

Vitamin A Prevention of Triamcinolone Acetonide Effects on Granuloma Growth: Lack of Effect on Prolyl Hydroxylase¹ (39408)

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The continued extensive use of potent corticosteroids necessitates an understanding of the mechanism of action of this class of drugs. Clinically, some of the side effects which are the result of altered collagen metabolism are: ecchymoses, atrophy, telangiectasia, and stria. While there have been many investigations concerning clinical and biochemical parameters of glucocorticoid therapy, the mechanism(s) of these phenomena to date remain unclear. Ragan *et al.* (1) demonstrated a decrease in granulomatous tissue in ear wounds of rabbits administered cortisone acetate. Other investigators have demonstrated antianabolic effects of glucocorticoids on collagen metabolism (2-13).

Ehrlich and Hunt (14) reported that vitamin A overcame the inhibitory effects of cortisone on tensile strength in the early stages of healing. These workers also reported that the reduction of inflammatory cell infiltration, fibroplasia, capillary budding, and deposition of collagen fibers following glucocorticoid administration is reversed by concurrent vitamin A therapy (15). These studies suggested that vitamin A administration reversed the antianabolic effects of glucocorticoids on collagen synthesis.

Administration of glucocorticoids to rats results in a decrease of prolyl hydroxylase in normal tissues and in sponge-induced granulomatous tissue (16-18). The present stud-

ies were undertaken to determine if concurrent vitamin A treatment would reverse the effect of glucocorticoid on prolyl hydroxylase activity in granulomatous tissue.

Materials and methods. 4-³H]Proline (15 Ci/mmol) and 2,3-³H]proline (30 Ci/mmol) were obtained from New England Nuclear. Polyvinyl sponges (3.8 × 1.5 cm) were purchased from Scientific Products. Triamcinolone acetonide (T.A.) and vitamin A were obtained from Squibb & Co. and USV Pharmaceuticals, respectively. Eagle's minimal essential medium with Hanks' balanced salt solution (pH 7.4) 2 × glucose and 2 × NaHCO₃ and penicillin-streptomycin solution were obtained from Grand Island Biologicals.

Four-week-old male Sprague-Dawley rats (100 g) were anesthetized with diethyl ether. All operations were carried out using sterile technique. A sterilized sponge was implanted in the dorsothoracic region using a trocar which was inserted into an incision 2 cm above the tail. The trocar was removed and the wound was closed with stainless steel autoclips. Immediately after sponge implantation, intraperitoneal administration of drugs was initiated. Animals received daily injections for the next 3 days. Six to eight rats per group were treated with either 0.2 ml of 0.9% (w/v) saline, T.A. (20 mg/kg), vitamin A (150,000 I.U./kg) or T.A. plus vitamin A. The control group was injected with the vehicle. The animals were killed on Day 4, and the tissue-covered sponges were dissected free from the surrounding tissues. At autopsy, there were no visible signs of infection of granulomatous tissue. The granulomatous tissue was stripped away from the sponge, weighed, frozen in liquid nitrogen, pulverized, and a 10% (w/v) tissue homogenate in 0.25 M

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sucrose was prepared using the polytron ST system. The granuloma represents only the tissue surrounding the sponge. The 16,000 g supernatant was prepared and used to measure enzyme activity by a modification (18) of the method of Hutton *et al.* (19). An aliquot of the original homogenate was hydrolyzed in 6 N HCl and assayed for hydroxyproline content by the method of Stegeman and Stalder (20). Total protein was determined by the method of Lowry *et al.* (21). Deoxyribonucleic acid content was quantified by the method of Burton (22). Additional aliquots were extracted with chloroform-methanol for lipid content (23).

The rate of proteinaceous hydroxyproline formation in granulomatous tissue was determined by a modification of an *in vitro* tissue mince system previously described (24). The granulomatous tissue was rinsed twice in minimum essential medium (Eagle, F-12) containing 10,000 units of penicillin and 10,000 μ g of streptomycin. Samples of

granulomatous tissue from the animals were incubated in 10 ml of minimal essential medium which was made 0.28 mM with sodium ascorbate. 2,3- 3 H]Proline (500 μ Ci) were added and incubated under air at 37° for 60 min. After incubation each mixture was separated from the media by filtering through two layers of cheesecloth. The tissue was rinsed twice with 25 ml of 10% (w/v) trichloroacetic acid (TCA) at 0–3° and homogenized in 10% (w/v) TCA with a Polytron-ST homogenizer for 45 sec. The sample was centrifuged at 10,000 g for 15 min. The tissue pellet was resuspended and washed three times with 5% TCA. Ten milliliters of 6 N HCl was added to the tissue pellet and the sample was hydrolyzed for 18 hr in an autoclave. Hydroxyproline was isolated by Dowex cationic-exchange chromatography as described previously (18). The formation of proteinaceous hydroxyproline was linear for at least 90 min.

Results. A dose of 150,000 I.U./kg of vitamin A was required to prevent the weight loss of granuloma tissue in animals treated with 20 mg/kg T.A. (Table I). The amounts of DNA, protein, and proteinaceous hydroxyproline per granuloma were reduced after T.A. treatment when expressed per total granuloma (Table II). Since these differences were not observed when the data were expressed either on a DNA or protein basis, the decreases in DNA, protein, and hydroxyproline result from the general antianabolic effect of glucocorticoid on granuloma growth. Vitamin A treatment prevented the reduction of DNA, protein, and hydroxyproline contents. The amount of lipid per granuloma in both T.A. and T.A. + vitamin A groups was twice as much as either saline control or vitamin A alone. Concurrent vitamin A

TABLE I. THE DOSE OF VITAMIN A REQUIRED TO REVERSE THE TRIAMCINOLONE-MEDIATED DECREASE OF GRANULOMA WEIGHT.^a

Treatment	Granuloma weight (mg)
Saline	707 \pm 66
Vitamin A (50,000 I.U./kg)	746 \pm 14
Vitamin A (150,000 I.U./kg)	788 \pm 88
T.A.	411 \pm 40*
T.A. + vitamin A (50,000 I.U./kg)	530 \pm 55*
T.A. + vitamin A (150,000 I.U./kg)	887 \pm 143

^a The values represent the mean \pm SE of four to eight samples. Animals were either treated with saline, vitamin A at the doses indicated, triamcinolone (T.A. 20 mg/kg), or vitamin A plus T.A. for four daily injections. The granulomatous tissue was dissected from the animal and weighed as described in the text.

* Significantly different from control at $P \leq 0.05$.

TABLE II. VITAMIN A REVERSAL OF GRANULOMA WEIGHT, HYDROXYPROLINE, DNA, AND PROTEIN CONTENT IN TRIAMCINOLONE-TREATED RATS.^a

Treatment	Δ Body weight (g)	Granuloma weight (g)	Hydroxyproline (μ g)	DNA (μ g)	Protein (mg)	Lipid (mg)
Saline	+ 35 \pm 1.9	0.85 \pm 0.6	1.79 \pm 0.26	1.18 \pm 0.17	60.0 \pm 5.8	18.9 \pm 3.7
Vitamin A	+ 27 \pm 4.2	0.84 \pm 0.10	1.92 \pm 0.27	1.08 \pm 0.14	64.3 \pm 9.2	19.9 \pm 3.4
T.A.	- 17 \pm 1.6*	0.29 \pm 0.03*	0.67 \pm 0.12*	0.51 \pm 0.10*	22.0 \pm 1.16*	31.2 \pm 3.8**
Vitamin A plus T.A.	- 20 \pm 1.5*	0.69 \pm 0.07	1.52 \pm 0.18	1.51 \pm 0.04**	56.5 \pm 3.78	32.2 \pm 2.1**

^a Values represent the mean \pm SE of four to eight samples. Triamcinolone acetone (T.A. 20 mg/kg) and vitamin A (150,000 I.U./kg) were injected ip for 4 consecutive days from the day of sponge implantation. The granuloma tissues were prepared and assayed for protein, DNA, and hydroxyproline content as described in the text. Hydroxyproline, DNA, and protein values represent the amounts per granuloma.

* Significantly lower than control at $P \leq 0.05$.

** Significantly greater than control at $P \leq 0.05$.

treatment did not prevent the reduction of body weight by T.A.

The effects of vitamin A treatment on collagen synthesis of granuloma tissue following T.A. treatment was quantified by measuring both prolyl hydroxylase activity and proteinaceous hydroxyproline formation. The prolyl hydroxylase activity was significantly decreased from the control value in both T.A. and T.A. + vitamin A groups (Table III). Concurrent vitamin A treatment did not alter the effect of T.A. on prolyl hydroxylase activity. The T.A. group was not significantly different from the vitamin A plus T.A. group. Proteinaceous hydroxyproline formation was decreased in both T.A. and T.A. plus vitamin A groups (Table III). The steroid-induced decrease of both prolyl hydroxylase activity and the rate of proteinaceous hydroxyproline formation in granulomatous tissue was not altered by concurrent vitamin A treatment.

Discussion. The treatment of either wounds or sponge-induced granuloma with corticosteroids affects several parameters of regeneration and repair including reduction of inflammatory cell infiltration, fibroplasia, and collagen synthesis (2-13). In the present study a reduction in weight and vascularity of the granuloma tissue was noted in the steroid-treated animals, as previously reported (3-5, 7, 8). Concurrent vitamin A

prevented the decreases of several parameters by T.A. Although granuloma weight and the amounts of DNA, protein, and proteinaceous hydroxyproline were all significantly lower than control values in the T.A. group, all of these parameters returned to control levels when vitamin A was given concurrently.

Glucocorticoid administration to rats results in a decreased prolyl hydroxylase in both normal and granulomatous tissues (16-18). The dose of T.A. used significantly decreased prolyl hydroxylase activity in granulomatous tissue. Although Stein and Keiser (25) demonstrated that vitamin A is able to prevent the glucocorticoid-mediated decrease of this enzyme in wounded skin, we did not observe this in sponge-induced granulomatous tissue. Both prolyl hydroxylase activity and the rate of proteinaceous hydroxyproline formation were decreased in animals treated with glucocorticoid plus vitamin A. The dose of vitamin A was the lowest dose which prevented the granulomatous weight reduction by T.A.

Our data indicate that concurrent vitamin A treatment prevented the effect of this steroid on granuloma vascularity and weight but did not block the specific effects of T.A. on prolyl hydroxylase activity and hydroxyproline formation. The degree of vascularity was visually estimated at autopsy. The decrease in proteinaceous hydroxyproline formation in T.A. plus vitamin A groups may represent either a decrease in collagen polypeptide synthesis and/or prolyl hydroxylation step of collagen biosynthesis. Although hydroxyproline formation was decreased in the vitamin A plus T.A. group, total granulomatous proteinaceous hydroxyproline was the same as control. This is quite possible since the decrease in prolyl hydroxylase in tissues occurs after several daily steroid injections (17), whereas the decrease in protein synthesis is evident within hours after initiation of glucocorticoid treatment (7, 8, 10-12, 18, 26). A longer treatment period may be required to observe decreases in total proteinaceous hydroxyproline.

The data indicate that although vitamin A is able to prevent the antiinflammatory activity of glucocorticoids, this vitamin does not block the specific effect of glucocorti-

TABLE III. PROLYL HYDROXYLASE ACTIVITY AND HYDROXYPROLINE FORMATION IN GRANULOMATOUS TISSUE OF RATS TREATED WITH TRIAMCINOLONE AND/OR VITAMIN A.^a

Treatment	Prolyl hydroxylase activity (dpm × 10 ⁻³ /mg protein)	[³ H]Hydroxyproline formed (dpm × 10 ⁻⁴ /mg protein)
Saline	6.3 ± 1.3	18.8
Vitamin A	5.3 ± 1.5	26.0
T.A.	1.9 ± 0.7*	3.4
Vitamin A plus T.A.	0.6 ± 0.3*	5.2

^a Values for prolyl hydroxylase activity represent the mean ± SE of six samples. Triamcinolone (T.A. 20 mg/kg) and vitamin A (150,000 I.U./kg) were injected ip for 4 consecutive days. The granulomatous tissue was excised and prepared for assay of prolyl hydroxylase activity as described in the text. Samples of granulomatous tissue from the rats of each group were pooled and incubated *in vitro* with [³H]proline as described in the text.

* Significantly different at $P \leq 0.05$.

coids. The data suggest that the antiinflammatory activity of this class of drugs is not mediated through a selective inhibition of collagen synthesis.

Summary. Vitamin A administered concurrently with triamcinolone acetonide prevented the effects of steroid on granuloma wet weight, vascularity, hydroxyproline, protein, and DNA. Although vitamin A blocked the general inhibitory effects of glucocorticoid treatment on granuloma growth, decreases of both prolyl hydroxylase activity and proteinaceous hydroxyproline formation were observed. These results indicate that vitamin A did not prevent the specific effects of glucocorticoids on prolyl hydroxylase activity and hydroxyproline formation.

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