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Influence of Testosterone and Dihydrotestosterone on Bone-Matrix Induced Endochondral Bone Formation

Shakti P. Kapur[1] and A. H. Reddi[2]

[1]Department of Anatomy and Cell Biology, School of Medicine, Georgetown University, Washington DC, USA; and [2]Bone Cell Biology Section, National Institute of Dental Research, National Institutes of Health, Bethesda, Maryland, USA

Summary. A bone matrix-induced endochondral bone development model has been used to study the effects of androgens on different stages of bone development in castrated young adult rats. Androgen treatment, especially with dihydrotestosterone (DHT) for 7 days, inhibited $^{35}SO_4$ incorporation by the developing cartilage in the induced plaques. Castrated control animals maintained for 11 days after implantation of bone matrix showed significantly lower calcium levels in the induced implant than was observed earlier in the unoperated controls. DHT treatment for 11 days caused dramatic increases in levels of calcium in the implants. Testosterone had little effect. When androgen treatment was continued for 21 days, while levels of alkaline phosphatase in the implants were unaffected, levels of calcium in the implants were significantly higher than on day 11 for both castrated control and androgen-treated animals. Peak alkaline phosphatase activity (day 10) is known to precede peak calcium mineralizing activity (day 12) in this model and it is also known that calcium levels remain high thereafter. Evaluation of calcium and alkaline phosphatase levels in the proximal tibial metaphyses of castrated control and androgen-treated groups of animals showed no changes after 11 days treatment. Prolonged treatment (21 days) elevated the levels of alkaline phosphatase whereas no change was observed in calcium levels in the tibial metaphyses. These findings demonstate that androgens stimulate mineralization and that DHT is more active when used for short periods of time and in early stages of bone development in matrix-induced implants.

Key words: Matrix-induced endochondral bone formation — Alkaline phosphatase — Calcium — Testosterone — Dihydrotestosterone.

Androgens are considered important in skeletal maturation of growing animals, including closure of the epiphyseal plate, deposition of new osteoid, and its mineralization [1]. Significant correlations exist between androgens, total calcium content, and alkaline phosphatase. Increases in alkaline phosphatase levels precede increases in mineralizing calcium levels so that these two parameters of bone development do not peak coincidently [2, 3]. Further, evidence of androgens' effect on bone is provided by the observations that osteoporosis in hypogonadal adults or in castrated males is reversed by androgen treatment [4]. Postponement of mineralization in delayed idiopathic puberty further reinforces such correlations [5]. However, the exact interaction between androgens and cartilage which plays an important role in skeletal growth remains unclear. On one hand, androgens are reported to directly stimulate sulfate uptake by cartilage [6, 7], but another set of studies indicate that androgens have no effect on sulfation of glycosaminoglycans secreted by chondrocytes [8]. Further, androgens promote cell proliferation while also causing increased hypertrophy and mineralization of matrix [9]. Such diverse effects may be related to age of the animal used in a given study [6]; for example, androgen receptor binding activity is known

Send reprint requests to A. H. Reddi, NIDR/NIH, Building 30 Room 211, Bethesda, MD 20892, USA.

to show age-dependent fluctuations, peaking in the case of growing boys at age 10–11, with additional spikes of binding rates at age 4–5 [10].

The difficulty in understanding such diverse effects of the androgens on different stages of skeletal growth and maturation is partly due to the complexity of endochondral differentiation. The growth plate is a heterogenous assemblage of different cell populations at various stages of differentiation and this makes it difficult to separate various cellular targets at different levels of their development. The use of demineralized bone matrix (DBM)-induced endochondral bone differentiation system potentially circumvents these complexities and permits a study of the discrete stages of endochondral bone development. Implantation of DBM leads to appearance of mesenchymal cells and their proliferation (day 3), followed by chondrogenesis (days 5–7), hypertrophy and mineralization (day 9), appearance of osteoblasts and osteoid formation (days 11–14), and formation of a hemopoietic marrow cavity with remodeling bone (days 19–21) [11–13]. Biochemical parameters used to quantitate these responses at different stages of endochondral bone development are well established in this model. The incorporation of $^{35}SO_4$ into proteoglycans on day 7 is an index of chondrogenesis [12]. The levels of alkaline phosphatase and total calcium content reflect the level and extent of mineralization [12, 14, 15]. The experimental model is amenable to a systematic study of hormonal factors regulating bone formation and has been successfully used to investigate the influence of estrogen and progesterone [16], corticosteroids [17], diabetes and insulin [18, 19], and effects of hypophysectomy, growth hormone, and thyroid-stimulating hormone [20]. In the present study we have examined the effect of two androgens—testosterone and dihydrotestosterone—on endochondral bone development using this model.

Materials and Methods

Preparation of DBM

Dehydrated diaphyseal shafts of rat femur and tibia were pulverized in a CRC micromill (Technilab Instruments, Vineland, NJ) and sieved to a particle size of 74–420 μm. Such particle size with an extensive surface area is considered essential for providing a suitable substratum for anchorage-dependent proliferation and differentiation in this model [21, 22]. Liquid nitrogen was used to freeze the bone shafts prior to and during pulverization to avoid heat denaturation of proteins. Demineralization of this bone powder was carried out by using 0.5 N HCl, extracted with water, ethanol, and ether [12].

Castration and Bone Matrix Implantation of Male Rats

Male rats (Long Evans strain, 28–31 days) weighing between 80 and 100 g were surgically castrated under ether anesthesia and allowed to recover for 1 week. Approximately 25 mg of DBM was bilaterally implanted subcutaneously at two sites in the thoracic region of each animal. They were then divided into nine groups of 5 animals each. Groups 1 and 2 were each administered subcutaneously 200 μg of testosterone (TES) or dihydrotestosterone (DHT) in 200 μl of sesame oil daily for 7 days. A control group (#3) received sesame oil alone. These three groups were used to study the effect of androgens on cartilage growth using $^{35}SO_4$ incorporation in DBM-induced growing implants. Groups 4 and 5 were also administered daily the same dose of androgens for an 11-day period accompanied by a castrated control group (#6) that received sesame oil only. Groups 7 and 8 received daily androgens in the same dose and manner for 21 days, accompanied by a control group (#9).

Animals were killed by CO_2 asphyxiation on days 7, 11, and 21. Weight of each animal was recorded at the beginning and end of each experiment. Ventral prostates were removed and weighed to examine the effects of castration and androgen treatment. Blood samples were taken by cardiac puncture for serum calcium analysis prior to sacrifice. Serum calcium was measured by atomic absorption spectrophotometry after suitable dilution in 0.5% (v/v) HCl containing 0.1% lanthanum oxide (wt/vol) [23].

Measurement of Alkaline Phosphatase and Calcium Levels in Matrix-Induced Plaques and Tibial Metaphyses

DBM-induced button-like implants were removed from 11 and 21 day treated and control groups of animals, separated from adhering tissues, weighed and homogenized in 2 ml of ice cold 0.15 M NaCl/3mM $NaHCO_3$. Following centrifugation, the alkaline phosphatase activity and protein content of the supernatant and the calcium content of the acid soluble fraction of the centrifuged sediment were determined as indices of bone formation following the method of Reddi and Sullivan [20]. Proximal tibial metaphyses of all rats used in this study were also removed and served as normal in vivo markers of possible changes in mature bone tissue.

Extraction of Proteoglycans

Day 7 implants were removed from animals, minced, and labeled with sodium [^{35}S]sulfate, 50 μCi per implant in Dulbecco's Modified Eagle Medium (DMEM) for 3 hours at 37°C. The labeled samples were extracted for proteoglycans at 4°C by combining with 4 M guanidine HCl containing 0.1 M aminohexanoic acid, 5 mM benzamidine HCl, 0.05 M sodium acetate, pH 5.8, overnight with constant stirring. The tissue samples were then centrifuged at 12,000 rpm at 4°C for 30 minutes in a JB-3 rotor using hard centrifuge tubes. The supernatant was then removed and a second extraction was carried out for 3 hours at 4°C with constant stirring, centrifuged, and the supernatant pooled with the first supernatant.

Aliquots (2 ml) of the 4 M guanidine extract were eluted on