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Osteoblast Gene Expression in Rat Long Bones: Effects of Ovariectomy and Dihydrotestosterone on mRNA Levels

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Abstract. The steroid sex hormones exert major effects on bone formation although the molecular events associated with their activity remain unclear. We have investigated the effects of ovariectomy and dihydrotestosterone (DHT) administration to both sham-operated and ovariectomized (ovx) rats on the bone mRNA levels of osteoblast genes. Rats were randomly allocated to either sham or ovariectomy operations and were administered either vehicle or 40 mg/kg body weight DHT by silastic tube implants at the time of operation for 8 weeks, at which time they were killed and total RNA was extracted from the long bones. Northern blot analysis indicated that the mRNA levels of the bone cell genes α1(I) collagen, alkaline phosphatase, osteocalcin, and osteopontin were markedly increased in ovx rats between 6- and 30-fold. DHT administration to ovary-intact, estrogen-sufficient rats increased the mRNA levels of α1(I) collagen, alkaline phosphatase, osteopontin, and osteocalcin between 3- and 9-fold. In contrast, DHT did not alter levels of these mRNA species in ovx rats. The data demonstrate that estrogen deficiency increased mRNA levels of genes expressed during osteoblast development and suggest an interplay between estrogen and androgen action in regulating the expression of a number of bone cell genes.

Key words: Osteoblast — Ovariectomy — Rat — mRNA — Bone formation.

Ovariectomy in the adult rat produces a rapid increase in bone cell activity with increased osteoblast number and activity preceding increased osteoblast number and bone formation rate [1, 2]. The resultant bone loss arises from trabecular perforation and dissolution of trabecular structures. The lag time between increased bone resorption and bone formation possibly contributes to this bone loss but the major cause is more likely to be increased resorption depth in the estrogen-deficient state, although this effect has not yet been unequivocally proven in this model [3]. Whether estrogen deficiency impairs bone formation remains controversial although many of the existing studies, particularly in the ovariectomized (ovx) rat model, suggest there is no such impairment [4].

In contrast, the administration of androgens to adult female rats, at least at low doses, has an anabolic effect on bone formation with apparently no effect on bone resorption. Treatment of osteopenic ovx rats with dihydrotestosterone (DHT) increases bone formation rate with a concomitant increase of trabecular bone volume and bone mineral density [5, 6]. DHT treatment in estrogen-sufficient and estrogen-deficient rats increases serum alkaline phosphatase (ALP), a biochemical marker of osteoblast activity [7]. Furthermore, when high doses of DHT are administered to osteopenic ovariectomized (ovx) rats, bone resorption is inhibited with decreased osteoclast surface and numbers [5] and decreased urine deoxypyridinoline excretion [7].

The observed changes in bone cell metabolism following ovariectomy and DHT administration in ovary-intact, ovx, and osteopenic ovx rats have been mainly limited to morphometric and biochemical measurements. The molecular events involved in the stimulation of bone formation remain unclear. A model for bone formation, derived largely from *in vitro* experimentation, has been proposed involving the regulated, sequential expression of a series of osteoblast genes associated with the proliferation, synthesis of matrix protein, and mineralization activities of these cells [8]. We have previously reported the effects of DHT on the bone-related biochemical variables in ovary-intact and ovx rats [7]. The present study investigates the effect of ovariectomy and DHT administration on the bone levels of mRNA for a number of genes expressed during osteoblast development in these rats.

Material and Methods

cDNA Probes

The cDNA clones employed as probes were rat c-*fos* [9] provided by Dr. G. Wittert, Royal Adelaide Hospital, Adelaide, Australia, rat α1(I) collagen [10], rat ALP [11], rat osteopontin [12], and rat osteocalcin [13] provided by Dr. Jon N. Beresford, Bath Institute for Rheumatic Diseases, Bath, UK; also human carbonic anhydrase II [14] and rat tartrate-resistant acid phosphatase [15] provided by Dr. M. H. Zheng, The University of Western Australia, Australia, and rat glyceraldehyde 3-phosphate dehydrogenase (GAPDH) [16]. cDNA inserts from these plasmids were isolated and radiolabeled as described below. The size of the mRNA for each of the genes are as follows: rat α1(I) collagen, 4.7 kb [10]; rat

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ALP, 2.5 kb [17]; rat osteopontin, 1.5 kb [18]; rat osteocalcin, 0.6 kb [19], and rat c-*fos*, 2.1 kb [9].

Animals

As described previously [7] adult female Sprague Dawley rats (265–357 g, 8 months old) were supplied by the Gilles Plains Animal Resource Centre (Gilles Plains, South Australia). Each rat was meal fed 20 g per day commercial rat chow containing 0.7% calcium, 0.6% phosphorus, and 200 U/kg vitamin D (Milling Industries Pty. Ltd., South Australia), and tap water was supplied *ad libitum*. The animals were housed at 26°C with a 12-hour light-dark cycle. All procedures were approved by the Institute of Medical and Veterinary Science and the University of Adelaide Animal Ethics Committees.

Experimental Procedure and Design

A total of 12 rats were used for mRNA analyses that were randomly allocated to either a sham or ovariectomy operations performed under halothane anesthesia. Within each operation group, rats were further randomized to receive either vehicle (silastic tubing) or 40 mg/kg body weight DHT (Sigma Chemical Company, St. Louis, MO) administered by silastic tubing implants (Dow-Corning Medical Silastic tubing, Dow-Corning, Midland, MI) 1.5 cm long at the time of operation. Serum DHT levels obtained with this procedure have been published [7]. At 8 weeks postoperation, animals were killed and both tibiae and one femur were excised and the surrounding soft tissue was removed. Following excision of the ends of the long bones, bone marrow was removed by flushing with ice-cold saline and discarded. The remaining bone was crushed with surgical pliers, then, after combining samples from each rat, were homogenized (Ultraturrax, Janke and Kunkel, Staufen, Germany) in RNase inhibiting solution (4 M guanidine isothiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% sarcosyl, 0.1 M 2-mercaptoethanol) [20]. The homogenates were stored at −70°C prior to total RNA extraction.

Preparation of Total RNA from Bone and Northern Blot Analysis

To determine the optimum method for extracting RNA from rat bones, three techniques were compared. The guanidine isothiocyanate solution (10 ml) was used for extraction by (1) flushing the marrow cavity, (2) crushing bones with surgical pliers, or (3) homogenizing bones. Total RNA was isolated by phenol chloroform extraction based on the method of Chomczynski and Sacchi [20] as reported by Ohta et al. [21]. The protocol was modified by the introduction of a final step where RNA was precipitated in 3 volumes of 4 M sodium acetate, pH 7.0. RNA preparations were denatured at 95°C for 5 minutes prior to analysis by electrophoresis in 1% agarose in 10 mM Tris-acetate, pH 8.2 containing 1 mM EDTA and stained with ethidium bromide.

For Northern blot analyses, total RNA (15 μg/lane) was separated in a 1.2% formaldehyde agarose gel and transferred to a Nylon membrane (Boehringer Mannheim-GmBH, Mannheim, Germany) by capillary action in 20 × SSPE, pH 7.4 (0.36 M NaCl, 0.02 M Na_2HPO_4, 0.002 M EDTA). The RNA was immobilized on the membrane by exposure to UV radiation for 30 seconds (UV Crosslinker, Ultra-Lum, Adelaide, Australia). Nylon membranes were prehybridized at 42°C for 2 hours in a buffer solution containing 50% formamide, 5 × Denhardt's solution (0.1% bovine serum albumin, 0.1% polyvinyl pyrrolidone, 0.1% Ficoll 400), 0.1% sodium dodecyl sulfate (SDS), 5 × SSPE, pH 7.4, 0.05% tetrasodium pyrophosphate ($Na_4P_2O_7$), pH 7.4, and 200 μg/ml of sheared denatured salmon sperm DNA. The cDNA probes were labeled with [α^{32}P-dATP] by random primer extension (Megaprime™ DNA labeling kit, Amersham, Sydney, Australia) according to the manufacturers instructions. The probes were purified by carrier precipitation, added to the prehybridization solution, and incubated at 42°C overnight. Filters were washed twice at 42°C in 2.5 × SSPE, pH 7.4, 0.01% SDS, and 0.01% $Na_4P_2O_7$ for 10 minutes and washed twice at 65°C in 5 × SSPE pH 7.4, 0.01% SDS, and 0.01% $Na_4P_2O_7$ for 10 minutes. Filters were exposed overnight to a PhosphorImager™ screen (Molecular Dynamics, Sunnyvale California, USA), scanned, and quantified using a Molecular Dynamics PhosphorImager™ and ImageQuant computer software, version 5.25 on a personal computer.

Calculations

All mRNA levels were normalized for GAPDH mRNA levels by dividing the signal obtained from the ImageQuant program for the mRNA species of interest by the signal for the GAPDH mRNA for each rat. For each Northern blot, total RNA was isolated and analyzed from the bones of one rat from each experimental group. The normalized mRNA levels were expressed as a fold increase relative to sham rats receiving vehicle for each individual Northern blot analysis to allow for direct comparison of mRNA levels between Northern blot analyses. The effect of ovariectomy and the administration of DHT were analyzed by unpaired *t*-tests performed on a personal computer. A value of $P < 0.05$ was considered significant.

Results

Initial studies were undertaken to optimize the extraction of total RNA from the long bones of adult rats. The highest yields of RNA required homogenization in the guanidinium isothiocyanate solution (128 μg RNA/femur) compared with simply crushing bones with surgical pliers (19 μg RNA/femur) or flushing the bone marrow cavity with this solution (8 μg RNA/femur). The quality of the total RNA analyzed on agarose gels was greatly improved by a final precipitation with 4 M sodium acetate (Fig. 1A). This precipitation step is commonly employed to remove glycogen from RNA [22] and has been used for the purification of RNA from cultured bone cells [23]. The nature of the material responsible for the smearing effect observed in Figure 1A is unknown. Phenol/chloroform/isoamyl alcohol extractions and washing with 70% ethanol did not improve these RNA preparations. The changes in RNA electrophoretic mobility were typical of all preparations of RNA from bone and therefore this precipitation step was included in the following experiments. The effects of ovariectomy and DHT on the levels of osteoblast- and osteocyte-specific mRNA types (α1(I) collagen, ALP, and osteocalcin) and on osteopontin mRNA in rat long bones were determined by Northern blot analysis using specific probes and expressed relative to GAPDH mRNA.

A representative Northern blot for detection of α1(I) collagen is shown in Figure 1B. A single band of RNA was detected using the cDNA probe for α1(I) collagen and this band corresponded in size to the endogenous mRNA, as judged by molecular weight markers. The levels of this major band varied according to the treatment but the levels of GAPDH mRNA remained unaltered during the treatments (Fig. 1B). Similarly, the other mRNAs were detected using specific cDNA probes and major single bands were observed corresponding to the expected molecular sizes, and GAPDH did not alter during the treatments. Three separate rats were included in each treatment group and the mean data are presented in Figure 2 where each mRNA is expressed relative to GAPDH mRNA.

At 8 weeks postovariectomy the mRNA levels of α1(I) collagen and osteocalcin were increased by 30-fold and 13-fold, respectively ($P < 0.05$, $P < 0.001$). Alkaline phospha-

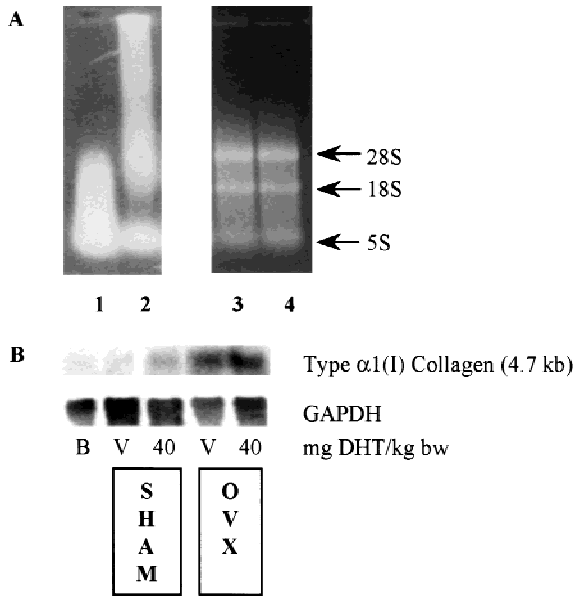

Fig. 1. (A) Electrophoretic analyses of total RNA extracted from homogenized bone. Lanes 1 and 2 contain typical samples processed by phenol-chloroform extraction. Lanes 3 and 4 contain these same samples further processed by sodium acetate precipitation as described in Materials and Methods. All RNA was stained with ethidium bromide and the positions of ribosomal RNA markers are shown. **(B)** Northern blot analyses for α1(I) collagen mRNA in bone total RNA extracted from rats killed at baseline (B) as well as ovary-intact (SHAM) or ovariectomized (ovx) rats treated with vehicle (V) or 40 mg DHT/kg body weight for 8 weeks (40). Levels of GAPDH mRNA in the same samples are presented for comparison.

tase and osteopontin mRNA levels were also increased as a result of ovariectomy by 30-fold and 6-fold, respectively, although these changes did not achieve statistical significance.

Treatment of ovary-intact and ovx rats for 8 weeks with the androgen, DHT also affected bone cell mRNA levels. Administration of DHT to ovary-intact, sham-operated rats produced a ninefold increase in α1(I) collagen mRNA ($P < 0.05$), a fourfold increase in osteocalcin mRNA ($P < 0.05$), a threefold increase in ALP mRNA (not significant (ns) and a fivefold increase in osteopontin mRNA (ns) (Fig. 1). In contrast, DHT administration to ovx rats at the time of operation had no effect on the ovx stimulation of α1(I) collagen, ALP, osteopontin, or osteocalcin mRNA levels.

Discussion

The results of Northern blot analyses show that following ovariectomy there is an increase in bone of mRNA for the osteoblast- and osteocyte-specific genes α1(I) collagen, osteocalcin, and although not achieving statistical significance, ALP. Osteopontin mRNA, which is produced by a variety of bone cells, also tended to be increased by ovariectomy, although again this did not achieve statistical significance. The data are consistent with the accompanying increase in bone resorption and bone formation following ovariectomy [1, 2]. In the present study, however, mRNA for the osteoclast genes carbonic anhydrase and tartrate-resistant acid phosphatase were undetectable by Northern blot analysis (data not shown). In contrast, Zheng et al. [14], using the same technique, demonstrated marked increases in the mRNAs for these genes following ovariectomy in the rat. A possible explanation for the variance in these results is that Zheng et al. extracted total RNA from whole tibiae whereas in the present study total RNA was extracted from the shaft of the long bones after the epiphyses, including the growth plate, and bone marrow was removed. It would appear therefore that a different profile of mRNA may be obtained from these latter areas particularly if they are rich in osteoclasts.

It was also observed in the present work that mRNA levels for c-*fos* gene, although detectable, were low and unaffected by ovariectomy (data not shown). This finding was unexpected since the c-*fos* gene, together with the c-*myc* gene, are expressed during the proliferation of various cell types including osteoblasts. Previous studies have found that in bone RNA extracts, mRNA for an accompanying gene, c-*myc* is elevated at 2 weeks following ovariectomy [24]. These authors also reported increases in mRNAs for α1(I) collagen and osteocalcin in the same extracts, although the levels were only 1.25-fold of the levels in sham rats. The mRNAs for c-*fos* and c-*myc* have half-life values as short as 15 minutes and are unstable, allowing for their rapid disappearance after induction [25]. In the present study mRNA levels were measured at 8 weeks following ovariectomy at a time when osteoclast and osteoblast numbers remain elevated [3]. However, it is likely that this time difference accounts for the failure to detect the ovariectomy-induced stimulation of c-*fos* expression and the significantly greater levels of induction for the osteoblast-specific mRNA species. Overall, our data are consistent with ovariectomy-induced stimulation of bone formation secondary to ovariectomy activation of osteoclasts [1, 2] stimulating osteoblasts over a wide range of developmental stages.

DHT treatment in ovary-intact rats increased mRNA levels of the bone cell genes α1(I) collagen, ALP, osteopontin, and osteocalcin after 8 weeks. The increase in ALP and osteopontin mRNA levels, however, did not reach statistical significance because of the individual variation observed in these mRNA levels. The serum levels of ALP, however, also vary greatly between individual rats [7]. c-*fos* mRNA is expressed during proliferation of the osteoblast *in vitro* [8]. No major stimulation on c-*fos* mRNA levels was observed in the current study, although a stimulatory effect of DHT on proliferation may have occurred prior to 8 weeks following treatment as DHT has been demonstrated to increase the proliferation of a variety of bone-derived cells [26–28]. The action of DHT treatment to increase the mRNA levels of α1(I) collagen, ALP, osteopontin, and osteocalcin suggests that androgens in the presence of ovarian hormones stimulate all stages of osteoblast differentiation.

The mechanism of this androgen effect on the mRNA levels of the osteoblast genes in the ovary-intact rats requires further investigation. The increase could reflect an increase in the rate of gene expression and/or an increase in the stability of the mRNA. Whether DHT acts directly or indirectly to increase mRNA levels *in vivo* is an important question. In this regard, it has been reported that the mRNA level of type α1(I) collagen was increased in cultured osteoblast-like cells following treatment with testosterone and DHT [26]. The number of differentiated bone cells, as indicated by positive staining for ALP, was also increased following treatment with DHT [27]. DHT has been shown to stimulate the proliferation of mouse calvarial cells [27],

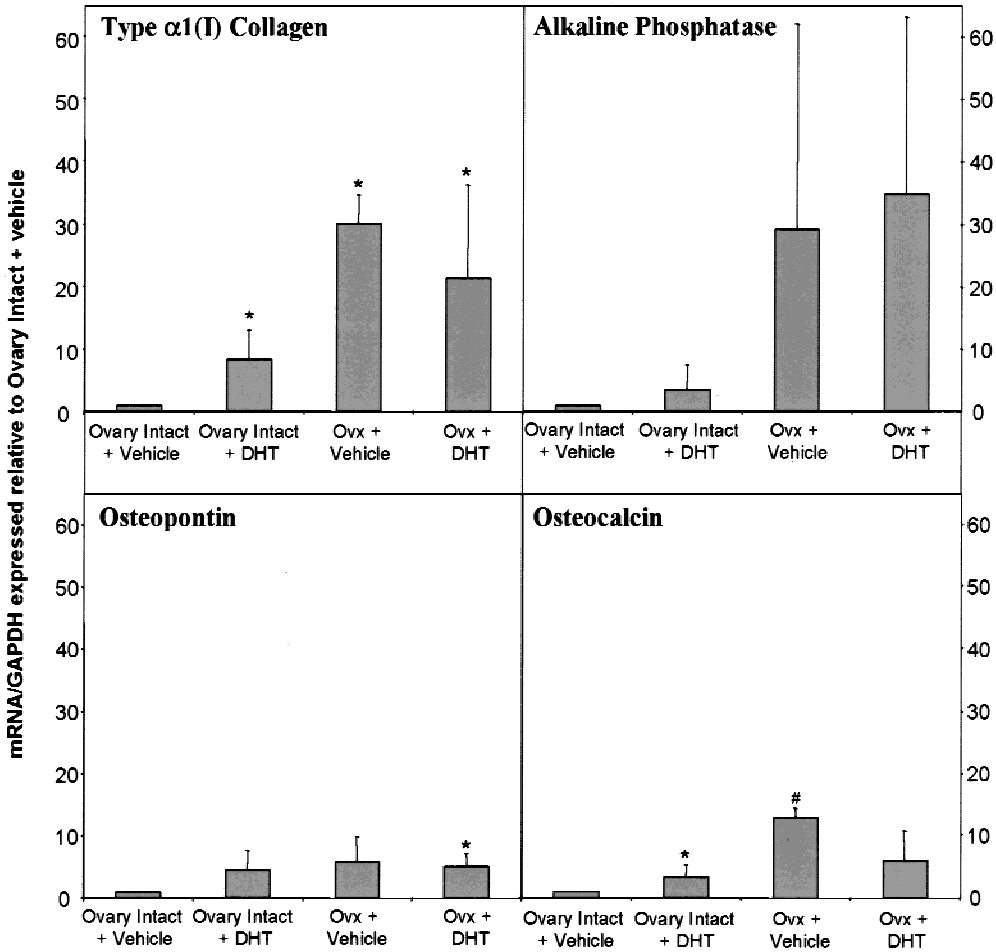

Fig. 2. Quantification from Northern blots of α1(I) collagen, alkaline phosphatase, osteopontin, and osteocalcin mRNA levels corrected for GAPDH and expressed as a fold increase relative to Ovary-Intact + Vehicle for Ovariectomized (Ovx) + Vehicle, Ovary-Intact and Ovx rats administered DHT from the time of operation. Values are mean ± SD, n = 3. *$P < 0.05$ versus Ovary-Intact + Vehicle, #$P < 0.001$ versus Ovary-Intact + Vehicle.

rat bone cells [28], and MC3T3-E1 osteoblast-like cells [29] *in vitro*. Furthermore, DHT and the synthetic androgen, RU1881, act directly on avian osteoclasts and human and mouse osteoclast-like cells to decrease resorption activity *in vitro* [30]. In contrast, previous studies have failed to demonstrate an effect of androgen on osteoclasts in culture [31, 32]. Overall, the data from these cell culture studies suggest that *in vivo*, the effect of DHT may reflect a direct response of both osteoblasts and osteoclasts to the hormone.

DHT administration to ovx rats immediately following operation had no effect on mRNA levels of α1(I) collagen and osteocalcin and only a marginal stimulation of ALP and osteopontin levels. At this time bone cell activity is known to be high [1, 2] and thus it appears that androgens have little, if any, effect under the conditions of ovariectomy-stimulated bone cells. These effects are in contrast to the stimulation of bone formation by DHT in osteopenic ovx rats when DHT was administered at 8–12 weeks following ovariectomy [5, 6]. Other reports have demonstrated that DHT and nandrolone decanoate treatment suppress serum osteocalcin levels in these animals [7, 33]. The association of osteocalcin with bone mineralization is unclear with the recent proposal, based on studies of the osteocalcin-knockout mouse, that osteocalcin functions to limit bone formation without impairing bone resorption or mineralization [34]. Furthermore, DHT appears to have some antiresorptive activity in ovx rats [5, 7] and this activity may also exert inhibitory effects on osteoblast gene expression. Further studies are required to identify the actions of DHT on osteoblast gene expression under the conditions of osteoclastogenesis at a later time after ovariectomy.

The current study has identified efficient methods for extraction and preparation of RNA from adult rat bones and demonstrated for the first time that at 8 weeks post-oophorectomy there is an increase in the mRNA levels of a number of osteoblast-specific genes examined during osteoblast development. The effects of DHT on osteoblast expression differ in estrogen-sufficient and estrogen-deficient rats and therefore the action of androgens on bone cell gene expression appears to be estrogen dependent. The data suggest an interplay between estrogen and androgen action in regulating the expression of the osteoblast genes which requires further investigation at the molecular level.

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