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In Vitro Inhibition of Cell Growth, Collagen Synthesis, and Prolyl Hydroxylase Activity by Triamcinolone Acetonide (40750)[1]

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Introduction. The generalized inhibition of protein synthesis by corticosteroids has been considered the reason for decreased collagen synthesis in dermal tissues following clinical or experimental use of these agents (1–7) and has provided the rationale for intralesional treatment of keloids and hypertrophic scars by corticosteroids (8). However, the specific effects of these agents on collagen metabolism remain unclear. For example, corticosteroids have been reported to stimulate as well as inhibit collagen synthesis both *in vivo* and *in vitro* (9–13). This laboratory reported that collagen synthesis was not diminished by corticosteroids in regressing keloids, chick calvaria cultures, or rat skin wounds, however inhibition was observed in 5-day rat wounds after the animals received long-term, massive doses of methylprednisolone (Depo-Medrol) (14).

In the present study, the direct effects of triamcinolone acetonide on fibroblast division, collagen metabolism, total protein synthesis, and prolyl hydroxylase activity were measured. The experiments were designed to determine whether this steroid has a differential effect on these parameters in keloid cultures compared to normal dermal cultures.

Materials and methods. Fibroblast isolation. Cell lines were derived from fresh biopsies of keloid or normal skin as previously described (15). Fibroblasts were subcultured by trypsinization (0.1% trypsin, Worthington Biochemical Corp., Freehold, N.J., in Hanks' balanced salt solution (pH 7.4) for 15 min at 37°). Cells in the third to ninth passage were used in these experiments. The cultures were maintained in Eagle's minimum essential medium (MEM, Gibco, Grand Island, N.Y.) supplemented with 10% fetal calf serum, penicillin (100 U/ml), streptomycin (100 μg/ml), and 25 m*M* Tricine buffer (pH 7.4) in a humidified atmosphere at 37°.

Triamcinolone acetonide preparation. Pure triamcinolone acetonide (9α-fluro-11β, 21-dihydroxy-2′, 2-dimethyl-16α, 17α-dio-1, 4-pregnadiene-3, 20-dione, M_r 434.49) was obtained from Squibb Institute (Princeton, N.J.) and a stock suspension was prepared in MEM (2 mg/ml) without fetal calf serum but supplemented with penicillin (100 U/ml), Mycostatin (50 U/ml), and streptomycin (100 μg/ml).

Experimental culture conditions. On Day 0 of each experiment, cultures of either keloid or normal skin fibroblasts were initiated at a density of 1.2×10^5 cells per 65-mm dish (Falcon Plastics, Oxnard, Calif.) in MEM supplemented with 2% fetal calf serum. After 48 hr, three groups of plates were established. One group was cultured with 4 ml of MEM containing 10% fetal calf serum and 50 μg/ml of the steroid, a second group received MEM, serum, and 1 μg/ml steroid while a control group received MEM, serum, and no steroid. The steroid was added to the cultures after 48 hr in order to avoid interference with cell attachment processes. The cells were allowed to grow under these conditions for 5 days with no refeedings and analyses were performed on nonconfluent cultures.

Collagen and protein synthesis measurements. Five days after addition of triamcinolone acetonide, 8 or 12 plates from each group were refed with 3 ml of MEM without serum but supplemented with freshly prepared 0.1 m*M* ascorbate and the steroid concentration appropriate to that group. After a 30-min preincubation period at 37°C, each plate received 5 μCi of L-[5-^{3}H]proline (hydroxyproline free, 43 Ci/m*M*, Schwarz–Mann, Orangeburg, N.Y.) for 4 hr at 37°. The labeling period was stopped by rapid freezing (−20°). Upon thawing, the cells and medium were har-

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vested together at 4° with a rubber policeman into centrifuge tubes. Four plates were pooled per sample and duplicate or triplicate samples were analyzed for each determination. The plates were washed with 5 ml cold deionized water and the washes were added to the appropriate pooled samples. Following sonication (Branson sonifier, Stamford, Conn.) at maximum amperage for 30 sec, the samples were maintained at 4°, and the protein from the combined medium and cell lysates was precipitated by the addition of trichloroacetic acid (TCA, 5% final concentration). TCA-Precipitable protein was washed free of unincorporated isotope and then digested with purified bacterial collagenase to determine absolute and relative collagen and noncollagen protein (16). The radioactivity released by collagenase and remaining in the supernatant after addition of 5% TCA and 0.25% tannic acid was measured by liquid scintillation counting and expressed as counts per 10^5 cells whereas noncollagen protein was precipitated and the radioactivity was measured separately and expressed as counts per 10^5 cells. Collagen synthesis was also expressed as a percentage of total protein synthesis. Relative collagen synthesis was calculated to correct for the enriched amino acid content of collagen (22%) compared to other proteins (4.1%) (17).

Cell counts and DNA determinations. Cell numbers were determined at the time of analysis by trypsinizing the monolayers from duplicate or triplicate plates and directly counting the cells in a hemocytometer. DNA was measured according to the method of Burton (18).

Prolyl hydroxylase determination. Duplicate cultures of keloid and normal skin fibroblasts were treated with triamcinolone acetonide according to the protocol described above. Prolyl hydroxylase was measured by a tritium release assay (19) and enzyme activity was expressed as cpm/mg protein (20) and cpm/10^6 cells.

Measurement of [^{14}C]proline specific activity in the cell pool. On Day 0, keloid-derived or normal skin fibroblasts were plated into 30 100-cm^2 culture dishes at a density of 3.5×10^5/dish in MEM supplemented with 2% fetal calf serum. After 48 hr the cultures were divided into three groups and treated with triamcinolone acetonide as described above. Five days later, eight plates from each group were labeled under the conditions described above but with [^{14}C]proline (2.5 μCi/dish, 261 mCi/m*M*, New England Nuclear, Boston, Mass.). After a 4-hr labeling period, the monolayers were washed free of the radioactive medium using phosphate-buffered saline (pH 7.4). The monolayers were then harvested into centrifuge tubes at 4°. Following sonication, the protein was precipitated in 5% TCA, and the samples were centrifuged 10,000*g* for 15 min at 4°. The supernatants were removed, lyophilized, and resuspended in 2 ml of deionized water. The 2-ml samples were desalted on a Dowex AG-50 column (0.5 × 7 cm), eluted with 15 ml of 2 *N* NH_4OH, and evaporated to dryness. Colorimetric (21) and radioactive (22) proline measurements were performed and results were expressed as μCi/μ*M* proline.

Collagen degradation. As an index of collagen degradation, TCA-soluble, radioactive hydroxyproline was measured in keloid and normal skin cultures grown and treated according to the experimental conditions described above. After treatment for 5 days with triamcinolone acetonide, cells were incubated 4 hr at 37° in MEM supplemented with 0.1 m*M* ascorbate, the appropriate steroid concentration, and [^{14}C]proline (5 μCi/dish) which had been purified of trace hydroxyproline contamination by ion-exchange column chromatography (1 × 30 cm, Dowex 50, H^+, 200–400 mesh) (23). At the end of the labeling period, medium and cells were harvested, sonicated, and precipitated with TCA (5% final concentration) at 4°C. The first two TCA supernatants (10,000*g*) were pooled, lyophilized, and hydrolyzed in 6 *N* HCl for 18 hr. Hydrolysates were decolorized (Norit A) and hydroxyproline was isolated by ion-exchange column chromatography (1 × 25 cm, Dowex 50, H^+, 200–400 mesh) with 1 *N* HCl as eluant (23). The fractions were monitored by scintillation counting and those representing the hydroxyproline peak were pooled and as-

sayed for [^{14}C]hydroxyproline by a modification (16) of the procedure of Peterkofsky and Prockop (22). Radioactive hydroxyproline was expressed as dpm per 10^6 cells.

The Student's t test was used in all studies for statistical analyses.

Results. Triamcinolone acetonide significantly inhibited the growth of normal skin fibroblasts ($P < 0.05$) but did not significantly inhibit the growth of keloid-derived fibroblasts (Table I). Furthermore, 50 μg/ml triamcinolone acetonide resulted in significant reduction of DNA content ($P < 0.05$) only in the normal skin cultures.

Relative collagen synthesis (%) was significantly inhibited ($P < 0.01$) by triamcinolone acetonide (Table II), and the inhibition was similarly dose-dependent in both cell types. However, the corticosteroid increased the specific activity of radioactive proline in keloid and normal skin cultures (Table III). Therefore, collagen, and noncollagen protein (cpm/10^5 cells, Table II) were corrected for the increased specific activity in the proline precursor pool which is assumed to be the true proline precursor pool − t RNA. When this was done, significant inhibition of collagen synthesis ($P < 0.05$) was observed in all cultures treated with 50 μg/ml triamcinolone acetonide (Table II). In two cultures (NS-2 and K-1, Table II), corrected values for isotope incorporation into noncollagen protein were increased ($P < 0.05$). Collagen synthesis relative to noncollagen protein was significantly inhibited by 50 μg/ml triamcinolone acetonide (Table II) in all cultures tested.

As an index of collagen degradation, TCA-soluble [^{14}C]hydroxyproline was analyzed in steroid-treated and untreated cultures (Fig. 1). TCA-Soluble [^{14}C]hydroxyproline was significantly increased ($P < 0.02$) in keloid-derived cultures by both steroid concentrations and significantly increased ($P < 0.02$) by 50 μg/ml steroid in the normal skin cultures.

Triamcinolone acetonide decreased prolyl hydroxylase similarly in both normal skin and keloid cultures (Table IV). Prolyl hydroxylase activity of both cell lines was reduced 55–67% after treatment with 50 μg/ml of the steroid ($P < 0.05$) and 34–61% following treatment with 1 μg/ml steroid (cpm/mg protein and cpm/10^6 cells).

Discussion. These studies provide direct evidence that triamcinolone acetonide specifically inhibited collagen synthesis and decreased prolyl hydroxylase activity in keloid and normal skin fibroblast cultures. We have previously reported increased collagen synthesis in keloids both in fresh biopsies (14) and in keloid cultures compared to normal skin (15), therefore, the present, comparative study was performed to determine if keloid-derived fibroblasts respond differently to this steroid than normal dermal fibroblasts.

Effects of triamcinolone acetonide on cell growth. Under standardized culture conditions, the growth kinetics of keloid-derived fibroblasts, normal skin, and normal scar fibroblasts are similar (15, 24). In the present study, the 31–38% decrease in cell numbers without a reduction in the DNA content at 1 μg/ml triamcinolone

TABLE I. EFFECT OF TRIAMCINOLONE ACETONIDE ON GROWTH AND DNA CONTENT OF KELOID AND NORMAL SKIN FIBROBLASTS

Cell line	Triamcinolone μg/ml[a]	Cells × 10^5/sample ± SE[b]	μg DNA/sample ± SE[c]
Normal skin	0	9.9 ± 1.2	13.5 ± 1.1
	1	6.9 ± 0.8 (31%)[d]	13.3 ± 0.6 (0)
	50	4.6 ± 0.7 (53%)[d]	8.8 ± 0.9 (35%)[d]
Keloid	0	8.5 ± 1.7	9.3 ± 1.2
	1	5.3 ± 1.2 (38%)	10.4 ± 1.7 (0)
	50	5.3 ± 0.8 (38%)	8.6 ± 1.5 (8%)

[a] Treatment for 5 days.
[b] Each value is the mean of six determinations; number in parentheses is the percentage inhibition.
[c] Each value is the mean of four determinations.
[d] Significantly different from nonsteroid-treated controls at $P < 0.05$.

TABLE II. EFFECT OF TRIAMCINOLONE ACETONIDE ON COLLAGEN AND NONCOLLAGEN PROTEIN SYNTHESIS[a]

Cell line	Triamcinolone μg/ml	Collagen cpm/10^5 cells ± SE	Noncollagen protein cpm/10^5 cells ± SE	Relative collagen synthesis (%)
Normal skin				
1	0	114 ± 4	518 ± 21	3.9 ± 0.2
	1	84 ± 13[b]	584 ± 24	2.6 ± 0.3[b]
	50	18 ± 4[c]	477 ± 19	0.7 ± 0.2[c]
2	0	125 ± 12	523 ± 15	4.3 ± 0.5
	1	245 ± 27[d]	1174 ± 30[d]	3.7 ± 0.3
	50	48 ± 3[c]	485 ± 6	1.9 ± 0.1[c]
Keloid				
1	0	626 ± 46	1027 ± 44	10.1 ± 0.3
	1	562 ± 14[b]	1751 ± 53[d]	5.6 ± 0.1[b]
	50	215 ± 21[c]	1309 ± 105	2.9 ± 0.1[c]
2	0	285 ± 17	705 ± 46	7.0 ± 0.1
	1	217 ± 11[d]	659 ± 43	5.8 ± 0.1[b]
	50	93 ± 14[c]	776 ± 51	2.1 ± 0.2[c]

[a] Collagen and noncollagen protein (cpm/10^5 cells ± SE) have been corrected for the increased specific activity in the proline precursor pool in the steroid-treated cultures (Table III); each sample represents the mean of three observations following steroid treatment for 5 days.
[b] Significantly different from 50 μg/ml at $P < 0.01$.
[c] Significantly decreased compared to nonsteroid-treated control and 1 μg/ml dose at $P < 0.05$.
[d] Significantly different from nonsteroid-treated control and 50 μg/ml dose at $P < 0.05$.

agrees with the findings of Ponec *et al.* (25), demonstrating that 5 μg/ml triamcinolone acetonide had no effect on DNA content in 3T3 fibroblast cultures and are similar to observations of other investigators (26–29). It has been postulated that there is a stage in the cell cycle following DNA replication which is especially receptive to the inhibitory action of low doses of corticosteroids (29). Therefore, it is possible that DNA replication occurs normally in the presence of the steroid but that cell division is inhibited. The present data suggest that keloid-derived fibroblasts are more resistant to the growth inhibitory effects of triamcinolone acetonide than normal dermal fibroblasts. There are several reports that cell lines such as human uterine fibroblasts or mouse fibroblasts have the capacity to enzymatically transform steroid hormones into less inhibitory metabolites or less biologically active forms (28, 29), but the mechanisms whereby keloid-derived fibroblasts may be more resistant to the high dose of steroid than normal dermal fibroblasts remain unclear. In contrast to these findings, Russell *et al.* (30) reported inhibition of keloid cell division and stimulation of normal cell growth following treatment with hydrocortisone. Because of differ-

TABLE III. EFFECT OF TRIAMCINOLONE ACETONIDE ON THE SPECIFIC ACTIVITY OF THE PROLINE POOL IN NORMAL SKIN AND KELOID FIBROBLASTS

Cell line	Triamcinolone[a] μg/ml	[^{14}C]Proline μCi/μM ± SE[b]
Normal skin	0	0.75 ± 0.15 (6)
	1	1.20 ± 0.13 (4)[c]
	50	1.20 ± 0.22 (4)[c]
Keloid	0	0.63 ± 0.06 (6)
	1	1.04 ± 0.14 (3)[c]
	50	1.01 ± 0.04 (4)[c]

[a] Treatment for 5 days.
[b] Number in parentheses is the number of observations.
[c] $P < 0.025$ compared to nonsteroid control.

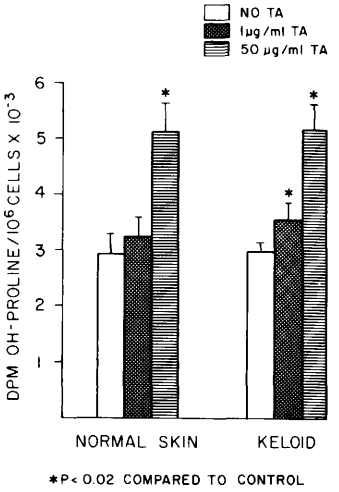

FIG. 1. Effect of 1 and 50 μg/ml triamcinolone acetonide treatment for 5 days on TCA-soluble [^{14}C]hydroxyproline in keloid and normal skin fibroblasts. The cultures were labeled for 4 hr with hydroxyproline-free [^{14}C]proline. Each point equals the mean of four observations.

ences in initial plating densities, refeeding schedules and continuous application of hydrocortisone to the cultures in studies by Russell *et al.* (30), direct comparisons with the present findings cannot be made. For example, in another study, dexamethasone had no effect on the growth of rat fibroblasts in medium with 2% serum but was inhibitory to the cells when the medium was adjusted to a 10% serum concentration (31). These studies suggest that culture conditions can influence the inhibitory action of corticosteroids in different studies.

Effects of triamcinolone acetonide on collagen synthesis. The present *in vitro* studies demonstrate that triamcinolone acetonide significantly and selectively decreased collagen synthesis in keloid and normal skin cultures in a similar, dose-dependent manner. Russell *et al.* (30) reported a differential inhibition of collagen synthesis when confluent keloid and normal fibroblasts were treated with hydrocortisone. It is possible that, similar to effects on cell proliferation, steroids may also inhibit collagen synthesis differentially in separate studies. For example, the differences in collagen synthesis noted by Russell *et al.* were in confluent cultures while the present study measured collagen synthesis in nonconfluent (log-phase) cultures (30). Furthermore, Russell *et al.* maintained and analyzed their cultures under conditions different from those used in the present study.

Effect of triamcinolone acetonide on cell pool proline. Because triamcinolone acetonide increased the uptake of radioactive proline into the intracellular pool, it was necessary to correct absolute collagen and noncollagen values for these increases. It remains unclear whether alteration in the cell pool by triamcinolone acetonide is due to the effects of the steroid on the transport of amino acids into the cell or are due to steroid-induced alterations on the utilization or availability of endogenous proline.

Effects of triamcinolone acetonide on collagen degradation. The findings of increased [^{14}C]hydroxyproline in

TABLE IV. EFFECT OF TRIAMCINOLONE ACETONIDE ON PROLYL HYDROXYLASE ACTIVITY

Cell line	Triamcinolone dose μg/ml	Prolyl hydroxylase[a]	
		Per mg protein[b]	Per 10^6 cells[b]
Normal skin	0	9836 ± 1438	365 ± 99
	1	6148 ± 1322 (37%)	240 ± 44 (34%)
	50	4031 ± 939[c] (59%)	158 ± 39[c] (57%)
Keloid	0	10654 ± 2175	373 ± 85
	1	4179 ± 1120[c] (61%)	200 ± 91 (46%)
	50	4206 ± 1636[c] (61%)	128 ± 27[c] (66%)

[a] cpm of ^{3}H released in 15 min ± SE; percentage decrease in parentheses.
[b] Each value equals the mean of five observations for normal skin and four observations for keloid following steroid treatment for 5 days.
[c] $P < 0.05$ compared to nonsteroid control.

triamcinolone-treated cultures are consistent with the reports of other investigators that steroids enhance collagen degradation (32–35). Although the observed increases in labeled hydroxyproline could be due to steroid-mediated increases in intracellular [^{14}C]proline, this is unlikely because hydroxyproline formation is decreased in connective tissues following corticosteroid administration (8, 36). Furthermore, soluble [^{14}C]hydroxyproline increased in a dose-dependent manner (Fig. 1) while the specific activity of the [^{14}C]proline pool did not. Therefore, it appears that triamcinolone acetonide simultaneously enhanced collagen degradation and selectively inhibited collagen synthesis by fibroblasts in culture. Further studies are required to define the effects of steroids on collagen degradation both *in vivo* and *in vitro*.

Prolyl hydroxylase. The findings of decreased prolyl hydroxylase by triamcinolone presented here agree with initial reports of glucocorticoids decreasing prolyl hydroxylase (37). Recently, Newman and Cutroneo (36) reported a dose-dependent decrease in newborn rat dermal prolyl hydroxylase activity and collagen synthesis following treatment with triamcinolone diacetate.

Conclusion. The inhibition of collagen synthesis by triamcinolone acetonide in this *in vitro* system may explain some of the changes in connective tissue which are observed following clinical use of many corticosteroids. Furthermore, both keloid-derived and normal dermal fibroblasts respond similarly to the steroid except that keloid fibroblasts appear to be more resistant to the growth-inhibitory effects of the steroid. Decreased collagen synthesis as well as enhanced collagen degradation appear to be similar in both fibroblast types.

Summary. The effects of triamcinolone acetonide on fibroblasts isolated from normal dermis and keloids were tested by measuring cell division, DNA content, collagen synthesis, and prolyl hydroxylase activity following steroid treatment. In the present study, the DNA content and cell division of normal fibroblasts were inhibited by 50 μg/ml steroid to a greater extent than keloid fibroblasts. Collagen synthesis was inhibited by triamcinolone acetonide in a similar, dose-dependent manner in both keloid-derived and normal skin fibroblasts. Although triamcinolone acetonide decreased prolyl hydroxylase in all cultures, this inhibition did not correlate with the steroid-induced inhibition of collagen synthesis. In addition, triamcinolone acetonide increased the specific activity of radioactive proline in the amino acid cell pool 37–38% in all cultures. Triamcinolone acetonide enhanced TCA-soluble, [^{14}C]hydroxyproline production in both cell types, suggesting that collagen degradation was increased by this corticosteroid.

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