↓ 20%	31
hFOB/AR-6 cell line	DHT	↓ 30%	44

T, testosterone; ↑ increase in per cent over control, ↓ decrease in per cent over control.

is similar to the effects of 17 β -estradiol on the hFOB/ER-9 cells (103), which represent the ER counterpart cell line (104). These findings are also consistent with two other reports in the breast cancer cell line, MCF7 (105), and the prostate carcinoma cell line, PC-3 (106), where activation of the transfected AR in cell lines that had previously been androgen-insensitive inhibited proliferation.

The effects of androgens on osteoblast proliferation appear to be modulated by other osteotropic steroid hormones. For instance, while 5 α -DHT and testosterone stimulated proliferation of neonatal rat calvarial osteoblastic cells, co-treatment with 1,25-dihydroxyvitamin D₃ blunted these mitogenic effects (102). Androgen responsiveness of osteoblastic cell cultures may also be modulated by prior prenatal exposure of the donor animal to sex steroid hormones (107) and its nutritional vitamin D status (101).

Effects of androgens on osteoblast differentiation

The osteoblastic phenotype is defined by the ability to express the ectoenzyme alkaline phosphatase (AP), to synthesize various extracellular matrix proteins, including type I collagen (which is the major extracellular matrix protein), osteocalcin, and osteonectin, and to form mineralized matrix when cultured under appropriate conditions.

The regulation of these osteoblastic differentiation markers by androgens is complex and controversial, as reviewed in Table 3. The first evidence that androgens stimulated the differentiation of osteoblasts was the observation that 5 α -DHT specifically increased the percentage of AP-positive cells of various osteoblastic cells (primary mouse neonatal calvaria cells, primary human adult osteoblasts, and the osteosarcoma cell line, TE-89) (98). However, the significance of this

observation was dampened by the fact that the percentage of AP-positive cells was suboptimal (and therefore not all of the cells were truly mature osteoblasts) and that AP-negative cells could potentially represent non-osteoblastic cell populations derived from the preparation procedure. Subsequently, AP activity of osteoblasts was shown to be increased (99), not affected (35, 102), or decreased (44) by various androgens. DHEA and 5 α -DHT were similar in stimulating both the percentage of AP-positive cells and AP activity by primary human osteoblastic cells, without prior conversion of DHEA and its metabolites by 3 β -hydroxysteroid dehydrogenase or 5 α -reductase (99).

Data on androgen regulation of secreted osteoblast-specific markers are similarly ambiguous. While some authors have reported induction of type I collagen mRNA levels (31) or synthesis (102) by 5 α -DHT, others have found no effects (33, 108, 109). Using the hFOB/AR-6 cell line, 5 α -DHT inhibited both baseline and 1,25-dihydroxyvitamin D₃-induced type I collagen synthesis in a dose-dependent manner (44). Osteocalcin secretion has been reported to be stimulated (99) or not affected (35, 44) by androgens.

Finally, two studies suggest that androgens may exert their anabolic effect by stimulating bone mineralization. In TE-85 osteosarcoma cells, 5 α -DHT increased mineralization of extracellular bone matrix as assessed by calcium incorporation in a time- and dose-dependent fashion (35). Consistent with this, Kasperk *et al.* (99) demonstrated that both 5 α -DHT and DHEA stimulated mineralization of extracellular matrix by primary human osteoblastic cells.

Indirect effects of androgens in bone cells – the role of cytokines and growth factors

The role of paracrine and autocrine factors in mediating estrogen action on bone has been extensively studied.

Table 3 Effects of androgens on osteoblast differentiation markers. For a detailed characterization of osteoblastic cell models see Table 1.

Marker of osteoblast differentiation	Androgen(s)	Change	Reference no.
Alkaline phosphatase (AP)			
Percentage of AP-positive cells	DHT	↑	98
AP activity	DHT, DHEA	↑	99
	DHT, T	→	35, 102
	DHT, T	↓	44
Type I collagen			
mRNA levels	DHT, T	↑	31
		→	33
Protein secretion	DHT, T	↓	44
[³ H]proline incorporation	DHT	↑	102
	DHT	→	108, 109
Osteocalcin secretion	DHT, T, DHEA	↑	99
	DHT, T, DHEA	→	35, 44
Mineralization of extracellular bone matrix	DHT, DHEA	↑	35, 99

T, testosterone; ↑ increase, → no change, ↓ decrease.

Table 4 Paracrine mediators of androgen actions on bone cells.

Cytokine	Androgen(s)	Change	Method	Reference no.
TGF- β	DHT, T	↑	mRNA levels	31, 126
	DHT, T, DHEA	↑	Activity	127
	DHT	↓	mRNA levels	44
TIEG	DHT	↓	mRNA levels	44
IGF-I	DHT, T	↑	mRNA levels	136
IGF-IIR	DHT	↑	mRNA levels	126
		↑	Affinity for IGF-2	137
IGFBP-3	DHT	↑	mRNA levels, Western ligand blot	136
IGFBP-4	DHT	↓	mRNA levels, Western ligand blot	136
IL-1 β	T	↑	mRNA levels, protein production	146
IL-6	DHT, T, DHEA	↓	Reporter construct	19
PGE ₂	DHT, T	↓	mRNA levels, protein production	143
	DHT, T	↓	Protein production	109

T, testosterone: ↑ increase, → no change, ↓ decrease.

Thus, in addition to various direct effects on bone, kidneys, and the gut (110, 111), estrogen modulates the expression and activity of various cytokines and growth factors that are produced locally in bone (112–114). Potential mediators of the effects of estrogen on bone include interleukin (IL)-1 β (115), IL-6 (116, 117), tumor necrosis factor- α (118), macrophage colony-stimulating factor (119), and prostaglandin (PG) E₂ (120), the synthesis of which is suppressed by estrogen, as well as IL-1 receptor antagonist (121), transforming growth factor- β (TGF- β) (122), insulin-like growth factor-binding protein (IGFBP)-4 (123), and bone morphogenetic protein-6 (124), which are stimulated by estrogen. In an analogous fashion some of these factors have also been implicated as mediators of the effects of androgens on bone, as summarized in Table 4.

TGF- β

Bone represents the body's largest reservoir for TGF- β , which is stored largely bound to its binding protein, TGF- β latency-associated peptide. TGF- β is a potent mitogen for osteoblastic cells and inhibits bone resorption by suppressing osteoclast recruitment and activity (125). Estrogen is known to stimulate both *de novo* synthesis of TGF- β and activation of latent TGF- β (110, 122, 125). Several groups have demonstrated that androgens (5 α -DHT, testosterone, and DHEA) increase TGF- β gene expression (31, 126) and activity (127), suggesting that at least part of the positive effects of androgens may be mediated by an osteoblast-derived increase in TGF- β production and activity. In support of this hypothesis are the findings of an *in vivo* study conducted on male rats (128). Orchiectomy resulted in an 80% reduction of the skeletal concentrations of all three TGF- β isoforms, whereas concomitant treatment with testosterone was capable of preventing it (128). One study, however, demonstrated decreased TGF- β and

TGF- β -induced early gene (TIEG) mRNA levels in osteoblasts following treatment with 5 α -DHT (44). TIEG has been recognized as a transcription factor, that mediates some effects of TGF- β (129, 130). Of interest, the decrease in TGF- β and TIEG mRNA levels paralleled growth inhibition of 5 α -DHT in this cell line (44), indicating that TGF- β may indeed mediate the effects of androgens on osteoblast proliferation (44, 126).

Insulin-like growth factors (IGFs)

Other candidates for mediating androgen effects on bone are the closely related IGF-I and -II which, together with their receptors, IGF-IR and IGF-IIR, and their six binding proteins, IGFBP-1 to -6, form a complex autocrine/paracrine system (131, 132). Some components of the IGF system are abundantly expressed in human bone (IGF-II, IGFBPs), whereas others (IGF-I) are less abundant (131). IGF-I and IGF-II are both potent mitogenic peptide growth factors which also enhance the differentiation of various mesenchymal cells, including osteoblasts (131, 133, 134). Rodent bone cells differ from human bone cells in the high abundance of IGF-I and an altered IGFBP profile (131).

While some studies have found that the mRNA levels of IGF-I and IGF-II by osteoblastic (126, 135) and pre-osteoblastic cells (33) were not affected by androgen treatment, Gori *et al.* (136) have demonstrated that, in the hFOB/AR-6 cell line, IGF-I mRNA levels were up-regulated sixfold by 5 α -DHT. Thus, at least part of the anabolic effect of androgens on bone may be due to increased IGF-I production by osteoblasts (133, 134).

Modulation of the response of target cells to IGFs by interacting with the IGF receptors represents an additional mechanism by which androgens may modulate the IGF system. Kasperk *et al.* (126, 137) have reported that 5 α -DHT enhanced the mitogenic effects of

IGF-II by increasing the number of IGF-IIR and its affinity for IGF-II.

Finally, all six IGFBPs have been detected in osteoblastic cells and have been found to be differentially regulated by systemic and local factors involved in bone metabolism (131). In the hFOB/AR-6 cell line, 5 α -DHT increased the stimulatory IGFBP-3 (at both the mRNA and protein level), and decreased IGFBP-4, which acts as a protease and is inhibitory for IGF-I action (136). These changes occurred in a time- and dose-dependent fashion, were specific for androgens, were absent in a negative control cell line (hFOB) (138), and were detected both at the mRNA and protein levels (136). Since estrogen increased the production of IGFBP-4 in an estrogen-responsive cell line (123), differential regulation of IGFBP-4 may indicate a fundamental difference between the actions of estrogen and androgens on the IGF system in osteoblasts. In summary, the stimulation of IGF production and the shift of the IGFBP profile towards a more 'stimulatory' IGFBP pattern by androgens is consistent with an anabolic effect of androgens on bone. Conversely, decreased serum IGF-I concentrations (139) and an 'unfavorable' IGFBP profile (140) have been implicated in some forms of male osteoporosis.

IL-6

IL-6 represents a pro-inflammatory cytokine which is produced in the bone microenvironment by osteoblasts and marrow stromal cells (112). IL-6 promotes osteoclastogenesis and increases bone resorption (116). The production of IL-6 is up-regulated by pro-inflammatory cytokines that are stimulated by estrogen deficiency (112, 113), its expression is suppressed by estrogen (116, 141), and intact IL-6 action is a prerequisite for estrogen deficiency-induced bone loss as demonstrated by the use of IL-6 knock-out mice (142) and the administration of neutralizing antibodies against IL-6 to normal mice (116).

Two studies have demonstrated that androgens may act on IL-6 production by osteoblastic lineage cells in an analogous fashion (19, 143). The first study using a murine marrow stromal cell line, which spontaneously expresses 336 ± 46 AR/cell, demonstrated suppression of an IL-6 promoter reporter construct by 5 α -DHT and testosterone by 80% to 90% (19). The second study employing the hFOB/AR-6 cells, which express ~4000 AR/cell, demonstrated inhibition of IL-6 mRNA and protein production by 70%–80% following treatment with 5 α -DHT and testosterone, but not DHEA (143). The findings that IL-6 is markedly suppressed by gonadal androgens (19, 143) by transcriptional control (19), and that androgens suppress both constitutive and cytokine-stimulated IL-6 production (143) suggest that suppression of IL-6 by androgens constitutes a physiologically relevant response in osteoblastic lineage cells. Moreover, the effects of androgens on IL-6 action might

be further enhanced by decreased production of the two IL-6 receptor subunits, gp80 and gp130, following treatment with 5 α -DHT, whereas sex hormone deficiency up-regulates these receptor subunits (144).

In further support of IL-6 as an anti-resorptive mediator of androgen action, two *in vivo* studies demonstrated that orchiectomy stimulated IL-6 secretion by bone marrow cells by 40% (145) and increased the number of osteoclast progenitor cells by two- to threefold (19). Moreover, the increase in osteoclast progenitor cells could be prevented by testosterone replacement therapy and/or by IL-6 neutralizing antibody. In addition, bone loss following orchiectomy did not occur in IL-6 knock-out mice (19). These data support the hypothesis that, at least in part, inhibition of IL-6 production by androgens in osteoblastic lineage cells accounts for the anti-resorptive effects of androgens on bone.

Other factors

Other androgen-responsive candidate cytokines that are produced by osteoblasts include PGE₂ and IL-1 β , both of which may play a role in osteoblast–osteoclast cross-talk, since both are potent stimulators of osteoclastogenesis and osteoclast activity. IL-1- or parathyroid hormone-stimulated PGE₂ production in mouse calvarial cells was suppressed by 5 α -DHT and testosterone by 50% to 70% (109). Finally, in the human immortalized HOBIT cell line, which is derived from primary adult trabecular osteoblasts, testosterone increased IL-1 β mRNA steady-state levels and protein release to an extent similar to 17 β -estradiol (146). These results are in contrast to the fact that IL-1 β represents one of the most potent inducers of IL-6, and that both androgens (19, 143) and estrogen (114, 116) suppress IL-6 production.

Recent studies have also demonstrated altered B cell development with an expanded number of bone marrow B cells in male mice following orchiectomy which was reversible by androgen replacement therapy. This raises the possibility that an altered cytokine pattern by immune cells induced by androgen deficiency may interact with marrow stromal cells, osteoblasts, and osteoclasts in the bone microenvironment (147). The significance of these findings on bone metabolism, however, remains to be defined.

Effects of DHEA on bone metabolism

The adrenal androgen, DHEA, is the most abundant androgen in both women and men (148, 149). The decrease of DHEA serum concentrations with aging (by 10% per decade) has been associated with various age- and aging-dependent diseases, including diabetes mellitus, obesity, cardiovascular diseases, and osteoporosis (150). DHEA has since earned a reputation as a 'fountain of youth' (151) to treat these various

ailments. While there are many animal studies using DHEA (148–150), these need to be interpreted with caution since animals, in general, lack an age-dependent decline in serum DHEA concentrations, and therefore treatment with DHEA essentially represents a pharmacological intervention rather than a physiological replacement therapy (150). A further problem with studies of DHEA is that it can be metabolized to estrogens and, thus, biological effects evoked by DHEA may result from activation of the AR or the ER (148, 149).

Specific DHEA-binding sites have been reported in T cells (152), although this has not been confirmed for bone cells. Nevertheless, DHEA has been demonstrated to stimulate both proliferation and differentiation of primary human osteoblastic cells in a manner similar to the gonadal androgens, testosterone and 5 α -DHT (99). DHEA also stimulated TGF- β activity by primary human osteoblastic cells in a manner similar to testosterone and 5 α -DHT (127) but, in contrast to testosterone and 5 α -DHT, inhibited steady-state mRNA levels of the proto-oncogene *c-fos* (127), suggesting a different mechanism of transcriptional activation of genes. In the hFOB/AR-6 cell line, however, DHEA, unlike 5 α -DHT, failed to activate an androgen-responsive element reporter construct (43), to inhibit proliferation (44), to modulate the IGF/IGFBP system (136), or to suppress cytokine-stimulated IL-6 production (143).

A recent uncontrolled clinical study conducted on 14 postmenopausal women reported promising results of DHEA on bone metabolism in the absence of stimulatory effects on the uterus (153). After 12 months of treatment, BMD at the hip was increased by 1.2%, bone resorption (as assessed by hydroxyproline excretion) decreased by up to 28%, and perhaps most promising, serum osteocalcin concentrations, a marker of bone formation, increased twofold (153). Clearly, more basic research and controlled clinical studies are required to elucidate the effects of DHEA on bone *in vitro*, and to determine its potential use in the clinical setting in order to safeguard the use of this over-the-counter drug.

Effects of the anti-androgen hydroxyflutamide (OHF) on bone metabolism

OHF and its precursor, flutamide, are non-steroidal AR antagonists which are highly specific for the AR (154, 155). Flutamide is converted *in vivo* to the more potent OHF by hepatic hydroxylation (156). While OHF is considered a pure AR antagonist in most tissues of the reproductive system, its effect on bone may be more ambiguous. Animal studies revealed that female rats given flutamide displayed reductions in bone formation, whereas bone resorption remained unaffected (157, 158). In addition, treatment of hyperandrogenic adolescent women with flutamide did not affect BMD, although biochemical markers were not determined in this study (159). Of interest, OHF which *in vitro*

antagonized all effects of 5 α -DHT on the hFOB/AR-6 cell line, including AR activation (43), proliferation (44) and differentiation (44), suppressed cytokine-induced IL-6 production to the same extent as 5 α -DHT and, when co-administered with 5 α -DHT, was not able to prevent 5 α -DHT-induced IL-6 suppression (143). Thus, OHF appears to act as an AR agonist for effects on IL-6 production by osteoblasts. Since regulation of IL-6 gene expression by estrogen has been reported to employ the non-classical pathway (see Fig. 1B), where the activated steroid receptor does not require DNA binding (24), it is conceivable that OHF may inhibit IL-6 gene expression by activating the AR and binding to, and competing for, transcription factors.

Of note, in the presence of the co-activator for AR, ARA70, OHF has been demonstrated to activate an androgen-responsive element reporter construct in a human prostate cancer cell line (160), suggesting that tissue-specific differences in the expression of ARA70 may determine whether OHF is an AR agonist or antagonist. Furthermore, heterogenous distribution of the AR subtypes, together with differences depending on the promoter and cell-specific context of target genes (28), could also explain the differential effects of OHF as an AR agonist or antagonist. Clearly, further studies are needed to address the possibility that OHF and related compounds, by functioning as AR agonists on bone cells, may in fact represent a novel class of drugs, selective AR modulators.

Conclusion

With the use of molecular and cell biology techniques, a variety of direct and indirect effects of androgens on bone cells has been identified. Androgens have potent effects on the proliferation, differentiation, and mineralization of osteoblastic lineage cells, and regulate the production of various autocrine and paracrine cytokines (IL-1 β , IL-6, IGFs, IGFBPs, PGE₂, TGF- β) in the bone microenvironment, which, at least in part, may account for their anabolic and anti-resorptive effects as observed *in vivo*. Some of the conflicting results in the literature on androgen effects on osteoblasts may arise from the considerable differences between the various osteoblastic cell systems. The use of cell systems with a well-defined osteoblastic phenotype may resolve some of these controversies, may further elucidate the role of adrenal androgens, and may facilitate the development and characterization of selective AR modulators with beneficial effects on bone but without untoward effects on other androgen target tissues.

Acknowledgements

The authors' experimental work was supported by grant AG-04875 from the National Institutes of Health (to SK) and grant Ho 1875/1–1 from the Deutsche Forschungsgemeinschaft (to LCH).

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Received 24 August 1998

Accepted 14 December 1998