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Testosterone Stimulates Growth of Tibial Epiphyseal Growth Plate and Insulin-Like Growth Factor-1 Receptor Abundance in Hypophysectomized and Castrated Rats

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Puberty is associated with an increase in the plasma concentration of sex steroids, growth hormone (GH), and insulin-like growth factor-1 (IGF-1). Gonadal steroid hormones are important for the normal pubertal growth spurt and skeletal growth. The mechanism by which gonadal steroids induces skeletal growth is still not fully understood. To study the GH-independent effect of testosterone on growth, we investigated the effect of testosterone injections on the tibial epiphyseal growth plate (EGP) in an in vivo model of hypophysectomized and castrated male rats. Four groups (six animals each) of 28-d-old male rats were studied. Groups A, B, and C were hypophysectomized and castrated and received 500 µg/(kg·d) of hydrocortisone and 15 µg/(kg·d) of levothyroxine sodium. Groups A and B were also treated with daily sc injections of 10 µg of testosterone/100 g of body wt and 100 µg of testosterone/100 g of body wt, respectively, for 7 d. Group C was injected with vehicle alone. Group D were intact animals injected with saline (controls). Animals were sacrificed on 8 d. As expected, serum GH levels were found to be very low (1.13 ± 0.1 ng/mL) in the hypophysectomized animals (group C, hypopit), and testosterone treatment did not change them significantly. Serum IGF-1 decreased from 502.9 ± 13 ng/mL in group D to 167 ± 41.4 ng/mL in group C ($p < 0.001$). Testosterone therapy had no stimulatory effect on serum IGF-1 levels in the hypopit + low-dose group (A) (220 ± 94.8 ng/mL) and had an inhibitory effect in the hypopit + high-dose group (B) (39.3 ± 17.5). Histomorphometric determinations demonstrated an EGP width of 472.3 ± 39 µm in the intact animals but only 336.9 ± 1.6 µm in the hypopit group (C) ($p < 0.01$). High-dose testosterone treatment (group B) significantly increased the EGP width (to 438.8 ± 27.8), ($p < 0.001$), whereas low-dose testosterone (group A) did not. Immunohistochemistry studies revealed that the levels of IGF-1 in the EGP of the control animals were almost negligible and that testosterone did not change them. However, testosterone increased in a dose-dependent manner the abundance of IGF-1 receptor EGP. We conclude that testosterone has a direct, local, GH-independent effect on the EGP growth and IGF-1 receptor abundance.

Key Words: Testosterone; IGF-1; epiphyseal growth plate.

Introduction

Normal puberty is associated with an increase in the plasma concentration of sex steroids, insulin-like growth factor-1 (IGF-1), and growth hormone (GH) *(1–3)*. The pubertal growth spurt is determined by the combined effects of these hormones, together with other endocrine, paracrine, and autocrine factors, on the epiphyseal growth plate (EGP) *(1–5)*. The precise mechanisms by which the gonadal steroids stimulate skeletal growth remain unclear, mainly because of the difficulty in isolating their effect from the various other hormones and growth factors. The concomitant increase in the levels of gonadal steroids and GH during the pubertal growth spurt supports the notion that GH may mediate the effect of sex hormones on growth *(6,7)*. Indeed, it has been demonstrated that androgens stimulate GH secretion, and thereby the increment in serum IGF-1 level *(8)*. It has also been demonstrated that androgens are able to stimulate GH secretion via their aromatization to estrogen *(9)*. However, some researchers claim that androgens directly stimulate skeletal growth independent of the involvement of the GH-IGF-1 axis. This is supported by findings of a pubertal growth spurt even in children with GH deficiency and precocious puberty *(10)*.

In an in vitro model, we have recently shown that testosterone stimulates growth and local production of IGF-1 and IGF-1 receptor (IGF-1R) in chondrocyte cell layers of an isolated organ culture of mice mandibular condyle (a model of endochondral ossification) and that part of

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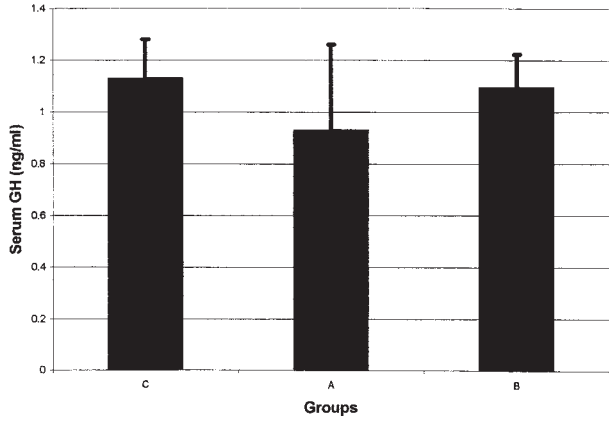

Fig. 1. Serum GH levels in hypophysectomized and castrated rats treated with saline alone (group C, hypopit), 10 µg of testosterone/100 g of body wt (group A, hypopit + low dose), or 100 µg of testosterone/100 g of body wt (group B, hypopit + high dose). Testosterone treatment had no significant effect on serum GH levels. Values are the mean ± SEM; $n = 6$ in each group.

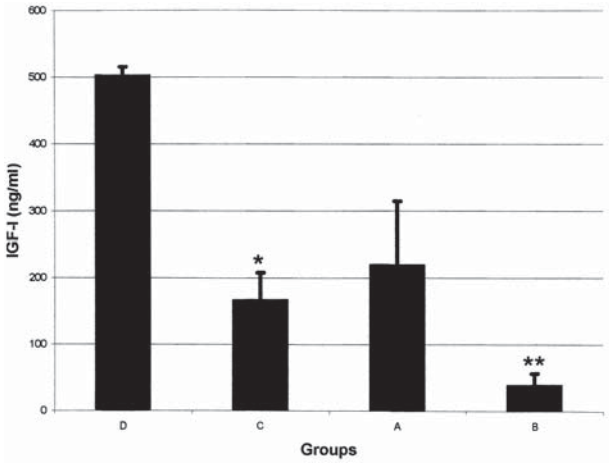

Fig. 2. Serum IGF-1 showed a significant decrease in group C (hypopit) compared with group D (controls) (*$p < 0.001$). Administration of 10 µg of testosterone/100 g of body wt (group A, hypopit + low dose) for 7 d had no significant effect on the serum IGF-1 levels. At a higher dose (group B, hypopit + high dose), serum IGF-1 levels dropped significantly (**$p < 0.03$; see double asterick. Values are the mean ± SEM; $n = 6$ in each group.

testosterone's effect is mediated by local IGF-1 and IGF-1R *(11)*. The present study was aimed at elucidating whether the direct stimulatory effect of testosterone observed in the in vitro model of cartilage growth is also valid in the in vivo model of hypophysectomized and castrated male rats.

Results

Effect of Hypophysectomy on Serum GH and IGF-1 Levels

As expected, serum GH levels were very low (1.13 ± 0.1 ng/mL) in the hypophysectomized animals (group C, hypopit). Testosterone treatment had no significant effect on serum GH levels in the hypophysectomized rats, which measured 0.9 ± 0.3 and 1.1 ± 0.137 ng/mL in groups A (hypopit + low dose) and B (hypopit + high dose), respectively (Fig. 1). Serum IGF-1 showed a significant difference between the intact animals (group D) and the hypopit group (C) (502.9 ± 13 vs 167 ± 41.4 ng/mL, $p < 0.001$). Administration of 10 µg of testosterone/100 g of body wt for 7 d (hypopit + low dose) had no significant effect on serum IGF-1 levels, which measured 220 ± 94.8 ng/mL, whereas 100 µg of testosterone/100 g of body wt (hypopit + high dose) induced a significant decrease to 39.3 ± 17.5 ng/mL ($p < 0.03$) (Fig. 2).

Effect of Testosterone on Width of Tibial EGP

Morphometric analysis of the testosterone-treated EGP was performed on hematoxylin and eosin (H&E)-stained sections. As shown in Fig. 3, the overall width of the EGP of the hypophysectomized rats (group C) was reduced by 28.8% compared with the width of the intact animals (group D) (472.3 ± 39 vs 336.9 ± 6 µm; $p < 0.001$). In rats treated with 100 µg of testosterone/100 g of body wt (hypopit + low dose), EGP width increased by only 6% compared with that of untreated hypophysectomized rats (group C) (p = NS). Treatment with 100 µg of testosterone/100 g of body wt (hypopit + high dose) increased EGP with further and by 30% (to 438.8 ± 27.8; $p < 0.01$), which was significantly higher than that of the untreated castrated and hypophysectomized rats, and an EGP width was reached that was not significantly different from that of the intact animals (group D).

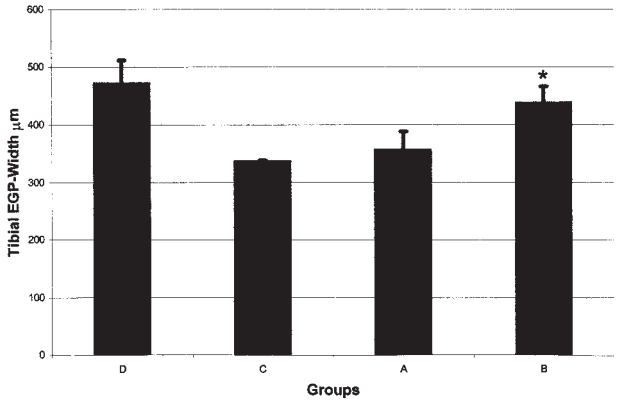

Fig. 3. Morphometric analysis of width of tibial EGP based on H&E-stained sections from hypophysectomized rats treated with 10 µg of testosterone/100 g of body wt (group A, hypopit + low dose) or 100 µg of testosterone/100 g of body wt (group B, hypopit + high dose) or saline alone (group C, hypopit) and intact rats (group D). *$p < 0.01$ compared to the untreated hypophysectomized rats (group C); **$p < 0.001$ for comparison between groups C and D. There was no significant differences between group B and group D (intact rats). Values are the mean ± SEM; $n = 6$ in each group.

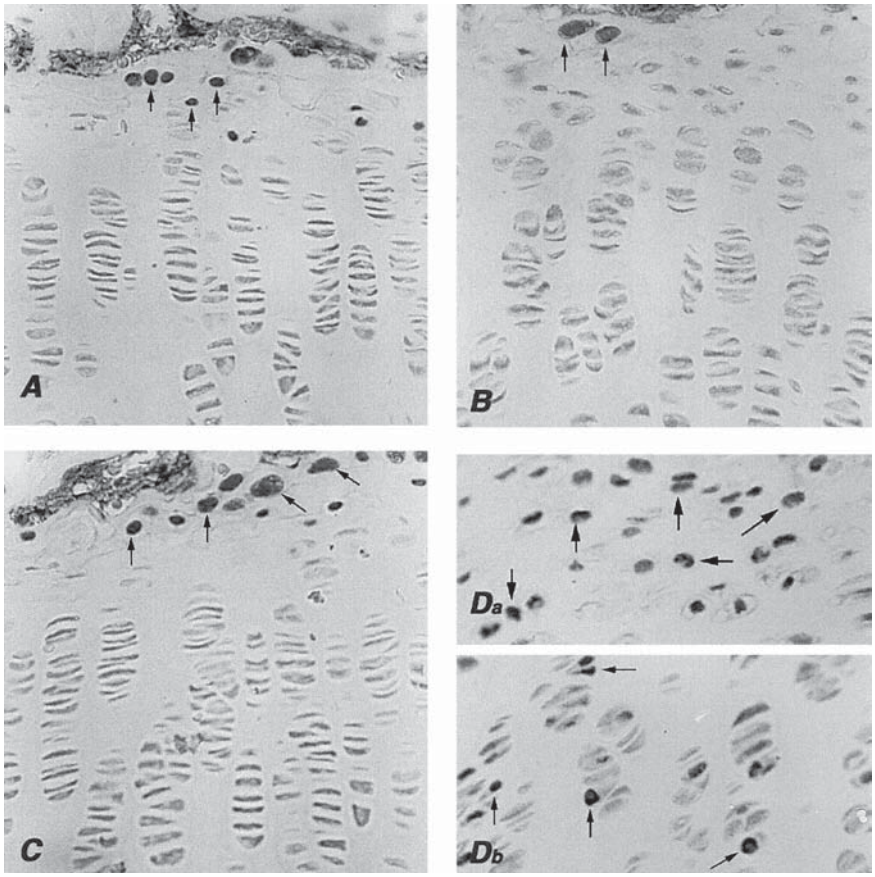

Fig. 4. Immunohistochemical localization of the IGF-1R in the tibial EGPs in sections derived from intact rats (**A**) and hypophysectomized rats (**B**) treated with 10 μg of testosterone/100 g of body wt (**C**) or 100 μg of testosterone/100 g of body wt (**Da,b**). Deparaffinized sections were incubated with rabbit anti-IGF-1R (α-subunit) and analyzed with biotin-avidin-peroxidase assay. Note that a high dose of testosterone significantly increased IGF-1R within resting cells (Da) and young and mature chondrocytes (Db); in the intact animals and the animals treated with lower doses of testosterone, positive staining was confined to the resting cells alone (original magnification ×240). Arrows indicate positive immunohistochemical reaction with IGF-1R.

Effect of Testosterone on IGF-1 and IGF-1-R

Testosterone therapy did not change the abundance of IGF-1 in the EGP of the hypophysectomized and castrated animals (data not shown), but it did affect the abundance of IGF-1R and IGF-1R mRNA. Immunohistochemical localization of the IGF-1R (Fig. 4) demonstrated a dose-response increment in IGF-1R abundance in the testosterone-treated hypophysectomized rats but not in the untreated hypophysectomized rats (group C, hypopit), in which almost no IGF-1R was detected (Fig. 4B). The EGPs of the animals treated with10 μg of testosterone/100 g of body wt (hypopit + low dose) showed a higher abundance of IGF-1R (Fig. 4C) than in the intact rats (Fig. 4A), but they were similarly confined to the resting zone. At a higher dose (100 μg of testosterone/100 g of body wt, hypopit + high dose), induced a significant increase in the abundance of IGF-1R in the resting zone (Fig. 4Da) as well as in the chondroblasts and chondrocytes (Fig. 4Db).

In situ hybridization (ISH) of IGF-1R mRNA showed that IGF-1R expression in the EGP of the hypophysectomized and castrated group (group C) was almost negligible (Fig. 5A). Testosterone at a dose of 100 μg/100 g of body wt (group B, hypopit + high dose) caused an increase in IGF-1R mRNA, mainly in the nonhypertrophic chondrocytes (Fig. 5B).

Discussion

In the present study, 7 d of testosterone injections in hypophysectomized and castrated rats increased EGP width. The stimulatory effect of androgens on growth independent of the presence of GH has been demonstrated in both human and animal models. Growth acceleration has been reported during puberty in children with GH deficiency owing to central nervous system lesions *(10)* and in children with GH insensitivity syndrome *(12)*. Furthermore, growth acceleration with anabolic steroid treatment has been demonstrated in patients with complete GH deficiency owing to GH gene deletion *(13)*. According to in vivo studies of hypophysectomized prepubertal lambs, testosterone can stimulate growth in the absence of GH *(14)*. Similar results were demonstrated after the administration of testosterone alone for 7 days to hypophysectomized castrated Sprague-Dawley rats *(15)*.

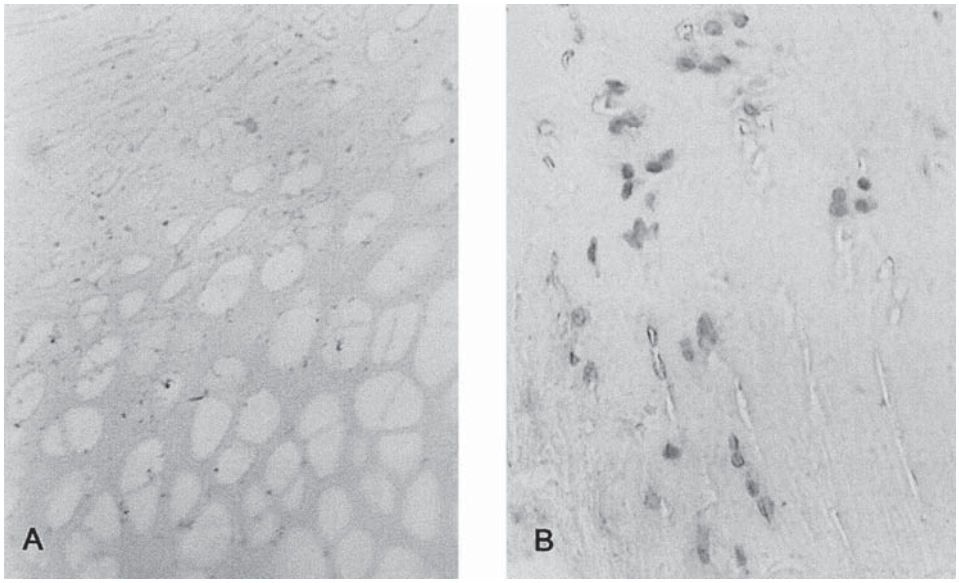

Fig. 5. Use of ISH to localize the IGF-1R mRNA within the EGPs in sections from hypophysectomized rats treated with vehicle alone (group C, hypopit) (**A**) or 100 μg of testosterone/100 g of body wt (group B, hypopit + high dose) (**B**). Assay was performed on deparaffinized sections of routinely processed tibial bones using digoxigenin-labeled antisense IGF-1R mRNA and peroxidase-conjugated antibodies. Note that hypophysectomy completely abolished the expression of IGF-1R (A). Testosterone (100 μg/100 g of body wt) (B) induced an increase in the abundance of the receptor throughout the whole chondrocytic population (original magnification ×240).

The mechanism by which testosterone exerts its GH-independent effect on growth in vivo was not fully elucidated. One possible mechanism is direct testosterone stimulation of the liver, the major source of circulatory IGF-1, to increase the secretion of this growth factor. Our group, however, already ruled out this possibility in an earlier study *(16)*. Similarly, in the present study, serum IGF-1 levels in the hypophysectomized animals were significantly lower than in the intact animals, and they did not increase after testosterone injection. Indeed, injections of 100 μg of testosterone actually significantly suppressed the serum IGF-1 level much like the effect observed previously by our group in castrated, not hypophysectomized, rats *(17)*. In that study, we suggested the possibility of a direct local stimulatory effect of testosterone on bones, which was supported by the unilateral increase in tibial EGP width under direct testosterone administration *(18)*. Further support was provided by our recent in vivo observation of a GH-independent direct stimulatory effect of testosterone on cartilage growth in mouse mandibular condyle, which serves as a model of endochondral ossification *(11)*. We were also able to show that this direct stimulatory effect of testosterone is mediated, at least partially, by local upregulation of IGF-1R gene expression.

In the present study, we found that testosterone increases, in a dose-response manner, the responsiveness to IGF-1, which is reflected by an increase in the abundance of the IGF-1R. In the untreated hypophysectomized rats, IGF-1R was hardly demonstrated at all, whereas in the animals treated with10 μg of testosterone/100 g of body wt (hypopit + low dose), IGF-1R was clearly demonstrated in the EGP localized to the resting zone cells. A higher dose of testosterone (100 μg/100 g of body wt, hypopit + high dose) induced a significant increase in the abundance of IGF-1R in the resting zone, and also in the chondroblasts and chondrocytes. This effect was preferentially expressed in the young chondrocytic population of the growth plate. The expression of IGF-1R reflects the sensitivity of tissue cells to IGF-1 activity *(19)*.

Although IGF-1R gene is expressed constitutively, in most cells, its expression is also highly regulated by a wide spectrum of factors. It is upregulated by various hormones and factors that exert anabolic effects *(20,21)*, and by certain pathologies such as cancer *(22,23)*. It varies dramatically with developmental changes *(24,25)*. Being a regulator of IGF-1 activity, IGF-1R can also be downregulated in order to buffer over IGF-1 activity, as described in cases of massive treatment with GH of constitutionally short children *(26)*. IGF-1R regulation has been widely reviewed by Sepp-Lorenzino *(27)*. The biologic activity of testosterone, an anabolic hormone, is connected with certain developmental stages, such that it exerts its stimulatory effect on skeletal growth by upregulating the expression of IGF-1R with the EGP.

These results suggest that testosterone has a GH-independent stimulatory effect on skeletal growth that is exerted directly on the EGP. The mechanism of this effect is probably mediated by increasing responsiveness to IGF-1. This is reflected by the increase in the level of IGF-1R.

Materials and Methods

Animals

Male Sprague-Dawley rats, 4 wk of age, were purchased from Harlen (Jerusalem, Israel). Animal breeding complied with the National Institutes of Health guide for the care and use of laboratory animals and was authorized by the committee for the ethical care and use of laboratory animals of the Ben Gurion University. Animals were housed in standard laboratory cages and fed normal rat chow ad libitum with free access to unlimited supplies of tap water. The rats were divided into four groups of six animals each. Groups A, B, and C were hypophysectomized and castrated by the supplier (DVM Nava Nevo, Weizmann Institute of Science, Rehovot, Israel). They received replacement therapy consisting of 15 μg/(kg·d) of levothyroxine sodium (Knoll, New York) and 500 μg/(kg·d) of hydrocortisone (Abic, Netanya, Israel) *(16,17)* and were provided salt-supplemented water containing 9.0 g of NaCl/L. Groups A and B also received daily sc injections of 10 μg of testosterone/100 g of body wt and 100 μg of testosterone/100 g of body wt (Fluka, Chemika-BioChemika, Buchs, Switzerland), respectively, for 7 d. Group C received 7 sc injections of the vehicle alone. Group D consisted of intact animals, which received sc injections of saline for 7 d and served as controls. Groups were designated as A = hypopit + low dose, B = hypopit + high dose, C = hypopit, and D = intact. All rats were sacrificed 7 d after the first injection. Trunk blood was collected, and serum was separated and frozen at –20ºC for later measurements of GH and IGF-1 levels. Two tibias from each rat were carefully removed and fixed in 4% paraformaldehyde for histomorphologic assessment, immunohistochemistry and ISH studies.

Serum IGF-1

Serum IGF-1 was measured by the functional separation method *(28)* in which excess IGF-II blocks the interference of IGF-binding proteins (IGFBPs). Serum (10 μL) was diluted 1:100 in acidic buffer (0.02 *M* NaH_2PO_4; 0.1 NaCl; 0.2% bovine serum albumin [BSA]; 0.02% NaN_3; 0.1% Triton X-100, pH 2.8) for 1 h at room temperature. Anti-IGF-1 antiserum (Fujisawa, Osaka, Japan) was diluted 1:70,000 in assay buffer (0.1 *M* phosphate-buffered saline [PBS], 0.2% BSA; 0.02% NaN_3; 0.1% Triton X-100, pH 7.8). Thereafter, 100 μL of samples and recombinant human IGF-1 standards (Fujisawa) were incubated at 4ºC for 20 h with 50 μL of IGF-2 (PeproTech, Rocky Hill, NJ) (100 ng/mL), 400 μL of antiserum, and 25,000 cpm/50 μL of ^{125}I IGF-1). Bound and free antibody were separated by incubating with a pre-precipitated suspension of antirabbit IgG (Sigma, St. Louis, MO): normal rabbit serum (1%:0.1%) in 5% polyethylene glycol (PEG) (1 mL). IGF-2 (5 ng/tube) completely reversed the interference of human recombinant IGFBP-3 (Upstate Biotechnology, Lake Placid NY) (5 ng/tube). The sensitivity was 0.02 ng of IGF-1/tube; the intraassay coefficients of variation (%CV) for sera with IGF-1 levels of 80, 212, and 412 ng/mL were 8.9, 2.9, and 9.6%, respectively. All sera were run in the same assay.

Serum GH

Serum GH was measured with a modified radioimmunoassay *(29)*. Rat sera (150 μL) were incubated with antirat GH antiserum (donated by Dr. A. F. Parlow) diluted 1:125,000 in assay buffer (0.01*M* PBS; 0.02% NaN_3; 0.33% EDTA; 0.5% BSA; pH 7.5) containing 1% normal monkey serum (Harlan, Rehovot, Israel) (100 μL). Rat GH standards (donated by Dr. A. F. Parlow) (100 μL) and ^{125}I mouse GH (AFP-10783B; donated by Dr A. F. Parlow) (200 μL) were incubated for 22 h at room temperature. Bound and free antibodies were separated by incubating with antimonkey IgG diluted 1:2.5 (ICN, Aurora, OH) (100 μL) with the addition of ice-cold 5% PEG (1 mL) for 2 h at 4ºC. All sera were run in the same assay.

Morphology and Morphometric Studies

Paraffin sections (6 mm) were deparaffinized in xylene, hydrated in graduated ethanols, and stained in H&E. Stained sections served for morphometric studies. Histomorphometric determinations of the total length of the cartilaginous zone were performed with an Olympus Cue-2 image analysis system using appropriate morphometry software (Olympus, Lake Success, NY). The system consists of a Zeiss Universal R photomicroscope (×10 objective) fitted with a Panasonic WV-CD50 camera and a Sony 14-in. color monitor connected to an IBM-compatible PC. The width of the EGP was measured by drawing a straight line from the epical border of the reserve zone cells layer to the lower border of the mineralized cartilage. Each point represents the average of measurements done on four slides from each of three different experiments. Significance was determined according to the two-tailed student's *t*-test.

Immunohistochemistry

Deparaffinized paraffin sections were exposed for 2 h at room temperature to specific antibodies: sheep anti-IGF-1 polyclonal antibody (cat. no. AB1011; Chemicon) (no cross-reaction with IGF-2) and rabbit anti-IGF-1R (anti-IGF-1R) (anti α-subunit) (cat. no. SC-712; Biotechnology, Santa Cruz, CA). This was followed by incubating with an appropriate biotinylated second antibody, with streptavidin-peroxidase conjugate and aminoethylcarbozole (AEC) as a substrate (Histostain-SP kit; Zymed). Counterstaining was done with hematoxylin. Nonspecific binding was blocked with 10% nonimmunized serum (rabbit or goat). As negative controls, slides were incubated with the relative nonimmune serum, i.e., mouse serum for nonclonal antibodies and rabbit serum for polyclonal antibodies.

In Situ Hybridization

Paraffin sections (6 μm) were loaded on precleaned poly-L-lysine-coated slides, deparaffinized with xylene, hydrated

with graduated ethanols, and treated with 3% H_2O_2 in methanol to neutralize endogenous peroxidase. Sections were then treated for 15 min with 12.5 µg/mL of proteinase K, rinsed with 2 mg/mL of glycine, and acetylated in 0.5% acetic anhydride in 0.1 *M* Tris, pH 8.0. Sections were post-fixed with 4% paraformaldehyde/PBS and prehybridized for 10 min in 2X saline sodium citrate (SSC) followed by 1 h in hybridization buffer (50% formamide, 0.5 mg/mL of salmon sperm DNA, 4X SSC, 1X Denhardt's solution). Hybridization was done overnight (18 h) at 42ºC in maximal humidity with 5 ng/µL digoxygenin (Dig)-labeled probe. At the end of the incubation period, slides were rinsed in SSC under increasing stringency conditions and then with 0.1 *M* Tris and 0.15 *M* NaCl, pH 7.5. Hybrids were detected using anti-Dig antibodies conjugated with peroxidase (Boehringer Mannheim, Germany) and AEC as a substrate and counterstained with hematoxylin. As negative controls, we used parallel sections reacted with the same concentration of Dig-labeled pSPT18-Neo antisense RNA transcribed with T7 from *Pvu*II-linearized pSPT18-Neo DNA (supplied with the kit).

Dig-Labeled Antisense RNA Probes for ISH

We used probes for mouse IGF-1R cloned in pBluescript SK^+ amp^+ for mouse IGF-1 cloned in pGEM3 amp^+ (386 bp). After linearization, antisense RNA was transcribed with (Sp6/T7) a Dig-RNA labeling kit (Boehringer Mannheim) according to the manufacturer's instructions.

Statistical Analyses

One-way analysis of variance was used to evaluate differences among groups for multiple comparisons. A p value of <0.05 was considered significant. Means are given as ±SEM.

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