Dexamethasone Regulation of Glycosaminoglycan Synthesis in Cultured Human Skin Fibroblasts

Similar Effects of Glucocorticoid and Thyroid Hormones

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Abstract. The effects of dexamethasone on glycosaminoglycan accumulation were examined in confluent human skin fibroblasts in vitro. The glucocorticoid consistently inhibited the incorporation of either [^{3}H]acetate or [^{3}H]glucosamine into hyaluronate when added to culture medium 72 h before harvest. This effect was half-maximal at ~1 nM and maximal at 5–10 nM. Inhibition occurred within 5 h of hormone addition and was near maximal by 25 h. 11α-hydrocortisone (10 nM), deoxycorticosterone (10 nM), and progesterone (100 nM) failed to inhibit this accumulation; however, progesterone (2 μM), a known glucocorticoid antagonist at high concentration, could attenuate the response to dexamethasone by 57%. Cultures were pulse-labeled and then chase incubated for up to 68 h. No difference in the rate of [^{3}H]hyaluronate degradation could be demonstrated in steroid-treated cultures. Triiodothyronine (T_3) can also inhibit synthesis of hyaluronate in fibroblasts (Smith, T. J., Y. Murata, A. L. Horwitz, L. Philipson, and S. Refetoff, 1982, *J. Clin. Invest.*, 70:1066–1073). Both T_3 and dexamethasone could inhibit glycosaminoglycan accumulation in a dose-dependent manner. Maximal T_3 effects were achieved at 1 nM and those of dexamethasone at 10 nM. Saturating concentrations of T_3 and dexamethasone added alone inhibited [^{3}H]hyaluronate by 54 and 49%, respectively. When both hormones were added, accumulation was inhibited by 84%. Dexamethasone inhibits [^{3}H]hyaluronate accumulation in a time, dose-dependent, and stereo-specific manner. The rate of glycosaminoglycan degradation was unaffected, and thus, the steroid inhibited the rate of macromolecular synthesis. This effect was likely mediated through glucocorticoid receptors. Hyaluronate synthesis in skin fibroblasts appears to be regulated by both glucocorticoids and T_3 through different pathways.

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Introduction

Glycosaminoglycans (GAG)[1] are complex sugars that comprise dermal ground substance (1). While the mechanisms involved in the synthesis and the biological role of GAG remain controversial, some human diseases are associated with an abnormal accumulation of these substances: Marfan's syndrome (2), Graves' disease (3), and hypothyroidism (4).

Human skin fibroblasts are easily obtained, can be propagated in tissue culture, and synthesize large amounts of GAG, of which hyaluronate (HA) predominates (5). Abnormal GAG accumulation has been reported in fibroblast cultures from patients with osteogenesis imperfecta (6), Marfan's syndrome (7), and rheumatoid arthritis (8). My colleagues and I demonstrated recently that 3,3',5 triiodothyronine (T_3), the biologically active thyroid hormone, could inhibit accumulation of HA in skin fibroblast cultures incubated in medium supplemented with thyroid hormone-depleted serum (9). This inhibition was both time- and dose-dependent, saturable at a physiologic concentration of T_3, and was a consequence of a decreased rate of synthesis rather than an increased rate of GAG degradation (10). The skin fibroblast culture depleted of thyroid hormone may thus be an in vitro model of human myxedema.

1. *Abbreviations used in this paper:* Dex, dexamethasone (1,4 pregnadien-9-fluoro-16α-methyl-11β,17α,21-triol-3,20-dione); DOC, 11-deoxycorticosterone (21-hydroxy-4-pregnene-3,20-dione); GAG, glycosaminoglycan; HA, hyaluronic acid; 11α-hydrocortisone, epicortisol (11α,17,21-trihydroxy-4-pregnene-3,20-dione); T_3, 3,3',5-L-triiodothyronine; T_4, thyroxine, 3,3',5,5'-L-tetraiodothyronine; Tx-Bs, serum from thyroidectomized calves.

Glucocorticoids, like thyroid hormone, are capable of influencing human skin fibroblasts in vitro by altering cellular proliferation (11), collagen biosynthesis (12), and regulating specific protein synthesis (13). While the observation that glucocorticoids can inhibit GAG accumulation has been made previously (14, 15), little is known about the nature of this inhibition or whether GAG synthesis or degradation is altered.

The current report describes results of studies designed to determine the kinetics, specificity, and mechanism involved in the inhibition of HA accumulation by dexamethasone (Dex), a potent synthetic glucocorticoid, and to compare this inhibition with that of T_3. Such information may be useful in understanding hormonal regulation of complex sugar synthesis in human skin fibroblasts.

Methods

Materials

[^{3}H]Acetic acid (2.8 Ci/mmol sp act) and [^{3}H]glucosamine (19.7 Ci/mmol) were purchased from New England Nuclear, Boston, MA, and [^{3}H]leucine (50 Ci/mmol) was obtained from ICN Pharmaceuticals, Irvine, CA. Chondroitin sulfate, dexamethasone, progesterone, triiodothyronine, and pronase were purchased from Sigma Chemical Co., St. Louis, MO, and deoxycorticosterone and 11α-hydrocortisone were from Steraloids, Wilton, NH. Hyaluronate was extracted from human umbilical cord. Streptomyces hyaluronidase was obtained from Miles Laboratories, Inc., Elkhart, IN. All other chemicals used were of the highest purity commercially available.

Procedures

Cell culture. Human skin fibroblasts were obtained from American Type Culture Collection, Rockville, MD, and propagated in Eagle's minimal essential medium supplemented with Earle's salts, glutamine, penicillin/streptomycin, and amphotericin B, and enriched with 10% fetal calf serum. Cells were plated on 60-mm diam plastic petri dishes covered with 4 ml medium that was changed every 3–4 d. Initial plating density ranged from ~400,000 to 600,000 cells/plate and cultures were allowed to grow to confluence in a 37°C, humidified 5% CO_2 incubator before any hormonal manipulations. Hormones were added as concentrates dissolved in 95% ethanol. Control cultures were treated with equivalent volumes of diluent. Some experiments involved shifting cultures to medium supplemented with 10% thyroidectomized calf serum (Tx-Bs; Rockland Farms, Gilbertsville, PA). This serum contained <0.3 μg/dl thyroxine (T_4) and <20 ng/dl T_3; the lower limits of sensitivity of respective radioimmunoassays kindly performed by Dr. N. H. Scherberg, University of Chicago. Hormone-treated cultures were usually incubated for 48 h and then shifted to the same medium to which either [^{3}H]acetic acid (5–10 μCi/ml) or [^{3}H]glucosamine (5 μCi/ml) was added and allowed to label for 16–24 h. The labeling period was shortened to 5 h in the time course study and to 10 h in the pulse-chase studies. The chase incubation in the later study utilized unlabeled medium containing 1 mM sodium acetate. Cultures were incubated with [^{3}H]leucine (5 μCi/ml medium) in protein synthesis studies.

Quantitation of [^{3}H]GAG and [^{3}H]HA accumulation. Culture media were quantitatively collected and cell layers washed with phosphate-buffered saline. Cell layers were then solubilized in 0.1 N NaOH and scraped off the substratum with a rubber policeman. Following the collection of an aliquot for protein determination by the method of Lowry et al. (16), the cell layers, media, and wash were recombined and digested with pronase (1 mg/ml) in a 100-mM Tris buffer, pH 8.0, at 50°C overnight in the presence of hyaluronate and chondroitin sulfate (250 μg each), which were added as carriers. After cooling on ice, samples were precipitated with trichloroacetic acid (TCA) (5% wt/vol final concentration) for 30 min and were then centrifuged at 10,000 *g*. The supernatant was dialyzed extensively against water to remove free label and an aliquot was counted by liquid scintillation spectrometry and defined as total GAG. In some experiments, postdialysis samples were lyophilized to dryness and subjected to streptomyces hyaluronidase digestion as previously described (9). Approximately 90% of the total [^{3}H]GAG was hyaluronidase digestible, irrespective of hormone treatment. In early experiments, uronic acid content in postdialysis samples

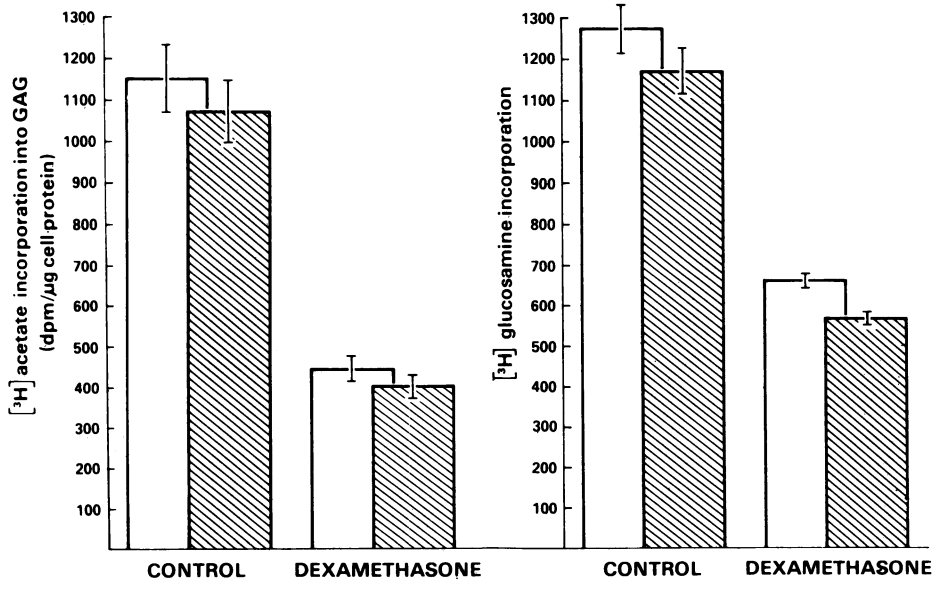

Figure 1. Effect of Dex on the incorporation of [^{3}H]acetate and [^{3}H]glucosamine into GAG (□) and HA (■). Confluent cultures were treated with Dex (100 nM) or diluent for 48 h, shifted to the same medium supplemented with either [^{3}H]acetate or [^{3}H]glucosamine (5 μCi/ml) for an additional 1 d, and then, harvested. Each column represents the mean of duplicate plates±range from one representative experiment. Incorporation is expressed either prior to (total GAG) or following streptomyces hyaluronidase digestion (hyaluronate).

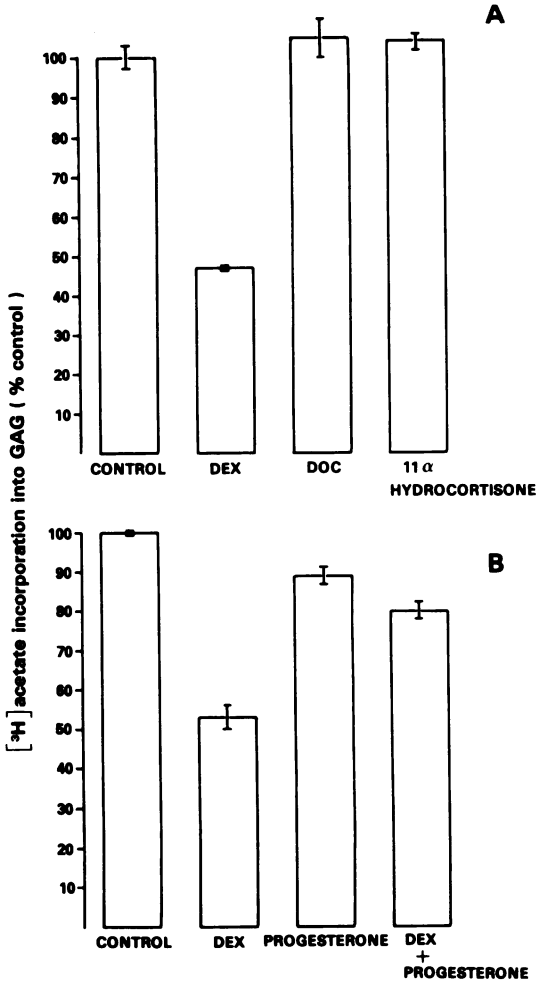

Figure 2. (*A*) Effects of Dex, DOC, and 11α-hydrocortisone on [^{3}H]GAG accumulation. Cultures were grown to confluence, shifted to medium enriched with a steroid (10 nM) or diluent alone (control) for 2 d, and then, labeled with [^{3}H]acetate (5 μCi/ml medium) for an additional 1 d. (*B*) Effects of Dex and progesterone on [^{3}H]GAG accumulation. Cultures were grown as in *A* and treated with either Dex (1 nM), progesterone (2 μM), both hormones, or diluent alone (control). Data are expressed as the mean±SEM of triplicate cultures from one representative experiment.

was determined by the method of Dische (17). Intersample recoveries were uniform. The digested material was separated by Sephadex G-50 (Pharmacia Fine Chemicals, Inc., Piscataway, NJ) column chromatography using a pyridine acetate buffer, pH 5.8, as the eluant. Details concerning the recoveries have been published elsewhere (9, 10).

Data are expressed either as mean±range of determinations in duplicate cultures or as the mean±SEM of triplicate cultures with statistical significance analyzed by Student's *t* test.

Results

The addition of Dex to confluent human skin fibroblast cultures resulted in a marked inhibition of both [^{3}H]GAG and [^{3}H]HA accumulation when present for 72 h before harvest (Fig. 1). Dex (100 nM) inhibited both [^{3}H]acetate and [^{3}H]glucosamine incorporation into GAG by 62 and 50%, respectively (Fig. 1). The absolute magnitude of this inhibition varied from 40 to 83% in other experiments. Lack of substrate specificity suggested that the inhibition of accumulation was not a consequence of precursor pool shifts. Results were similar whether expressed per milligram protein or cell number.

The dose dependence of the Dex inhibition was established by incubating cultures with various concentrations of the steroid (range 0.1–1,000 nM). A half-maximal response occurred at 1 nM and was saturable at 5–10 nM. Additions of up to 100-fold higher concentration had no further effects.

To assess whether the observed effects of Dex on [^{3}H]GAG accumulation could be produced by nonglucocorticoid steroidal hormones, some cultures were treated with Dex (10 nM); deoxycorticosterone (DOC, 10 nM), a mineralocorticoid devoid of glucocorticoid activity; or 11α-hydrocortisone (10 nM), an inactive steroid. As Fig. 2 *A* demonstrates, neither DOC nor 11α-hydrocortisone inhibited GAG accumulation when added at concentrations eliciting near maximal Dex inhibition. Progesterone, a steroid that has been previously shown to partially antagonize the action of glucocorticoids (18), also failed to influence the rate of [^{3}H]GAG accumulation at similar concentrations (data not shown). High concentrations of progesterone (2 μM), however, inhibited [^{3}H]GAG slightly (11%, $P < 0.005$) compared with Dex (1 nM, 47%, $P < 0.001$) (Fig. 2 *B*). When both hormones were added at these respective concentrations, the Dex effect on [^{3}H]GAG accumulation was attenuated by 57% ($P < 0.005$) (Fig. 2 *B*).

Fig. 3 demonstrates the time course of Dex inhibition of [^{3}H]GAG accumulation. When the labeling period was shortened, Dex inhibited [^{3}H]acetate incorporation within 5 h of hormone addition. There was a further decrease in accumulation over the duration of the study until near maximal effects were achieved at 25 h. Since confluence had been reached prior to the addition of hormone, protein content did not vary among plates.

To determine whether the effect of Dex on [^{3}H]GAG accumulation was a result of an inhibition of cellular uptake of labeled precursor or was due to a more specific effect on macromolecular metabolism, cultures were incubated under conditions that yielded a near maximal Dex response. Cell layers were then trypsinized, washed extensively, and analyzed for [^{3}H]acetate uptake. Control cell layers accumulated 45,600±4,400 dpm/100,000 cells (mean±SEM, $n = 3$) compared with 48,200±1,200 dpm/100,000 cells in Dex-treated cultures (NS). Alterations of cellular uptake, therefore, apparently were not responsible for the glucocorticoid inhibition of [^{3}H]GAG accumulation. Dex also failed to influence the incorporation of [^{3}H]leucine into TCA-precipitable material. In a study where [^{3}H]GAG accumulation was inhibited 58%, Dex-treated cultures retained 20,979±1,045 dpm [^{3}H]leucine/μg protein (mean±SEM, $n = 3$) in TCA-insoluble material compared with 21,283±565 dpm/μg protein in controls (NS).

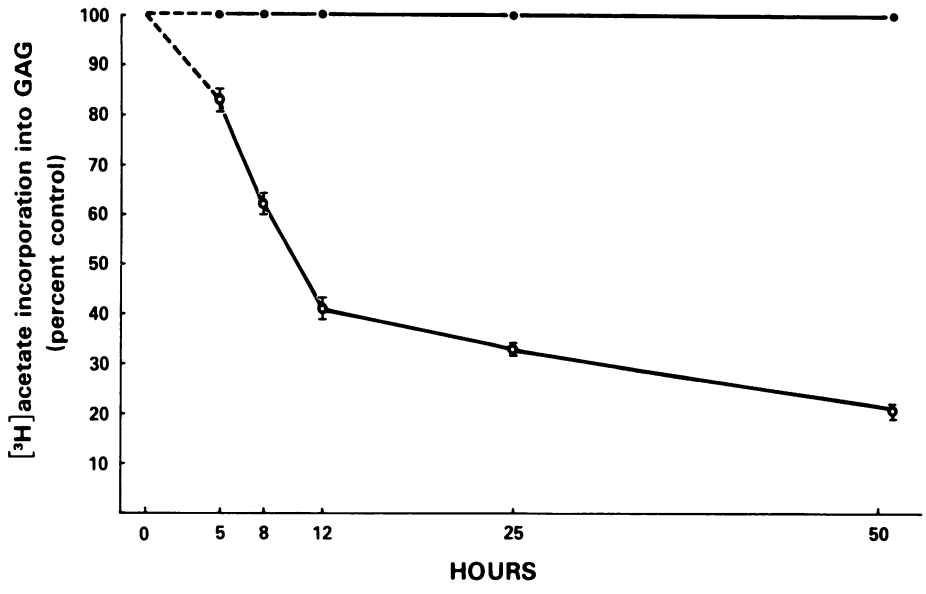

Figure 3. Time course of the effect of Dex on [^{3}H]GAG accumulation. Confluent cultures were incubated with Dex (100 nM) (○) or diluent (●) for the various durations of time indicated along the abscissa. All cultures were labeled for 5 h before harvest. Each datum point represents the mean±range from a single representative experiment.

To assess whether the observed inhibition by Dex of [^{3}H]GAG and [^{3}H]HA accumulation was a consequence of an altered rate of degradation, cultures were preincubated without or with the hormone (100 nM) and then pulse-labeled (10 h) followed by a chase incubation for various intervals in media containing unlabeled sodium acetate (1 mM). The results shown in Table I suggest that there was no effect of Dex on the degradation rate of either [^{3}H]GAG or [^{3}H]HA over the duration of the chase incubation (68 h), as would have been reflected by a change in the ratio of incorporation in cultures incubated with Dex vs. controls. In fact, there was no evidence of ^{3}H-labeled macromolecular degradation over the course of the chase incubation (data not shown), which is consistent with the previously reported absence of hyaluronidase in human skin fibroblasts (19). These data suggest that Dex inhibition is the consequence of an attenuated rate of synthesis paralleling those observations made previously of T_3 effects on GAG accumulation (10).

Table I. Effect of Dex on [^{3}H]GAG and [^{3}H]HA Degradation in a Pulse-Chase Experiment

	Duration of chase (*h*)			
	14.5	23	40	68
[^{3}H]GAG$\left(\frac{+\text{Dex}}{-\text{Dex}}\right)$	0.50	0.48	0.50	0.51
[^{3}H]HA$\left(\frac{+\text{Dex}}{-\text{Dex}}\right)$	0.49	—	—	0.50

Confluent cultures were incubated with either Dex (100 nM) or diluent for 2 d. All culture plates were then shifted to the same medium supplemented with [^{3}H]acetate (10 μCi/ml) and pulse-labeled for 10 h. After the labeling period, cell layers were incubated in medium containing unlabeled sodium acetate (1 mM) for the duration of time indicated (chase). The data are expressed as the ratio created by dividing the [^{3}H]GAG or [^{3}H]HA that accumulated in Dex-treated cultures by the accumulation in control cultures.

To compare the effect of Dex on [^{3}H]HA with that reported previously of T_3, confluent cultures were shifted to medium enriched with 10% Tx-Bs to which various concentrations of either Dex or T_3 were added. Both hormones inhibited [^{3}H]GAG accumulation in a dose-dependent saturable manner (Fig. 4). Dex inhibition was maximal at 10 nM, whereas that of T_3 saturated at 1 nM (Fig. 4). The magnitude of fractional inhibition by T_3 varied among individual experiments from 26 to 54%.

Having defined the concentrations of both T_3 and Dex that elicited maximal responses, a study was performed in which saturating concentrations of each hormone were added both individually and together. Compared with control cultures, those treated with Dex (100 nM) or T_3 (100 nM) accumulated 49 and 54% less [^{3}H]HA, respectively (Fig. 5). When cultures were incubated with both hormones, [^{3}H]HA accumulation was reduced by 84% (Fig. 5). Cultures incubated with both hormones invariably accumulated considerably less [^{3}H]GAG than did those incubated with high concentrations of either hormone alone. The effects of Dex and T_3 appear additive suggesting that the two hormones inhibit GAG synthesis through separate pathways.

Discussion

Skin fibroblast cultures are an established target for the action of steroid hormones. Receptors for testosterone (20), glucocor-

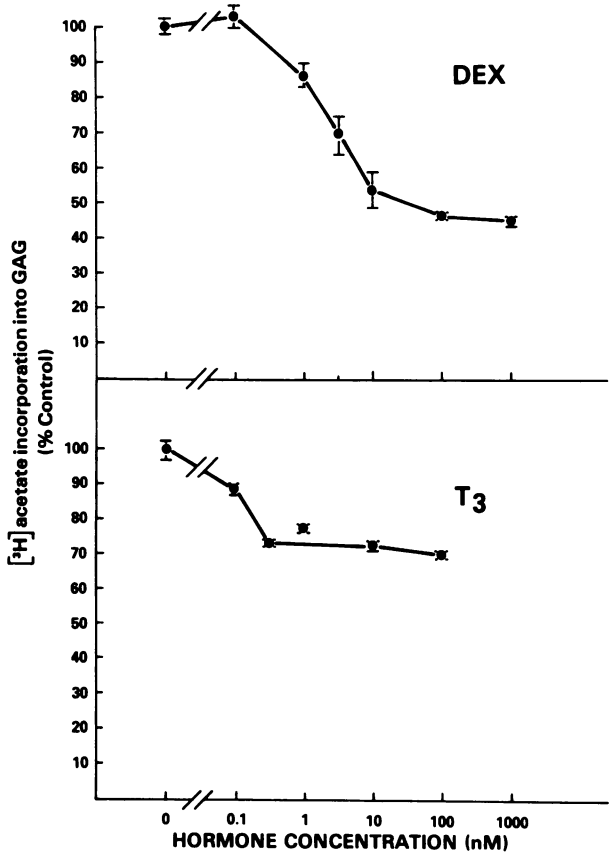

Figure 4. Effect of Dex and T_3 concentrations on [^{3}H]GAG accumulation in medium supplemented with 10% thyroidectomized calf serum. Confluent cultures were shifted to medium enriched with Tx-Bs (10%) and supplemented with various concentrations of Dex (top) or T_3 (bottom) for 2 d. The cultures were then labeled for 24 h and harvested. Each datum point represents the mean±range of duplicate culture plates.

ticoids (21), and 1,25-dihydroxycholecalciferol (22) have been demonstrated. In addition, fibroblasts possess high affinity receptors for nonsteroidal hormones such as T_3 (23, 24), insulin (25), and growth hormone (26), and are known to be responsive to a variety of hormones (27, 28) and growth factors (29). Defects in in vitro binding and/or action in skin fibroblasts from patients with hormone resistance have been shown to reflect the clinical derangements found in vivo. These include resistance to thyroid hormone (23, 30, 31), glucocorticoids (32), androgens (20), and vitamin D (22).

Various steroid hormones can influence GAG metabolism. Testosterone stimulates the accumulation of GAG in chick combs while hyaluronate content in surgically castrated cockerel combs falls after the discontinuation of hormone replacement (33). Similarly, estrogen enhanced the HA content in mouse skin (34). These effects could be blocked by antiestrogens such as tamoxifen and clomiphene (34). Earlier studies have demonstrated glucocorticoid inhibition of HA accumulation in various human synovial and skin fibroblasts (14, 15). In most of these studies, however, cultures were treated with very high concentrations of hormone (up to 100 μM), ignoring evidence that such high steroid concentrations might have nonspecific cellular effects (35). None addresses the issue of whether glucocorticoids affect GAG degradation rather than synthetic rates. The current report demonstrates that Dex, even at low concentrations (1 nM), can inhibit [^{3}H]HA accumulation without altering the rate of degradation. Although this effect could have resulted from intracellular precursor pool shifts, the equivalent results obtained from labeling cultures with either [^{3}H]acetate or [^{3}H]glucosamine suggest that this was not the case. Similarly, the lack of a Dex effect on cellular [^{3}H]acetate uptake suggests that substrate availability was unaltered.

Stereospecificity is a hallmark of receptor-mediated hormone action. The inhibitory effects of Dex reported here are apparently quite stereospecific because two structurally related steroids devoid of glucocorticoid activity failed to alter the rate of [^{3}H]HA accumulation. Progesterone, a compound possessing both antagonistic and glucocorticoid agonistic activities at high concentrations, had no effects at concentrations which yielded near maximal Dex inhibition. At higher concentrations ($\geq$2 μM), however, progesterone did inhibit slightly [^{3}H]GAG synthesis and partially blocked the effects of Dex. These results are entirely consistent with the observations of Samuels and Tomkins that progesterone could behave both as a suboptimal inducer of tyrosine aminotransferase and as a partial antagonist of glucocorticoid induction of that enzyme in hepatoma cells in vitro (18). This result implies further that Dex inhibition of HA synthesis was mediated through glucocorticoid receptors (36).

Dex inhibition of [^{3}H]GAG synthesis in human skin fibroblasts resembles that of T_3. Because addition of both hormones in concentrations that elicit maximal responses alone inhibit accumulation further, the two hormones appear to act through separate pathways. T_3 effects were maximal at approximately one order of magnitude lower concentration than those of Dex. Thus, the relationship between concentrations of the two hormones necessary to alter the rate of HA synthesis is similar to that necessary to demonstrate effects on growth hormone synthesis in vitro (37). In addition, the Dex concentrations required to elicit maximal inhibition in these studies is well within the range shown to inhibit endogenous glucocorticoid production in vivo (38).

Glucocorticoids and T_3 are known to coregulate a number of metabolic processes. For instance, both hormones stimulate the synthesis of growth hormone messenger RNA (mRNA) in vitro (37) and apparently facilitate the effects of each other (39, 40). Both glucocorticoids and insulin influence T_3-mediated augmentation of malic enzyme and α-glycerophosphate dehydrogenase in rat hepatocytes (41). T_3 and glucocorticoids in concert with testosterone and growth hormone are necessary to maintain cytosolic α_{2u} globulin mRNA in the male rat liver (42). It has been reported that corticosteroids and insulin, as

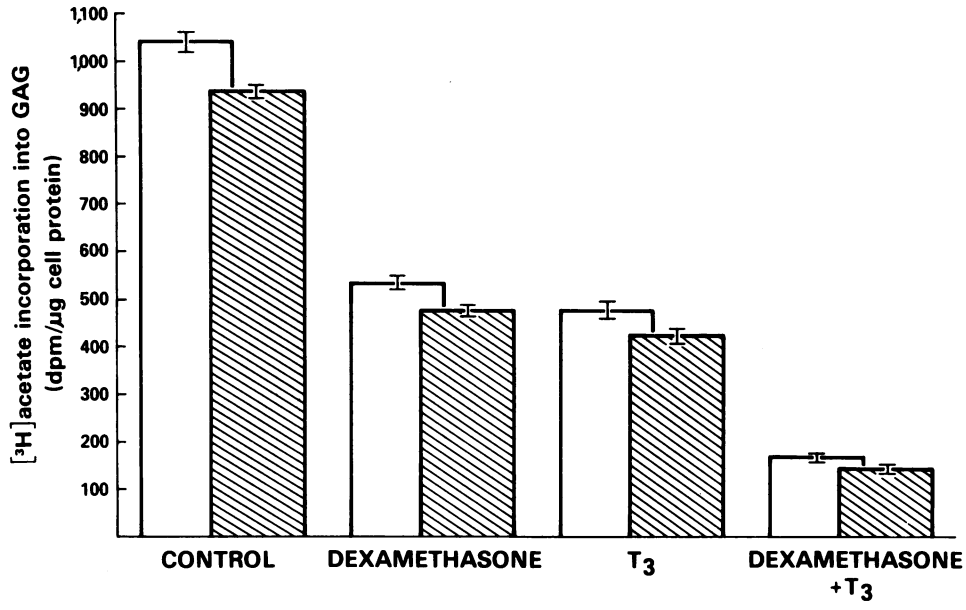

Figure 5. Comparison of Dex and T_3 effects on [^{3}H]GAG (□) and [^{3}H]HA (■) accumulation. Confluent cultures were shifted to medium enriched with Tx-Bs (10%), supplemented with diluent, Dex (100 nM), T_3 (100 nM), or both hormones, and incubated for 2 d. Cultures were then shifted to identical media and labeled for 24 h. Data are expressed as the mean±range of duplicate culture plates from one representative experiment.

well as thyroid hormone, exert permissive effects on the chemical and steroidal induction of δ-aminolevulinate synthase in chick embryo liver cells in culture (43). A recent study described effects of both T_3 and Dex on the synthesis of specific proteins in cultured pituitary adenoma cells (44). Using two-dimensional gel electrophoresis, Ivarie and co-workers demonstrated that each hormone could affect several proteins, either by enhancing or attenuating their rate of synthesis. In addition, some proteins appeared to be coregulated by the two hormones (44). An abundance of evidence thus exists to suggest multihormonal control of specific metabolic events. Apparently, the synthesis of hyaluronate is among these.

In summary, these studies demonstrate that Dex inhibits [^{3}H]HA synthesis in fibroblasts in a similar manner to other established effects of glucocorticoids. While the precise mechanism(s) through which Dex, as well as T_3, affects GAG synthesis remains incompletely understood, these results define a shared and perhaps physiologically important role for each hormone in the regulation of macromolecular synthesis in human skin fibroblasts.

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