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AGE-DEPENDENT RESPONSIVENESS OF RABBIT AND HUMAN CARTILAGE CELLS TO SEX STEROIDS *IN VITRO*

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Summary—Rabbit epiphyseal cartilage tissue has been shown to convert testosterone (T) to dihydrotestosterone (DHT). In this report, the metabolic conversion of T into DHT is shown to be age-dependent, being most active in cartilage from animal at the age of gonadal maturation. Human cartilage from newborn and prepubertal children is also shown to convert T into DHT and—to a lesser extent—to estradiol.

Low concentrations of DHT and 17β-estradiol (E_2) (10^{-11}–10^{-9} M) were also shown to stimulate *in vitro* cartilage cells from boys and girls respectively. As previously shown for cultured rabbit chondrocytes, the stimulating effects of both hormones on human chondrocytes was age-dependent. Cartilage cells derived from children up to one year old did not respond, while cells from boys and girls in the early phase of puberty responded best.

These data indicate that human cartilage tissue *in vivo*, contains both 5α-reductase and aromatase activities during post-natal skeletal growth. Androgens may act on cartilage after their metabolic conversion to estrogens. The mechanism of age-dependency of both cartilage androgen enzymatic activities and chondrocyte responsiveness to sex steroids *in vitro* remains to be explained.

INTRODUCTION

Gonadal steroids influence the skeletal growth of both sexes causing an increased growth rate at the time of gonadal maturation [1]. It is now generally agreed that the sex steroid effect on skeletal growth is due to an indirect action combined with a direct effect on skeletal tissue [2].

The indirect action is mediated by GH secretion. Both experimental and clinical evidence indicates that the increased steroid secretion at puberty is associated with stimulation of GH secretion, which in turn, stimulates IGF_1, at least partly accounting for the increased growth rate and bone maturation [3–5].

However, recent *in vivo* and *in vitro* observations suggest that sex steroids may have a direct affect on skeletal growth, independent of GH and IGF_1. Laron dwarfs, who suffer from a functional defective GH receptor gene and have high circulating values of GH but no endocrine generation of IGF_1, show a definite pubertal growth spurt in spite of their lack of circulating IGF_1 [6].

Other clinical data indicate that estrogens have a dual effect upon growth. Doses of ethynyl-estradiol as high as 400–800 ng/kg/day do not affect the bone growth rate of girls with Turner Syndrome, while the relatively low dose of 100 ng/kg/day of estrogens produces a maximal growth response, as evaluated by a doubling of the base-line ulnar growth rate, with no increase in the circulating IGF_1 [7].

We have, over the past few years, carried out *in vitro* studies to determine whether physiological concentrations of sex steroids could act directly on growth plate cartilage metabolism. Epiphyseal cartilage cells were isolated from prepubertal rabbits, and more recently from adolescent boys and girls, and cultured under serum-free conditions. Cartilage tissue from prepubertal animals was found to convert testosterone (T) mainly into its active metabolite, dihydrotestosterone (DHT), suggesting that cartilage is a target organ for androgens [8]. In primary cultures of rabbit epiphyseal cartilage T, DHT and 17β-estradiol (E_2) all stimulated

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chondrocyte sulfation, but the effect depended on the age of the animal donors [9]. Cartilage cells from rabbit fetuses and from post-natal animals at the age of gonadal maturation were most responsive to sex steroids. Recently Somjen *et al.* have shown that physiological concentrations of estrogens modulates the proliferation and energy metabolism of rat cartilage cells *in vitro* [10].

The present work provides *in vitro* data which confirm our previous findings that sex steroids act directly on chondrocyte metabolism in rabbit cartilage, and that the responsiveness of both rabbit and human chondrocytes is age-dependent. There appears to be a correlation between the maximum effect of sex steroids on human cartilage at the early phase of puberty and gonadal maturation.

EXPERIMENTAL

Animals

Prepubertal (15–25-day-old) and pubertal (40–60-day-old) Fauve de Bourgogne male and female rabbits were obtained from Ruvel France (Elevage de la Peulve, Happonvilliers 28 Thiron-Gardais, France).

Patients

Human cartilage biopsies from 24 girls and 19 boys aged between 4 months and 10 years were obtained during orthopedic surgery. The children were considered normal in term of skeletal growth. All of them were at a prepubertal stage as evaluated by clinical examination and in a few cases by the level of circulating sex steroid hormones.

Cartilage

Epiphyseal cartilage was carefully dissected from rabbit long bones or from human long bone, vertebra or nasal cartilage biopsies and used to prepare cartilage tissue for incubation or cultured chondrocytes.

Cartilage tissue incubation with [^{3}H]testosterone

Rabbit or human cartilage was cut into slices under sterile conditions. Known weights (80–150 mg) of cartilage were incubated in 3 ml FCS-free Dulbecco's medium, containing 1α, 2α-*N*-[^{3}H]testosterone ([^{3}H]T) (45–60 Ci/mmol, Amersham) in 30 μl propyleneglycol at a final concentration of 9–164 nM, for 3 h at 37°C. Radioactive material was then extracted from both the cartilage tissue and the incubation medium. Steroids were extracted with ethylacetate/cyclohexane v/v as described by Wright *et al.* [11]. The final radioactive steroid extract was suspended in 1 ml ethylacetate and stored at 4°C.

Each incubation was performed in triplicate for rabbit cartilage and in duplicate with human cartilage.

Separation of radioactive metabolites by celite column chromatography

Each radioactive steroid extract was evaporated to dryness and suspended in 500 μl pure hexane prior to chromatography. Total uptake of radioactivity was measured by counting an aliquot of the 500 μl hexane solution. A small column of celite (0.7 × 29 cm) was prepared according to Abraham *et al.* [12] and Thorneycroft *et al.* [13], but the elution was modified as follows to separate androgens and estrogens: 5 ml benzene/hexane 15/85; 15 ml ethylacetate/hexane 10/90; 10 ml ethylacetate/hexane 20/80. Columns were calibrated with 4[^{14}C]androst-4-ene-3, 17-dione ([^{14}C]A_4), 5α-dihydro-4-[^{14}C]testosterone ([^{14}C]DHT), 4[^{14}C]-testosterone ([^{14}C]T), and 4[^{14}C]estradiol ([^{14}C]E_2) (Amersham). The recovery of radioactivity was higher than 90%.

Eluate fractions of approx. 0.4 ml were collected and their radioactivity measured. The

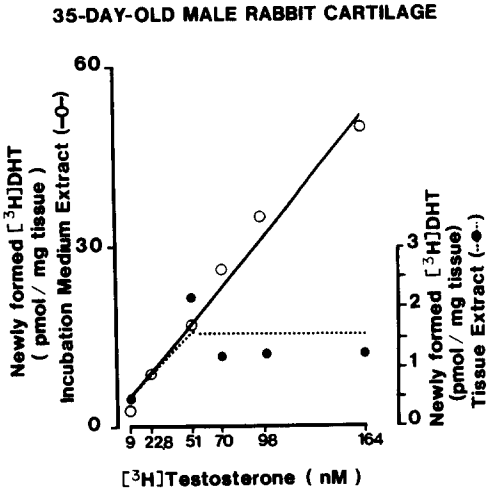

Fig. 1. Dose-dependent metabolism of [^{3}H]testosterone (9–164 nM) incubated for 3 h with rabbit epiphyseal cartilage. Total radioactivity was extracted from cartilage slices and incubation medium, and analysed by celite column chromatography. Each point corresponds to the amount of DHT radioactivity extracted from tissue slices (●—●) or corresponding incubation medium (○—○) region, as pmol per mg tissue.

radioactivity eluted under each newly-formed peak was calculated as a percent of the total radioactivity applied to the column and expressed as a mean percent of the total radioactivity extracted per milligram cartilage tissue or converted to pmole per milligram tissue.

Human chondrocyte cultures

Suspensions of chondrocytes were prepared by sequential enzyme digestion as previously described [9]. Cells were plated in 25 cm^2 tissue culture flasks at 1 to 2 × 10^5 cells per flask and incubated in Dulbecco's medium plus 10% FCS for 8 days at 37°C until confluency.

A few cells from each human chondrocyte preparation were placed in 9 cm^2 slide-flasks designed for microscopic examination of the phenotypic expression of cultured cells. Because human cartilage cells are known to rapidly dedifferentiate *in vitro*, only positive cultures, as determined by immunofluorescent staining of type II collagen (by using anti-type II collagen antibodies kindly provided by Dr J. Hartmann Lyon, France), were further studied for sulfation activity.

Effect of DHT and E_2 on sulfate incorporation into cultured human chondrocytes

Confluent cultured human chondrocytes were trypsinized and incubated at high density (10^5 cells/well) in 24-well tissue culture plates, using FCS-free, Phenol Red-free Dulbecco's medium for 24 h at 37°C. The medium was replaced with fresh FCS-free, Phenol Red-free and sulfate-free Dulbecco's containing 1.5 μCi/ml [^{35}S]sulfate plus 10^{-11} M–10^{-8} M DHT, or E_2. Controls contained the vehicle alone. Groups of 4 similarly-treated wells were prepared and incubated for 20 h at 37°C. The incorporation of radiolabeled sulfate was measured in 3 M Guanidinium extract from both medium and cell pellet as previously described [9]. Counts from each group of 4 wells were expressed as the mean ± SEM dpm/well and converted to dpm/μg DNA; the amount of DNA was measured in similarly-treated wells. Student's t-test was used for statistical analysis. Results are also expressed as percent response of the hormone-treated cells with respect to the basal incorporation by control cells.

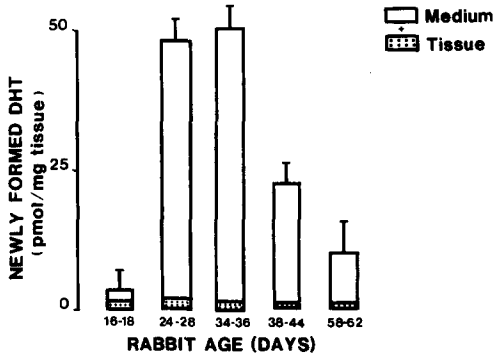

Fig. 2. Age-dependent [^{3}H]T metabolic conversion into [^{3}H]DHT in rabbit cartilage tissue slices. Cartilage from 3 rabbits was assayed at each age. Approximately 80 mg cartilage was incubated with 90 nM [^{3}H]T as in Fig. 1. The radioactivity was extracted and the amount recovered in the DHT region is expressed as pmol/mg tissue, mean ± SEM of 3 experiments.

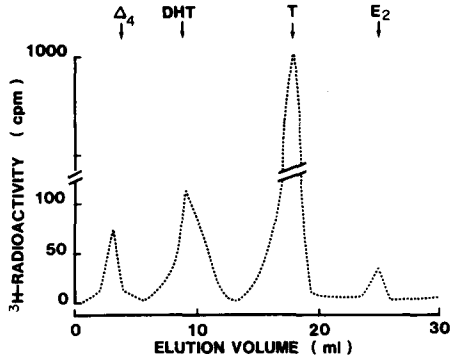

Fig. 3. Celite column elution profile of tritiated metabolites extracted from the vertebral cartilage slices (160 mg) of a 10-yr-old-boy which had been incubated with [^{3}H]T (90 nM), for 3 h. The elution profile is expressed as cpm per fraction from V_0 to V_t. Arrows indicate the positions of cochromatographed standards: [^{14}C]A_4 androstenedione, [^{14}C]DHT, [^{14}C]testosterone and [^{14}C]estradiol.

RESULTS

Testosterone metabolism in rabbit and human cartilage tissue

Prepubertal rabbit epiphyseal cartilage tissue transformed [^{3}H]testosterone ([^{3}H]T) into [^{3}H]-dihydrotestosterone ([^{3}H]DHT) (Fig. 1). This metabolic conversion depended on [^{3}H]T concentration, with maximum [^{3}H]DHT accumulation in cartilage tissue at relatively low concentrations of [^{3}H]T (50–164 nM).

The metabolic conversion occurred in cartilage from both males and females and depended on the age of the animal donor. Cartilage from 24–36-day-old rabbits contained 10 times more newly formed [^{3}H]DHT (50.0 ± 0.5 pmol/mg) than did cartilage from 16–18-day-old ones (5.0 ± 0.5 pmol/mg) (Fig. 2). The percent conversion of T into DHT decreased progressively between the age of 38 and 62 days.

The metabolic conversion of T into DHT can be related to the presence of 5α-reductase in cartilage during skeletal growth, with a maximum enzymatic activity during the very early phase of gonadal maturation.

Human epiphyseal cartilage tissue probably also contains 5α-reductase. Figure 3 shows the elution profile of radioactivity extracted from 160 mg cartilage isolated from a 10-yr-old boy, which had been incubated with 90 nM [^{3}H]T for 3 h. The major newly formed radioactive peak (10% of total radioactivity extracted) migrated in the DHT region. Two other minor peaks in the Δ_4-androstenedione and estradiol regions, represented 5 and 3.8% of the total radioactivity extracted. Similar results were obtained with cartilage from an 8-yr-old girl (data not shown).

Effect of T, DHT and 17β-estradiol (17β-E_2) on sulfate incorporation into human cultured chondrocytes

Surgical biopsies from prepubertal boys and girls were used to prepare chondrocyte cultures which were maintained in serum-free medium for 3 days. Low concentrations of both DHT and E_2 (10^{-11}–10^{-9} M) had a dose-dependent stimulating effect on [^{35}S]sulfate incorporation into macromolecules (Fig. 4).

The stimulating effect of both sex steroids varied with the age of the donor children. Cartilage cells from early neonatal children of both sexes (4–12 months after birth), did not respond to sex steroids (Fig. 5), but cartilage cells from 2–7-yr-old girls and 2–10-yr-old boys were very responsive. The cartilage samples from older children, were too small for culturing, thus no data on their responsiveness are available.

DISCUSSION

The data presented in this report support our previous findings in rabbits; they demonstrate that T, DHT and E_2 interact directly with human cartilage tissue and cells *in vitro*. They also indicate that the direct interaction of sex steroids with human and rabbit cartilage tissue and cells *in vitro* is age-dependent.

The metabolic conversion of T into DHT and androstenedione in approximately equal amounts was first shown in femoral epiphysis of growing rats by Jaffe [14] and in long bone epiphyseal cartilage tissue and cells of

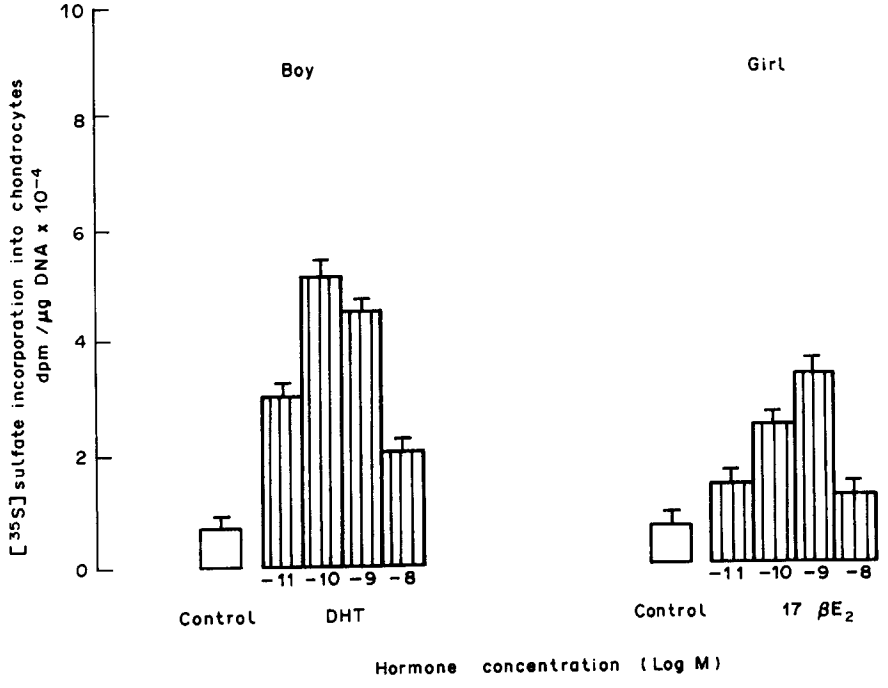

Fig. 4. Concentration-dependent effects of DHT and E_2 on [^{35}S]sulfate incorporation into cultured human chondrocytes. Epiphyseal cartilage cells from a 2-yr-old boy and a 2-yr-old girl were cultured to confluency and incubated in serum-free medium. Groups of 4 wells were incubated with 10^{-11} M–10^{-8} M DHT (male cartilage) or E_2 (female cartilage) plus 1.5 μCi/ml [^{35}S]sulfate for 20 h. Control groups were treated with 1% propyleneglycol. The radioactivity incorporated into chondrocytes was assayed as mean ± SEM dpm/group of 4 wells and expressed as mean ± SEM dpm/μg DNA measured in similarly-treated wells.

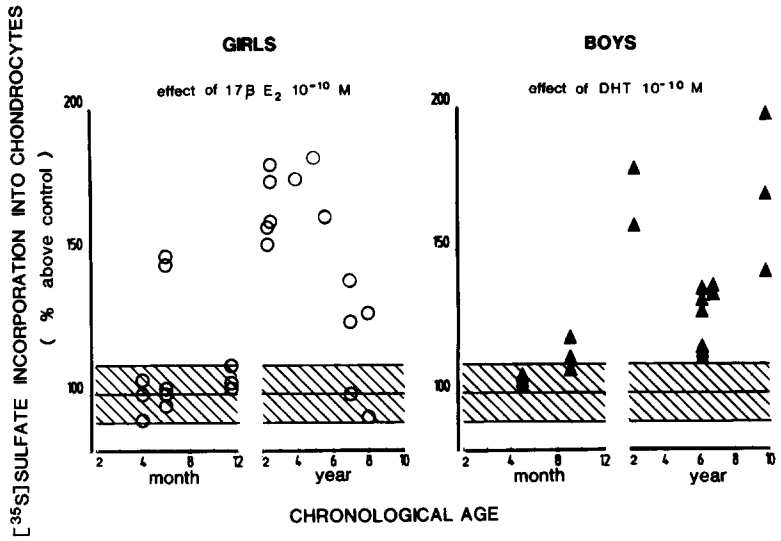

Fig. 5. Responsiveness of adolescent female and male human cartilage cells to 10^{-10} M E_2 and 10^{-10} M DHT. Epiphyseal chondrocytes from cartilage biopsies were grown to confluency and incubated with [^{35}S]sulfate plus E_2 or DHT for 20 h as described in Fig. 4. Each point represents the sulfate incorporation of the steroid hormone-treated group of cells as a percent of the activity in the corresponding control group.

prepubertal rabbits by Takahashi *et al.* [8]. The present study shows that this metabolic conversion occurs in human as well as in rabbit cartilage tissue. Human cartilage can not only form DHT and androstenedione but also convert testosterone into estradiol-like compounds, suggesting that cartilage not only contains 5α-reductase activity, but also aromatase activity capable of transforming androgens into estrogens.

In vivo, the pubertal growth spurt is generally attributed to the secretion of androgens in boys and estrogens in girls [1]. But, while estradiol is clearly the sex steroid associated with physiological pubertal growth in females, the abrupt increase in testosterone secretion in boys occurs only later in puberty, after an earlier increase in estradiol production [2]. Perhaps the growth promoting effects of androgens in males are partly mediated through their transformation into estrogens. Estrogens have more effect on skeletal growth both in males and females but there appears to be a low concentration of estrogen receptors in human osteoblast-like cells [15] as well as in cartilage from rabbits and dogs [16–18]. However, the evidence for androgen receptors in cartilage is still less convincing, despite recent reports of specific androgen binding sites in post-natal rabbit cartilage cells [19] and in human fetal cartilage [20]. Whether or not androgens act via the androgen receptor in cartilage or via the estrogen receptors remains questionable.

The age-dependency of the *in vitro* responsiveness of human and rabbit cartilage cells to sex steroids remains to be explained, but they do not appear to be due to differences in experimental manipulations. The previous *in vivo* exposure of cartilage cells to circulating androgens or estrogens may well be responsible for this age-dependency.

Human cartilage cells taken from children up to 1-yr old did not respond. There is an increase in the circulating concentrations of sex steroid hormones in both sexes during the first year after birth which is known to be small but significant. This could perhaps saturate the sex steroid receptors, which are present in cartilage at much lower concentrations than in other classical target tissues.

The estrogens may act on cartilage during skeletal growth in a manner analogous to the way E_2 regulates IGF_1 gene expression in classical target tissues such as uterus [21] or in bone cells *in vitro* [22, 23]. Embryonic chick cartilage [24] and post-natal rabbit chondrocytes [25] have been shown to produce local IGF_1 *in vitro*. Sex steroid hormones could thus regulate chondrocyte proliferation and/or differentiation via this local growth factor.

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