IN VITRO EFFECTS OF GLUCOCORTICOID HORMONES ON THE SYNTHESIS OF DNA IN CARTILAGE OF NEONATAL MICE

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1. Introduction

One of the most conspicuous effects of the glucocorticoid hormones is an inhibition of somatic growth in immature animals [1]. The administration of pharmacological doses of glucocorticoids to neonatal animals was found to suppress cartilage growth and bone formation [2,3]. Glucocorticoid-induced suppression of cartilage cell proliferation is significant within 24 h and is virtually complete within 72 h of a single dose of the hormone. Recent evidence has shown that mammalian chondrocytes possess high affinity glucocorticoid receptor proteins which bind dexamethasone [4]. This study presents evidence that in organ cultures of mouse neonatal condylar cartilage the presence of glucocorticoid hormone markedly suppresses cell proliferation and that the characteristic of this suppression is similar to that observed in neonatal mice cartilage in vivo. Moreover, this growth inhibitory effect is dose-dependent, specific to fluorinated corticoids, and is dependent upon an early stimulation of specific RNA and protein synthesis.

2. Materials and methods

2.1. *Organ culture system*

Condylar cartilage was microdissected under sterile conditions from the mandibular joint of newborn ICR (Institute Cancer Research, New York) mice. The cartilage was cleaned of all surrounding soft tissues and was stored in Hank's solution at 4°C. Isolated explants were then transferred to plastic disposable culture dishes (6.0 cm in diameter, Falcon) and were cultured (in a submerged form) in 4 ml of Gibco medium (MEM No. 410-1100) supplemented with 10% fetal calf serum (Gibco), 100 μg/ml ascorbic acid; 50 μg/ml glycine; 100 IU/ml penicillin; 100 μg/ml streptomycin and maintained at 37°C in an atmosphere of 95% air and 5% CO_2. The phosphate concentration in the medium was 3.0 mM and in order to maintain a stable pH (between 7.25–7.40) the medium was supplemented with 30 mM of sodium bicarbonate. Tissues were cultured for periods ranging from 2–74 h, and the medium was changed after 48 h.

Triamcinolone acetonide (Sigma) was initially dissolved in absolute ethanol and was subsequently added to the culture medium at concentrations ranging from 10^{-5}–10^{-10} M. Dose-response determinations were carried out on condyles that were cultured for 24 h. The solvent's (ethanol) final concentration was 0.1%, and control cultures contained the solvent at the above concentration. Dexamethasone, hydrocortisone, corticosterone, deoxycorticosterone, 17β-estradiol, progesterone and cortexolone were all purchased from Sigma, and were used for 'specificity' tests. All the above tests were performed on specimens that were cultured for 24 h.

2.2. *[^{3}H]Thymidine incorporation*

DNA synthesis was measured through the incorporation of [^{3}H]thymidine (5.0 Ci/mmol, Radiochemical Center Amersham into trichloroacetic acid (TCA) – precipitable fractions. Test and control condyles were labeled for 2 h in media that contained 2.0 μCi/ml of methyl-[^{3}H]thymidine. Following extraction with 5% TCA (30 min × 2; 4°C), tissues were dried with acetone (10 min), ether (10 min) and finally air dried. Individual specimens were then dissolved in 0.4 ml of

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soluene 350 (Packard) for 18 h at room temperature followed by 1 h at 60°C. Four ml of scintillation fluid (3 g PPO; 0.1 g POPOP and 2 ml glacial acetic acid in one liter of toluene) were added to each sample and were subsequently counted in a Packard Tri Carb/544 liquid scintillation spectrometer.

In order to determine the effects of RNA and protein synthesis inhibitors on the growth retardative effect of triamcinolone, condyles were initially preincubated for 2½ h in our routine medium and were subsequently cultured for 3 h in separate dishes that contained either triamcinolone acetonide (10^{-6} M), cycloheximide 2×10^{-4} M, puromycin 10^{-7} M or actinomycin D 4 μg/ml alone, or a combination of the hormone with one of the above inhibitors (all purchased from Sigma). [^{3}H]thymidine was added to all dishes 30 min prior to the termination of the cultures.

2.3. *[^{3}H]Thymidine autoradiography*

Test and control condyles that were cultured for 24 h were labeled with [^{3}H]thymidine during the last 2 h of culture (2.0 μCi/ml). Following fixation in 5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, at 4°C for 90 min they were dehydrated through graded ethanols and embedded flat in Epon. Thin (1 μm) sections for light microscopy were cut in the coronal plane using glass knives. Sections were mounted on glass slides, dipped in Ilford G-5 emulsion (diluted 1:1 with distilled water), warmed to 42°C. The latter were dried at room temperature and stored for 3 weeks at 4°C in light-tight boxes. Kodak D-19 was used for development, and after fixation the sections were stained with 0.1% toluidine blue.

3. Results

3.1. *Effects of triamcinolone on the incorporation of [^{3}H]thymidine: time-course response*

Two peaks of [^{3}H]thymidine incorporation were noted in control specimens: at 3 and 24 h (fig.1). Test condyles that were cultured in the presence of 10^{-6} M

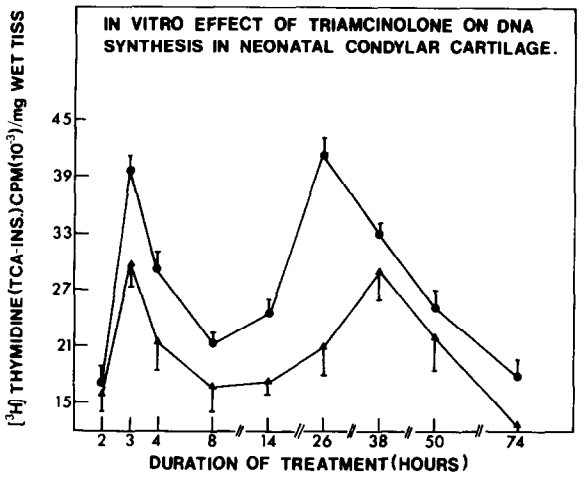

Fig.1. Mean curves indicating time-course effect of triamcinolone acetonide (10^{-6} M) upon the incorporation of [^{3}H]thymidine. Note the early inhibitory effect of the hormone, already 3 h after its application to the culture system. ●——● = control; ▲——▲ = hormone-treated.

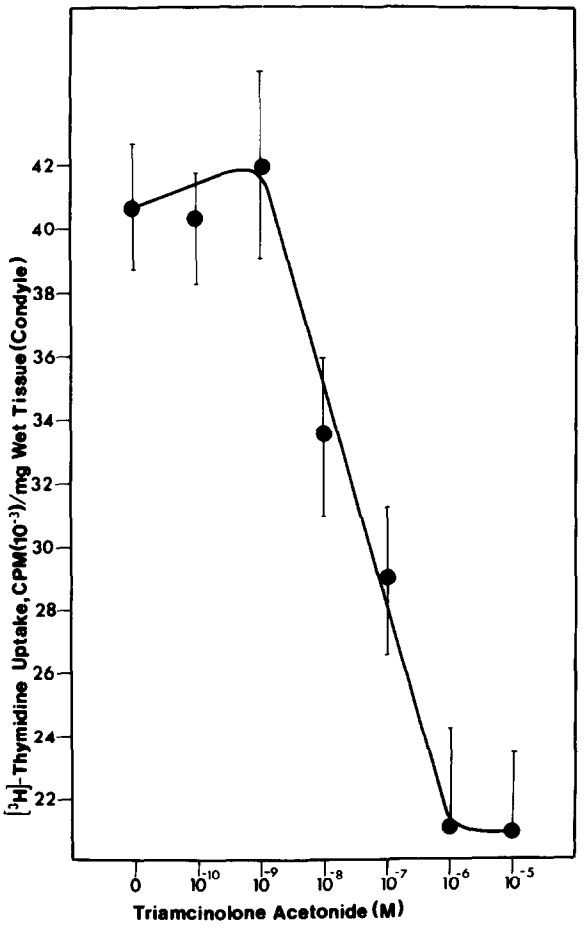

Fig.2. Effect of triamcinolone acetonide upon the incorporation of [^{3}H]thymidine by neonatal condylar cartilage: dose-reponse relationship. Tissues were cultured for 24 h and the isotope was added to the medium 2 h prior to the termination of the culture.

of triamcinolone acetonide incorporated significantly less [^{3}H]thymidine into TCA-precipitable material (fig.1). The hormone's retardative effect was first noted after 3 h when a decrease of 22% ($p < 0.01$) was recorded. The maximal effect occurred after 24 h when hormone-treated condyles revealed a deficit of 43% ($p < 0.001$).

3.2. *Effects of triamcinolone on the incorporation of [^{3}H]thymidine: dose-response relationship*

The effect of increasing doses of triamcinolone acetonide upon the incorporation of [^{3}H]thymidine is shown in fig.2. No significant differences were observed at concentrations of 10^{-9} and 10^{-10} M. With the use of a higher concentration (10^{-8} M), test condyles exhibited a deficit of 14% ($p < 0.002$); a feature that further intensified with increasing doses: 10^{-7} M led to a deficit of 23.1% ($p < 0.001$) whereas 10^{-6} M caused a reduction of 42.7% ($p < 0.001$). Thereafter, at a dose of 10^{-5} M, the tissue's response followed a plateau.

3.3. *Specificity of fluorinated synthetic analogues of glucocorticoids on the incorporation of [^{3}H]thymidine*

Triamcinolone and dexamethasone, both fluorinated glucocorticoids, were the only compounds that induced significant inhibitory effects upon the incorporation of [^{3}H]thymidine by cartilage cells at a dose of 10^{-8} M. Other non-fluorinated glucocorticoids (hydrocortisone and corticosterone) and non-glucocorticoid steroids (deoxycorticosterone, cortexolone, 17β-estradiol and progesterone) lacked a similar inhibitory effect at the above-mentioned concentration (table 1).

3.4. *Involvement of RNA and protein synthesis inhibitors on corticosteroid-induced suppression of [^{3}H]thymidine incorporation*

The addition of actinomycin D, cycloheximide and puromycin to the experimental system completely abolished the inhibitory effect of triamcinolone. Control cultures that contained each of the above inhibitors without the presence of triamcinolone, did not show any significant differences in the incorporation of [^{3}H]thymidine in comparison to 'nontreated' cultures.

3.5. *Cell-specificity for the antiproliferative activity of triamcinolone*

The present autoradiographic studies indicated that the target cells for the hormone's antiproliferative activity resided, exclusively, within the perichondrial layer which for the most part contained indifferentiated chondroprogenitor cells. Following the hormonal treatment the number of labeled cells along the zone of chondroprogenitor cells markedly decreased (fig.3,4).

Table 1
The effect of various steroid hormones on the incorporation of [^{3}H]thymidine in cultured condylar cartilage

Hormone used	Inhibition of [^{3}H]thymidine inc. (%)	Significance
Triamcinolone acetonide		
10^{-8} M	−18.0	<0.002
10^{-7} M	−31.0	<0.001
10^{-6} M	−48.0	<0.001
Dexamethasone		
10^{-8} M	−21.3	<0.002
10^{-7} M	−28.2	<0.002
10^{-6} M	−44.0	<0.001
Hydrocortisone		
10^{-8} M	− 3.3	N.S.
10^{-7} M	−13.4	<0.002
10^{-6} M	−14.4	<0.002
Corticosterone		
10^{-8} M	+ 0.96	N.S.
10^{-7} M	− 6.9	<0.02
10^{-6} M	−16.0	<0.005
Deoxycorticosterone		
10^{-8} M	+ 2.0	N.S.
10^{-7} M	− 4.7	N.S.
10^{-6} M	−11.9	<0.01
Estradiol		
10^{-8} M	− 0.03	N.S.
10^{-7} M	− 5.5	≈0.02
10^{-6} M	−14.7	<0.01
Progesterone		
10^{-8} M	− 0.66	N.S.
10^{-7} M	− 6.7	<0.02
10^{-6} M	−49.1	<0.001
Cortexolone		
10^{-8} M	+ 1.0	N.S.
10^{-7} M	+ 1.5	N.S.
10^{-6} M	− 2.0	N.S.

N.S. = not significant

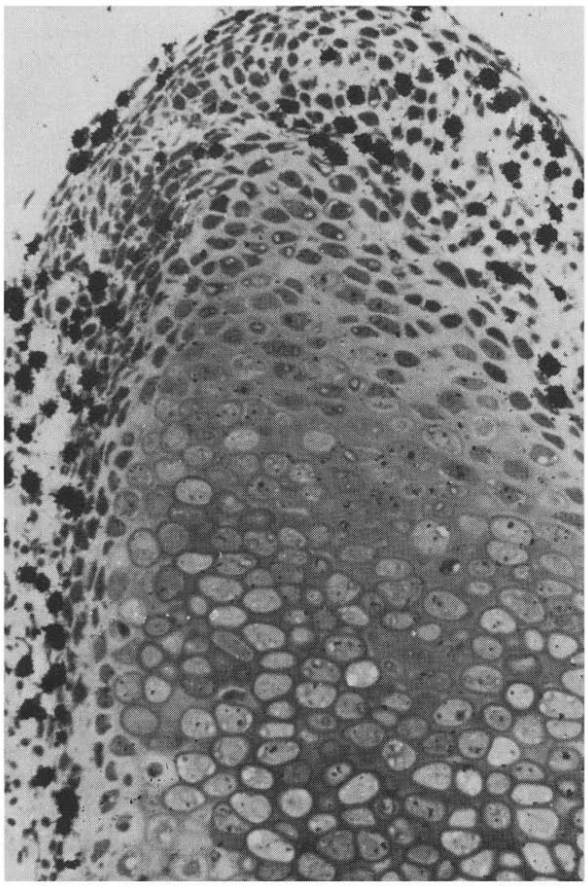

Fig.3. Coronal section through cultured condylar cartilage (24 h), that received one pulse (2 h) of [^{3}H]thymidine and subsequently processed for autoradiography. Note that almost all the labeled cells are concentrated within the perichondrium that surrounds the cartilage and contains all the cartilage progenitor cells. Toluidine blue. ×240.

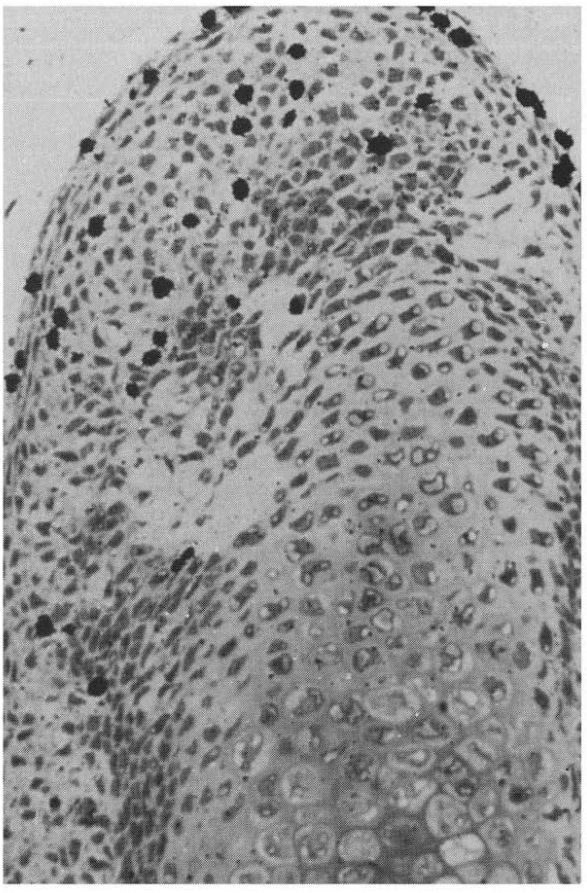

Fig.4. A similar section to that shown in fig.3 but obtained from a condyle that was cultured for 24 h in the presence of 10^{-6} M of triamcinolone acetonide. Note the marked decrease in the number of labeled cells throughout the zone of progenitor cells. Toluidine blue. ×240.

4. Discussion

The present study was undertaken to evaluate the effect of glucocorticoid hormones on the growth of chondrocytes in an in vitro experimental system. It became apparent that triamcinolone acetonide and dexamethasone, both synthetic fluorinated analogues induced a marked reduction in the incorporation of thymidine into proliferating cartilage cells, a feature that was dependent upon the novo synthesis of RNA and protein. There was a relatively short latent period, as by 3 h a significant decrease in thymidine incorporation was observed. The characteristic of this suppressive effect was very similar to that noted in neonatal mice condylar cartilage in vivo: suppression of both thymidine incorporation and cell proliferation very early after the exposure of the tissue to the hormone. Also, as in the intact animal, low concentrations (10^{-8} M) of hormone were required for this retardative effect [5].

The observations described here suggest that our organ culture system may serve as a useful experimental model for the problem of glucocorticoid-induced suppression of somatic growth. Recent and as yet unpublished studies in our laboratory imply that the above antianabolic effects of triamcinolone are due, at least in part, to a direct effect through a receptor-mediated mechanism.

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