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Effect of Retinoids and Estrogens on Testosterone-Induced Hyperplasia of Mouse Prostate Explants in Organ Culture (40654)[1]

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Carcinoma of the prostate is one of the leading causes of death from cancer in men over the age of 55. Treatment of human prostatic cancer includes surgery, chemotherapy, and radiation therapy (1, 2). Although several chemotherapeutic agents against prostatic lesions are being studied, estrogen treatment (with or without orchiectomy) remains the principal therapy for human prostatic cancer (1, 2). Several antiandrogens that might prove useful for prostatic lesions have also been proposed (1, 3–5).

Retinoids have been shown to inhibit or reverse carcinogen-induced epithelial lesions (e.g., hyperplasia and anaplasia) in mouse prostate organ cultures (6–10), and recently, we have shown that all-*trans*-(β)-retinoic acid inhibited and reversed testosterone-induced hyperplasia in mouse prostate organ culture (11). Since testosterone has been linked with human prostatic cancer and induces hyperplastic lesions of prostatic epithelium in organ culture (12, 13), this system seemed to provide an experimental approach to determine if retinoids, estrone, and 17β-estradiol could directly influence testosterone-induced prostatic hyperplasia.

Materials and methods. Organ cultures of mouse prostate were prepared as previously described (6, 7, 11). Ventral prostate from 8- to 10-week-old C3H mice were excised and teased into 1.5 × 1.5-mm explants. The explants were placed on lens paper supported by stainless-steel grids contained in 35-mm plastic petri dishes. The cultures were incubated at 37° in CMRL 1066 medium supplemented with 10% fetal bovine or horse serum, 100 IU per milliliter of penicillin and 100 μg/ml streptomycin using a gas phase of 50% O_2 + 45% N_2 + 5% CO_2. The retinoids and estrogens were dissolved in dimethylsulfoxide (DMSO) at 4 mg/ml and were diluted in culture medium to obtain the desired concentrations. A final DMSO concentration of less than 0.2% in the culture medium was used in all experiments. DMSO at the corresponding concentration was also added to the control cultures. All experimental work was carried out in subdued light.

Two series of experiments were carried out. For the first series, 13-*cis*-retinoic acid or the estrogens at different concentrations were added simultaneously with testosterone hemisuccinate (Sigma Chemical Co.) to determine if the induction of hyperplasia could be prevented. In the second series of experiments in which the reversal of hyperplasia was determined, explants were first made hyperplastic by treatment with testosterone for 8 days followed by simultaneous treatment with testosterone and different concentrations of either a retinoid or an estrogen for an additional 96 hr.

The changes in the rates of proliferation caused by testosterone or a test compound were estimated by the Colcemid metaphase arrest technique (14). Colcemid (2 μg/ml) was added to each culture dish 4 hr before the explants were fixed in buffered formalin. The explants were processed for histology, serially sectioned, and stained with hematoxylin and eosin as previously described (6, 7, 11). The number of arrested metaphases in 1000–2000 cells per explant was counted and the mitotic index (MI) for each explant was estimated. From each explant, 2–3 sections (8–10 sections per group) were scanned and the MI ± SD (standard deviation) calculated for each group (4–6 explants). The significance of the difference between the control and experimental groups was determined by the Student's t test. A P value less than 0.05 was considered significant.

Since a certain degree of variability occurred in the values of mitotic indices in different experiments, each experiment in-

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cluded a series of untreated explants (untreated control group) and a series of explants treated with testosterone (testosterone control group) for each time period. All experiments were repeated and similar results were obtained. The data included in each table represent a single experiment.

Results. Testosterone-stimulated epithelial proliferation at 4 and 8 days after treatment, but when explants were incubated simultaneously with testosterone and 13-*cis*-retinoic acid, the induction of hyperplasia was prevented (Table I). For instance, at 4 days, the MI in the control explants was 98 ± 38, whereas the corresponding value in the testosterone-treated explants was 700 ± 148. The MI in explants that were simultaneously treated with testosterone (1.3×10^{-5} *M*) and different concentrations of 13-*cis*-retinoic acid were 114 ± 59 and 151 ± 71 at 1.7×10^{-5} and 3.3×10^{-6} *M*, respectively. 13-*cis*-Retinoic acid produced a similar mitotic inhibition at Day 8.

In contrast to 13-*cis*-retinoic acid, estrone or 17β-estradiol at similar concentrations produced no mitotic inhibitory effect on testosterone-induced hyperproliferation (Table II). Further, estrone or 17β-estradiol had no significant effect on the MI of the explants as compared to the untreated control group of explants (Table II).

Testosterone stimulated mitoses for at least 12 days (Table III). When explants were treated with testosterone for 8 days followed by simultaneous treatment with testosterone and a retinoid at 1.7×10^{-5}, 3.3×10^{-6}, and 6.6×10^{-7} *M* for an additional 96 hr, the hyperplasia was reversed. For example, at 12 days, the MI in the control explants was 53 ± 25, whereas in those treated with testosterone, the corresponding value was 272 ± 57. The MI in explants that were first treated with testosterone then with testosterone and different concentrations of 13-*cis*-retinoic acid for an additional 96 hr were 96 ± 57, 100 ± 58, and 74 ± 36 at 1.7×10^{-5}, 3.3×10^{-6}, and 6.6×10^{-7} *M* concentrations, respectively. Retinoids themselves, at these concentrations, had no effect on the MI of explants as compared to the MI of untreated control groups of explants (data not shown; see Ref. (6, 7)).

A similar treatment of hyperplastic cultures with estrone or 17β-estradiol, however, did not produce a reversal of hyperplastic lesions (Table IV). In fact, 17β-estradiol appeared to act synergistically with testosterone as mitotic stimulation was observed in the explants.

Discussion. Synthetic retinoids have potential value in the prophylaxis and treatment of epithelial lesions (15–17), and experimental evidence indicates that structural modifications of the retinoid molecule may enhance activity with a concomitant decrease in toxicity (18, 19). An effective side-chain modification is the 13-*cis*-isomer of a retinoid. In this study, 13-*cis*-retinoic acid can directly inhibit and reverse testosterone-induced hyperplasia in mouse prostate explants (Tables I and III). As a geometric isomer of all-*trans*-(β)-retinoic acid, 13-*cis*-retinoic acid is active in altering epithelial differentiation and in reversing benzo(α)pyrene-induced metaplasia (8, 18, 20). The 13-*cis*-isomer of retinoic acid is less toxic than all-*trans*-(β)-retinoic acid (18, 21, 22), reduces the incidence of carcinoma in hamsters treated with benzo(α)pyrene–ferric oxide (18), and re-

TABLE I. INHIBITION OF TESTOSTERONE HEMISUCCINATE-INDUCED HYPERPLASIA BY DIFFERENT CONCENTRATIONS OF 13-*cis*-RETINOIC ACID

Days after treatment	Control (MI ± SD)[a]	Testosterone 1.3×10^{-5} *M* (MI ± SD)	13-*cis*-Retinoic acid concentration[b] (MI ± SD)		
			1.7×10^{-5} *M*	3.3×10^{-6} *M*	6.6×10^{-7} *M*
4	98 ± 38	700 ± 148[c]	114 ± 59[d]	151 ± 71[d]	250 ± 112[d]
8	150 ± 38	542 ± 112[c]	134 ± 44[d]	138 ± 51[d]	493 ± 98

[a] The number of arrested metaphases per 1×10^5 cells during a 4-hr period of Colcemid treatment. SD = standard deviation.

[b] Explants were simultaneously treated with testosterone hemisuccinate and different concentrations of 13-*cis*-retinoic acid for 4 or 8 days.

[c] The mitotic stimulation was significant as compared to the corresponding control group ($P < 0.05$).

[d] The mitotic inhibition was significant as compared to the corresponding group that only received testosterone hemisuccinate ($P < 0.05$).

TABLE II. LACK OF INHIBITION OF TESTOSTERONE HEMISUCCINATE-INDUCED HYPERPLASIA BY DIFFERENT CONCENTRATIONS OF ESTRONE OR 17β-ESTRADIOL

Hormone	Days after treatment	Control (MI ± SD)[a]	Testosterone 1.3×10^{-5} *M* (MI ± SD)	Estrogen concentration[b] (MI ± SD)		
				1.8×10^{-5} *M*	3.7×10^{-6} *M*	7.4×10^{-7} *M*
Estrone	4	142 ± 70		113 ± 26	161 ± 43	—
	8	139 ± 31		158 ± 37	193 ± 68	177 ± 47
Estrone + Testosterone	4	80 ± 21	190 ± 58[c]	458 ± 156	480 ± 65	460 ± 84
	8	113 ± 33	1005 ± 162[c]	954 ± 213	614 ± 76	711 ± 307
17β-Estradiol	4	142 ± 70		121 ± 47	116 ± 35	102 ± 16
	8	139 ± 31		169 ± 56	229 ± 65	226 ± 70
17β-Estradiol + Testosterone	4	127 ± 30	471 ± 120[c]	547 ± 236	457 ± 154	353 ± 113
	8	164 ± 63	419 ± 165[c]	349 ± 128	455 ± 147	809 ± 189

[a] The number of arrested metaphases per 1×10^5 cells during a 4-hr period of Colcemid treatment. SD = standard deviation.

[b] Explants were treated with either estrone or 17β-estradiol or were simultaneously treated with estrone plus testosterone hemisuccinate or 17β-estradiol plus testosterone hemisuccinate for 4 or 8 days.

[c] The mitotic stimulation was significant as compared to the corresponding control group ($P < 0.05$).

TABLE III. REVERSAL OF TESTOSTERONE-INDUCED HYPERPLASIA BY DIFFERENT CONCENTRATIONS OF RETINOIDS

Retinoid	Days after treatment	Control (MI ± SD)[a]	Testosterone 1.3×10^{-5} *M* (MI ± SD)	Hours after adding retinoid	Retinoid concentration[b] (MI ± SD)		
					1.7×10^{-5} *M*	3.3×10^{-6} *M*	6.6×10^{-7} *M*
13-*cis* Retinoic acid	8	149 ± 49	480 ± 120[c]				
	12	53 ± 25	272 ± 57[c]	96	96 ± 57[d]	110 ± 58[d]	74 ± 36[d]
The 1-methoxyethylcyclopentyl analog of retinoic acid	8	251 ± 55	436 ± 91[c]				
	12	134 ± 52	366 ± 89[c]	96	—	71 ± 13[d]	151 ± 47[d]
The trimethylmethoxyphenyl analog of retinoic acid	8	146 ± 63	619 ± 40[c]				
	12	135 ± 42	430 ± 107[c]	96	103 ± 40[d]	113 ± 43[d]	152 ± 58[d]
The trimethylmethoxyphenyl analog of retinoic acid ethylamide	8	146 ± 63	619 ± 140[c]				
	12	135 ± 42	430 ± 107[c]	96	122 ± 51[d]	157 ± 59[d]	101 ± 34[d]
The trimethylmethoxyphenyl analog of retinoic acid ethyl ester	8	251 ± 53	436 ± 91[c]				
	12	134 ± 52	366 ± 89[c]	96	152 ± 78[d]	82 ± 43[d]	275 ± 87

[a] The number of arrested metaphases per 1×10^5 cells during a 4-hr period of Colcemid treatment. SD = standard deviation.

[b] Explants were treated with testosterone hemisuccinate for 8 days followed by simultaneous treatment with testosterone hemisuccinate and different concentrations of retinoids for an additional 4 days.

[c] The mitotic stimulation ws significant as compared to the corresponding control group ($P < 0.05$).

[d] The mitotic inhibition was significant as compared to the corresponding group that received only testosterone hemisuccinate ($P < 0.05$).

duces the incidence and extent of bladder cancer in rats induced with *N*-methyl-*N*-nitrosourea (23) and *N*-butyl-*N*-(4-hydroxybutyl)nitrosamine (24). Peck and Yoder (15) have recently reported that in the treatment of patients with keratinizing dermatosis, 13-*cis*-retinoic acid may be more effective and less toxic than all-*trans*-retinoic acid.

The four retinoids (Chart 1) in which the β-ionone ring of all-*trans*-(β)-retinoic acid was replaced with a five- or six-carbon ring were active in reversing testosterone-induced hyperplasia in mouse prostate explants (Table III). Two of these retinoids were trimethylmethoxyphenyl analogs also modified at the terminal carboxyl group of the side chain (the ethylamide and the ethyl ester, Table III). Other reports have also indicated that the unaltered β-ionone ring of the retinoid molecule is not necessary for altering epithelial differentiation (18–20, 25) or for reversing hyperplasia of mouse prostate explants induced by *N*-methyl-nitro-*N*-nitrosoguanidine (7) or 3-methylcholanthrene (9,

10). The ethyl ester and ethylamide analogs of the trimethylmethoxyphenyl retinoids have also been reported to be active in reversing keratinization (18–20, 25), increasing RNA in epidermal cell cultures (18, 19), and in promoting the lysis of the extracellular matrix of cartilage in organ culture (9, 10).

Specific structural alterations of the retinoid molecule can affect different biological activities. Thus while the cyclopentenyl retinoid is more effective than all-*trans*-(β)-retinoic acid in modulating epithelial differentiation (20, 25) and highly active in inhibiting and reversing the effects of carcinogens on mouse prostatic epithelium *in vitro* (7–10), it is virtually devoid of vitamin A growth-promoting activity (26) and is highly effective in promoting the lysis of cartilage extracellular matrix in organ culture (26).

Estrone or 17β-estradiol had no effect on the MI of mouse prostate explants in this system (Table II). The effect of estrogens on prostatic epithelium may be dependent on the age of the explanted tissue. For instance in organ cultures of prostates from 6-week-old mice, estrone- and stilbesterol-induced

TABLE IV. LACK OF REVERSAL OF TESTOSTERONE HEMISUCCINATE-INDUCED HYPERPLASIA BY DIFFERENT CONCENTRATIONS OF ESTRONE OR 17β-ESTRADIOL

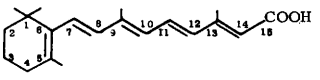
Estrogen	Days after treatment	Control (MI ± SD)[a]	Testosterone 1.3×10^{-5} *M* (MI ± SD)	Hours after adding estrogen	Estrogen concentration[b] (MI ± SD) 1.8×10^{-5} *M*	3.7×10^{-6} *M*	7.4×10^{-7} *M*
Estrone	8	118 ± 57	409 ± 149[c]				
	12	143 ± 49	513 ± 113[c]	96	782 ± 143	960 ± 134	610 ± 161
17β-Estradiol	8	119 ± 33	413 ± 141[c]				
	12	146 ± 13	594 ± 239[c]	96	1215 ± 161	1669 ± 578	1687 ± 458

[a] The number of arrested metaphases per 1×10^5 cells during a 4-hr period of Colcemid treatment. SD = standard deviation.

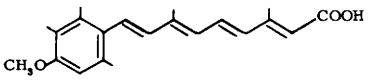
[b] Explants were treated with testosterone hemisuccinate for 8 days followed by simultaneous treatment with testosterone hemisuccinate and different concentrations of estrone or 17β-estradiol for an additional 4 days.

[c] The mitotic stimulation was significant as compared to the corresponding control group ($P < 0.05$).

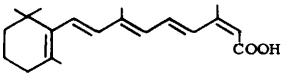
Structure of Retinoids



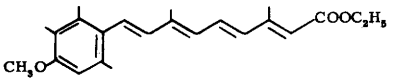
All-trans-retinoic acid



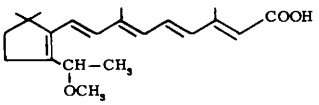
Trimethylmethoxyphenyl (TMMP) Analog of Retinoic Acid



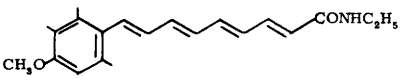
13-cis-Retinoic Acid



Trimethylmethoxyphenyl Analog of Retinoic Acid Ethyl Ester



The 1-Methoxyethyl Cyclopentenyl Analog of Retinoic Acid



Trimethylmethoxyphenyl Analog of Retinoic Acid Ethyl Amide

The retinoids were obtained from Hoffmann-La Roche, Inc. (Nutley, NJ and Basal, Switzerland) through the Lung Cancer Segment, NCI, Bethesda, MD.

hyperplasia of the alveolar epithelium (12, 27) while, in prostate explants from 5- to 6-month-old mice, estrone produced atrophy and necrosis of the epithelium (12). The failure of estrogens to stimulate the explants derived from 8- to 10-week-old C3H mice could be due to differences in susceptibility between the mice used in the study and those used by other investigators or to the influence of other hormones, presumably present in the serum used to supplement the medium. However, the prostate explants were evidently quite sensitive to stimulation with testosterone hemisuccinate (1.3×10^{-5} *M*, Tables I–IV).

The data show that estrogens failed to inhibit or reverse testosterone-induced hyperplasia in mouse prostate explants (Tables II and IV). In agreement with these results, other reports have indicated that *in vivo* 17β-estradiol had no significant effect on testosterone-stimulated DNA synthesis in the prostate of castrated rats (2, 28). Estrogens, however, cause remission of prostatic lesions in man (1, 29). The mechanism by which estrogens cause remission of these lesions has not been completely defined. Estrogens may interfere with the metabolism of testosterone. For example, Farnsworth (30) showed that 17β-estradiol or estrone inhibited the conversion of testosterone to dihydrotestosterone (DHT) in homogenates of prostate obtained from patients with benign prostatic hyperplasia. Lee *et al.* (28) showed that 17β-estradiol inhibited 5α-reductase activity in the prostate of intact rats, although no effect on the enzyme activity was observed in the prostate of castrated rats injected with testosterone and 17β-estradiol. Estrogens are believed to influence the testosterone titers indirectly via the pituitary gonad axis. Our results showing that estrogens had no effect on testosterone-induced hyperplasia in mouse prostate explants (Tables II and IV) suggest that estrogens do not have any direct effect on testosterone metabolism in this system and therefore support the view that an estrogenic effect in the remission of prostatic lesions in man may involve the pituitary–gonad axis. In this regard, Alder *et al.* (31) have suggested that estrogens may decrease testicular testosterone production by inhibiting pituitary gonadotrophin production. This is probably true since estrogens inhibit prostatic growth of the intact animal by reducing serum testosterone levels, whereas they do not block testosterone-induced growth in castrated rats (32).

Recently (33) Burns *et al.* showed that estradiol (5×10^{-9} *M*) inhibited the binding of DHT in the cytosol preparations of the seminal vesicle of guinea pig while diethylstilbestrol (5×10^{-9} *M*) had no apparent effect on this binding, although both estrogens caused regression of seminal vesicles when injected in the intact animal. These authors thus suggested that at least in the case of seminal vesicles, estradiol, in addition to its influence via the pituitary–gonad axis, may also have a direct effect at the tissue level (34). It should, however, be pointed out that in the case of prostate cytosol preparations, estradiol at significantly higher concentrations (10^{-6} *M*) had no effect on DHT binding (34, 35).

In contrast to estrogens, retinoids can directly inhibit and reverse testosterone-induced lesions in the mouse prostate explants. The mechanism by which retinoids cause these antiandrogenic effects is also not known, although several possibilities can be suggested. For instance, it may act by blocking the uptake of testosterone by competing with the hormone at the appropriate sites. Recently, B. P. Sani (personal communication) showed that testosterone does not compete with all-*trans*-retinoic acid for binding with retinoic acid-binding protein of chick embryonic skin, thus suggesting that testosterone and all-*trans*-retinoic acid bind independently to their receptor proteins. However, it is possible that the 13-*cis*-retinoic acid–protein complex inhibits the binding between DHT–protein complex and nuclear protein. Fang and Liao (36) have demonstrated that cyproterone acetate, an antiandrogen, inhibited the transport of labeled DHT from the prostatic cytoplasm to the nucleus, and these results were confirmed by other investigators (37–39).

Summary. Testosterone produced epithelial hyperplasia in mouse prostate explants in organ culture. Simultaneous treatment of explants with testosterone and 13-*cis*-retinoic acid inhibited the induction of hyperplasia. When explants were first made hyperplastic by treatment with testosterone for 8 days and

followed by simultaneous treatment with testosterone and a retinoid for an additional 96 hr, the following retinoids reversed the hyperplasia: 13-*cis*-retinoic acid, the 1-methoxyethylcyclopentenyl analog of retinoic acid, the trimethylmethoxyphenyl analog of retinoic acid, the trimethylphenyl analog of retinoic acid ethylamide, and the trimethylmethoxyphenyl analog of retinoic acid ethyl ester. The data indicate that these retinoids prevent androgen-induced hyperplasia in prostatic tissue in this system by a direct influence on the tissue.

Since estrone or 17β-estradiol had no effect on testosterone-induced hyperplasia in the prostate explants, it is concluded that these estrogens may act by way of an indirect mechanism (pituitary–tesis axis) in preventing androgen-induced hyperplasia of the prostate.

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