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9-*cis*-Retinoic Acid but Not 4-(Hydroxyphenyl)retinamide Inhibits Prostate Intraepithelial Neoplasia in Noble Rats[1]

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ABSTRACT

In most previous studies, the incidence and multiplicity of chemically induced prostate tumors have been used as end points for assessing the efficacy of various chemopreventive agents. In this study, we used prostate intraepithelial neoplasia (PIN) in Noble rats as an intermediate end point to examine the chemopreventive efficacy of two retinoids, 9-*cis*-retinoic acid (9cRA) and 4-(hydroxyphenyl)retinamide, which in previous studies have shown promising inhibitory effects on various carcinogenesis models. We found that 80–100% of Noble rats treated for 36 weeks with testosterone + 17β-estradiol developed multiple PIN lesions predominantly in the dorso-lateral prostate, which appears relevant to the place of origin of PIN and carcinoma in the human prostate. 9cRA at 50 or 100 mg/kg diet significantly decreased the multiplicity of PIN, whereas 4-(hydroxyphenyl) retinamide at 392 or 784 mg/kg diet, did not have an inhibitory effect on PIN. Thus, we provide for the first time evidence that the testosterone + 17β-estradiol-induced PIN in Noble rats could be used as a potential intermediate end point in assessing the efficacy of retinoids and possibly of other agents on prostate carcinogenesis, and that 9cRA alone or in combination with other agents may have clinical promise in preventing the development of prostate cancer in men.

INTRODUCTION

Among various agents that have been tested for prevention of prostate cancer, only a few have shown promising results, and these are mainly the inhibitors of testosterone synthesis and/or signaling, and retinoids (1, 2). Among the retinoids, promising results were obtained with 9cRA[3] on chemically induced prostate carcinogenesis in Wistar-Unilever rats (3), whereas the efficacy of 4-HPR, another synthetic retinoid that has been extensively used for prevention of breast cancer, is still controversial (4–6). It has been speculated that the differences in the efficacy of 9cRA and 4-HPR in inhibiting prostate carcinogenesis are a result of their effects on RARs (RARα, -β, -γ) or RXRs (RXRα, -β, -γ), which are expressed in prostate epithelial cells (7). 9cRA is a ligand for both RARs and RXRs (8, 9), whereas 4-HPR apparently does not affect the above receptors. Over the last several years new classes of retinoids have been developed that affect specific RARα, -β, -γ or RXRα, -β, -γ (10). The chemopreventive efficacy of these retinoids on prostate carcinogenesis is still unknown.

In most previous prostate chemoprevention studies, MNU + testosterone treatment has been used for induction of prostate carcinogenesis in rats, and the incidence and multiplicity of tumors have been used as end points of efficacy. However, in these studies the incidence and multiplicity of tumors is low, their latency is more than 1 year, and most importantly, the tumors arose in SVs and AP (11–13), which do not have corresponding counterparts in human prostate (14). Extensive studies (15–17) over the last 20 years have shown that Noble rats treated with T + E developed multiple prostate dysplastic lesions with characteristics of PIN. PIN occurred as early as 16 weeks after initiation of hormone stimulation, preferentially affects the DLP, and in morphology appears relevant to PIN of the human prostate (14).

In the present study, we used Noble rats and hormone stimulation (T + E) to induce PIN and to assess the chemopreventive efficacy of 9cRA or 4-HPR on prostate carcinogenesis. Both retinoids were given at doses that in previous studies have shown inhibitory effects on prostate and on other carcinogenesis models (6, 7). Here, we provide for the first time evidence that 9cRA, but not 4-HPR, inhibits PIN in Noble rats and that PIN could be used as a potential intermediate end point for assessing the efficacy of retinoids on prostate carcinogenesis.

MATERIALS AND METHODS

Animals and Hormone Stimulation. Male Noble rats were obtained from the Biological Testing Branch of the National Cancer Institute. After 1 week of quarantine and at the age of 12 weeks, the animals were randomized by weight and put on Teklad 4% Rat/Mouse chow (meal). The initial weight of the animals was 250–275 g. All animals were weighed weekly and observed twice daily for signs of illness. PIN was induced by continuous stimulation of prostate cell proliferation with T + E (17). Both hormones were administered s.c. via silastic capsule implants. Two 2-cm long capsules were prepared from silastic tubing (catalogue no. 602–205, 1 mm 1.d. × 2.2 mm o.d., lot HH125357; Dow Corning, Midland, MI). The capsules were filled with testosterone (T1500; Sigma Chemical Co., St. Louis, MO), and one 1-cm long capsule was filled with 17β-estradiol (E8875; Sigma Chemical Co.). Control rats received implants of unfilled (empty) silastic capsules. All silastic capsules were removed and replaced at 2-month intervals throughout the study.

Chemopreventive Agents. 9cRA was purchased from Sigma Chemical Co. 4-HPR was obtained from R.W. Johnson Pharmaceutical Research Institute (Springfield, PA). A Teklad (4%) Rodent Chow diet supplemented with menadione sodium bisulfite (12 mg/kg) for both 4-HPR and 9cRA to prevent hypoprothrombinemia was used. 9cRA was given in the diet at 50 or 100 mg/kg; 4-HPR was also administered at two concentrations, 392 or 784 mg/kg diet. In previous studies, the above doses of 9cRA and 4-HPR did not show toxicity in rats (3–6). The administration of both retinoids began 1 week before hormone implants and continued until the end of the experiment, 36 weeks after initiation of hormone treatment.

Necropsy and Prostate Examination. After sacrifice of the animals, the abdominal cavity was opened and the VP was dissected from the rest of the prostate complex and fixed in 10% buffered formalin. DLP, AP, and the intraprostate portion of SVs were cut longitudinally, fixed in formalin, and embedded in paraffin. Serial paraffin sections (4 μm each) were obtained at three separate tissue levels 200 μm apart and stained with H&E for evaluation of prostate lesions (11).

Statistical Evaluation. The differences in the incidence and frequency of PIN between control and retinoid-treated animals were assessed by the χ^2 test (Mentel-Haenszel) and by the two-sided Student's *t* test, respectively. Differences with a value of $P < 0.05$ were considered significant.

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[3] Abbreviations: 9cRA, 9-*cis*-retinoc acid; DLP, dorso-lateral prostate; AP, anterior prostate; SV, seminal vesicle; VP, ventral prostate; PIN, prostate intraepithelial neoplasia; 4-HPR, 4-(hydroxyphenyl)retinamide; RAR, retinoic acid receptor; RXR, retinoid X receptor; T + E, testosterone + 17β-estradiol; CIS, carcinoma *in situ*; MNU, N-methyl-N-nitrosourea.

Table 1 *Effects of 9cRA on the incidence and multiplicity of PIN in Noble rats*

Parameter	Basal diet	9cRA	
		50 mg/kg diet	100 mg/kg diet
Animals (no.): at start/at end	35[a]/19	20/17	20/17
T + E	+	+	+
Treatment (wk)	36	36	36
Survival (%)	100	85	85
Body weight (g), T + E	352	352	336
DLP			
Incidence (%)	100%	95	95
Multiplicity	3.2 ± 0.9[b]	2.1 ± 1.0[b]	1.8 ± 0.8[c]
AP + SV			
Incidence (%)	42	20	20
Multiplicity	0.4 ± 0.4	0.2 ± 0.4	0.2 ± 0.4
VP			
Incidence (%)	30	10	15
Multiplicity	0.4 ± 0.7	0.2 ± 0.5	0.2 ± 0.4
Body weight (g), basal diet	468[d]		

[a] Five animals from the control group (T + E) were killed 20, 24, 26, and 30 weeks after initiation of hormone treatment to assess the development of PIN in various prostate glands.

[b,c] A significant difference was found in multiplicity of PIN between control and 9cRA-treated animals: [b] $P < 0.01$ for 50 mg/kg diet and [c] $P < 0.001$ for 100 mg/kg diet, respectively.

[d] The body weight of the animals on the basal diet only (no hormone implants) was about 30% higher than of the animals with hormone implants, $P < 0.01$, two-sided *t* test.

RESULTS

One hundred fifty-five Noble rats were used in this study (Tables 1 and 2). Twenty animals/group were examined, with exception of the control group (treated with T + E only) in which 35 animals were examined. In this group, five animals were sacrificed 20, 24, 26, and 30 weeks after initiation of hormone treatment, and their accessory sex organs were examined to make sure that PIN develops before the terminal sacrifice. Starting from the 20th week, lesions with characteristics of PIN were consistently found in the DLP and AP (data not shown). In both experiments, T + E treatment reduced the animals' weight, which, at 36 weeks, was approximately 30% lower than in the animals on basal diet (Fig. 1, *A* and *B*). However, neither 9cRA nor 4-HPR at both dosage levels significantly decreased the body weight as compared with the control group of animals treated with T + E only, indicating that 9cRA and 4-HPR are not toxic.

In T + E-treated animals the incidence of PIN in the DLP varied between 78.5% and 100%, and the multiplicity varied between 1 and 5. 9cRA decreased the multiplicity of PIN in the DLP in a dose-dependent manner, from 3.2 ± 0.9 in control animals to 2.1 ± 1.0 and to 1.8 ± 0.9 in the animals treated with 50 or 100 mg/kg diet of 9cRA ($P < 0.01$ and $P < 0.001$), respectively (Table 1). 9cRA may have inhibitory effects in the development of PIN in AP and SVs and in VP as well, although the differences in the values with the control animals were not significant ($P > 0.05$). 4-HPR at both doses did not affect PIN in either the DLP or in the other accessory sex organs (Table 2). In two animals treated with 4-HPR, in addition to PIN, lesions with characteristics of CIS were also found.

Most PIN lesions were identified in the periurethral ducts (Fig. 2*A*). Two morphological types of PIN were distinguished among the control (T + E treated) and retinoid-treated animals: one that is composed of a multilayer of dysplastic cells that did not form pseudoacinar structures (Fig. 2*A*, *arrow*), and another one that is apparently a more advanced stage of the dysplastic process, where dysplastic cells form alveolar or papillary structures (Fig. 2*A*, *arrowhead*). In PIN, the cells lost their polarity, the nucleus was enlarged with dispersed chromatin, and mitotic figures were frequent (Fig. 2, *B* and *C*). PIN lesions in AP and VP were similar in morphology to those described above (Fig. 2*D*). PIN in the animals treated with 9cRA in morphology and origin was similar to those of control animals. However, in some 9cRA-treated animals, PIN formed cystic structures and in their lumen desquamated death cells were frequently observed (Fig. 2*F*, *arrow*). PIN lesions were frequently surrounded by a sheet of collagen, inflammatory cells, and connective tissue elements (Fig. 2*E*). 4-HPR did not induce significant alterations in morphology of accessory sex organs.

DISCUSSION

The primary goal of this study was to determine the feasibility of PIN in Noble rats as a potential intermediate end point for assessment of the chemopreventive efficacy of retinoids. Noble rats were used because: (*a*) they develop high incidence (up to 100%) and multiplicity (1–5/gland) of PIN; (*b*) the latency of PIN is relatively short (16–20 weeks); (*c*) PIN occurs predominantly in the DLP, which is relevant to the site of origin of PIN and carcinoma in human prostate; and (*d*) PIN is induced by hormone stimulation (T + E) only, without the need of a carcinogen to initiate the neoplastic process. Therefore, the induction of PIN in Noble rats after T + E treatment simulates a potential mechanism(s) of the prostate cancer development in man, where hormonal factors are predominantly involved (2). In most previous studies MNU plus hormone stimulation have been used to induce prostate carcinogenesis (3–5). However, MNU is not the cause of human prostate cancer, the latency of chemically induced prostate tumors is too long, the incidence and frequency of tumors is relatively low, and, most importantly, tumors occur not preferentially in the DLP but in AP and SV, which do not have relevant counterparts in the human prostate (17).

9cRA and 4-HPR were used as chemopreventive agents because in most previous studies they were efficacious in inhibiting mammary and other carcinogenesis models in rats and mice (1, 3, 6, 9). We found that: (*a*) of the animals treated for 36 weeks with T + E, between 80% and 100% developed multiple PIN in the DLP; (*b*) 9cRA decreased in a dose-dependent manner the frequency of PIN; and (*c*) 4-HPR did not suppress the development of PIN. In two animals treated with T + E and 4-HPR, in addition to PIN, two lesions with characteristics of CIS were also observed. Therefore, 4-HPR may not protect but potentate the neoplastic process in the DLP and the AP. To our best knowledge, this is the first study where the hormone-induced PIN in Noble rats was used as an intermediate end point for assessing the chemopreventive efficacy of 9cRA and 4-HPR on prostate carcinogenesis. 9cRA has also shown inhibitory effects on the development of prostate cancer in Wistar-Unilever rats, whereas 4-HPR was not efficacious (3, 6), supporting our hypothesis that PIN

Table 2 *Effects of 4-HPR on the incidence and multiplicity of PIN in Noble rats*

Parameter	Basal diet	4-HPR	
		392 mg/kg diet	784 mg/kg diet
Animals (no.): at start/at end	20/20	20/20	20/20
T + E	+	+	+
Treatment (wk)	36	36	36
Survival (%)	97.1	80	90
Body weight (g): T + E only	341	342	333
DLP			
Incidence (%)	78.5	85	90
Multiplicity	1.4 ± 0.9	1.5 ± 0.9[a]	1.6 ± 1.0[a]
AP + SV			
Incidence (%)	30	10	10
Multiplicity	0.4 ± 0.4	0.2 ± 0.4	0.1 ± 0.5
VP			
Incidence (%)	0	10	5
Multiplicity	0	0.1	0.05
Body weight (g), basal diet	439[b]		

[a] Two lesions with characteristics of CIS was found in 4-HPR-treated animals; there was no significant difference in the values between 4-HPR-treated and control (T + E) animals, $P > 0.05$.

[b] The body weight of the animals on the basal diet only (no hormone implants) was about 30% higher than of the animals with hormone implants, $P < 0.01$, two-sided *t* test.

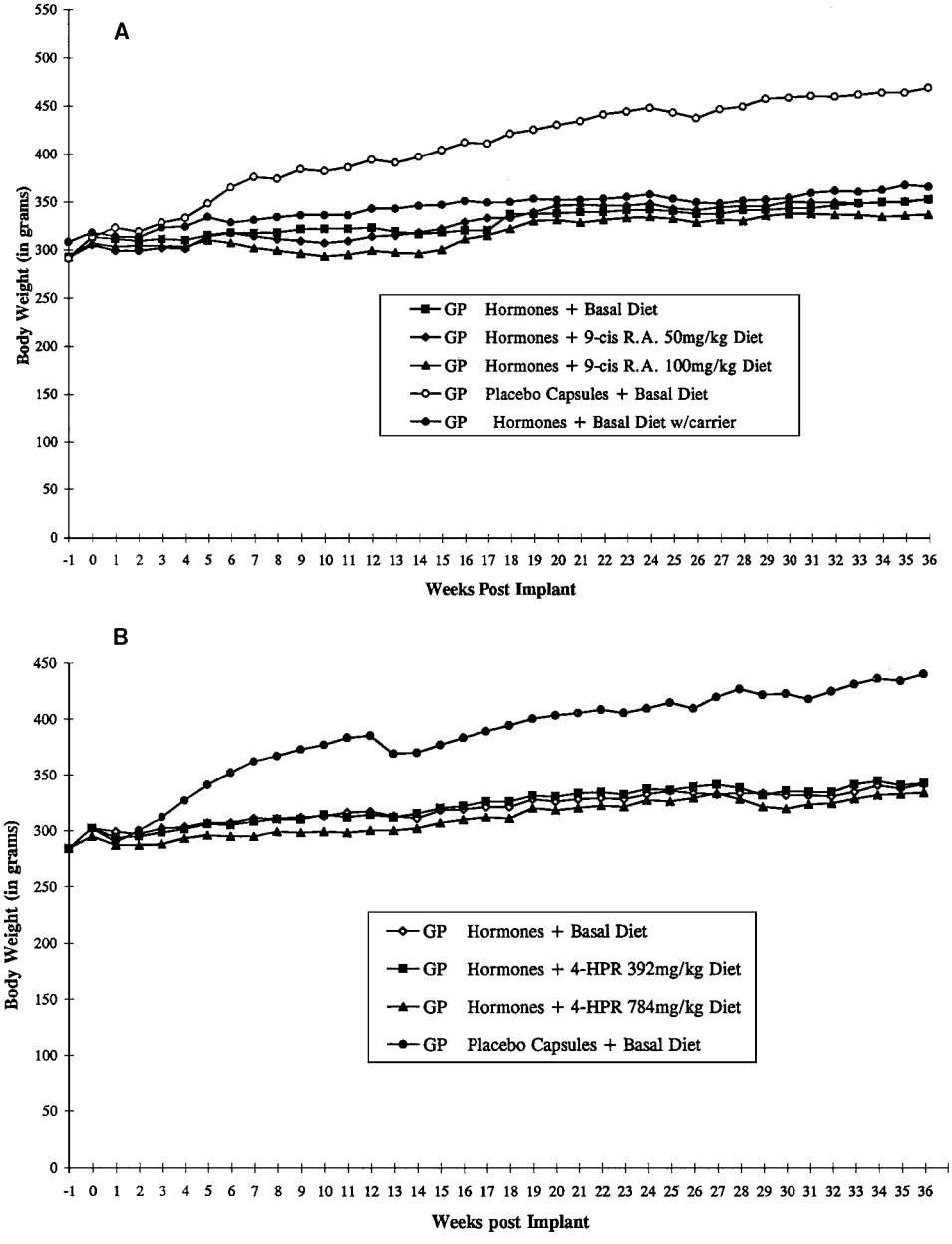

Fig. 1. *A*, changes in the animals' body weight in the course of treatment with T + E or with T + E + 9cRA. Note that T + E treatment causes a significant decrease in the body weight ($P < 0.001$) as compared with the animals on basal diet (the most upper curve). 9cRA at both dose levels did not significantly affect the body weight as compared with the animals treated with T + E. *B*, changes in the body weight of the animals treated with T + E or with T + E + 4-HPR. At the end of experiment, the body weight of T + E-treated animals was about 30% lower than that of control animals ($P < 0.01$, two-sided *t* test). 4-HPR did not affect the body weight, which was similar to the weight of T + E-treated animals.

is a valuable intermediate end point in assessing the chemopreventive efficacy of retinoids.

Although we obtained consistent data indicating that PIN could be used for assessing the efficacy of retinoids on prostate carcinogenesis, additional studies are needed to assess the biology of PIN and its progression toward malignant tumors. PIN may not only progress but eventually regress and disintegrate after withdrawal of the hormone stimulation (18). When already established, PIN may differentially respond to the above retinoids as well as to other chemopreventive and antitumor agents. Our data on the efficacy of 9cRA and 4-HPR in inhibiting PIN in Noble rats supports recent studies by McCormick *et al.* (3), demonstrating that 9cRA at 50 or 100 mg/kg diet decreased the incidence of prostate tumors in Wistar-Unilever rats treated with MNU + testosterone and that 4-HPR at doses of 392 or 784 mg/kg diet was not an efficacious inhibitor of prostate carcinogenesis (6). The lack of efficacy of 4-HPR on PIN in our study is also in agreement with the results published previously by Lucia *et al.* (5) and Slayter *et al.* (13), where prostate tumors have been induced in Lobund-Wistar rats by MNU + testosterone treatment. Clinical studies with 4-HPR in patients with prostate cancer also did not find significant alterations in prostate tumor cells (19). The differences in the efficacy of 9cRA and 4-HPR in inhibiting PIN and prostate tumor development is most probably associated with differences in their pharmacokinetics, metabolism, and effects on RARs and RXRs (20). Previous data have shown that 9cRA is a ligand for both RARs and RXRs (7–10), whereas it is still not known whether 4-HPR uses some of the above receptors to exert an effect. 9cRA forms heterodimers with other nuclear receptors and, thus, affects various signaling pathways and cell functions, which 4-HPR is apparently not able to modulate (6, 7).

In conclusion, in this study we obtained data indicating that PIN induced in Noble rats by continuous T + E stimulation could be used as an intermediate end point for assessing the efficacy of 9cRA, 4-HPR, and possibly of other chemopreventive agents on prostate carcinogenesis. Furthermore, the hormone pathogenesis, the high multiplicity, short latency, preferential location in the DLP, the similarity

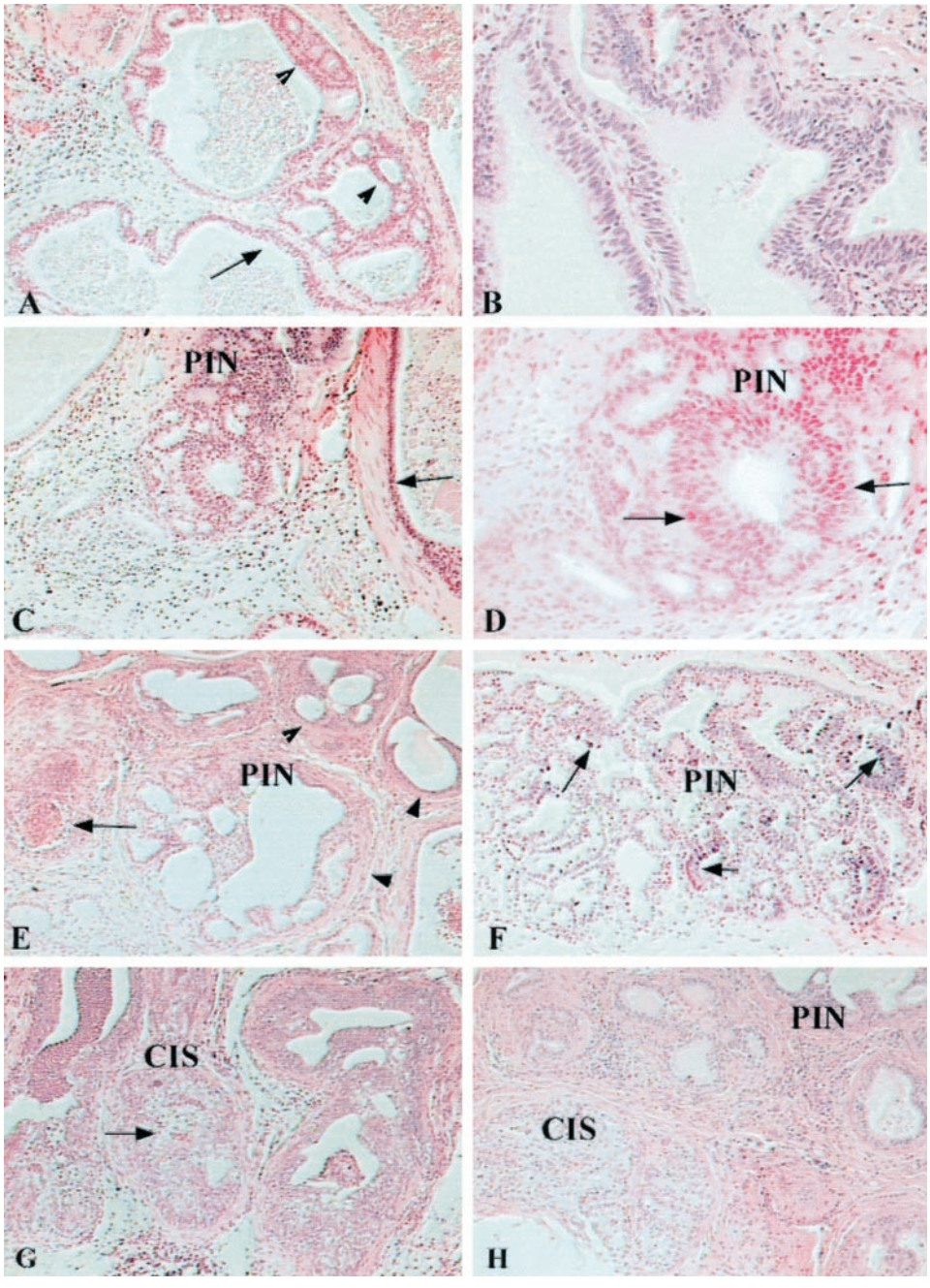

Fig. 2. *A*, PIN in the DLP of a control Noble rat treated for 36 weeks with T + E. Multilayer of dysplastic cells occupies isolated ductal areas (*arrow*) or form alveolar structures (*arrowhead*). H&E, ×100. *B*, PIN in the DLP of a Noble rat treated with T + E. Dysplastic cells cover the wall of a duct. Dysplastic cells are arranged in layers and exhibit a loss of polarity. The cells appear crowded together and piled on one another. Note the nuclear and cellular atypia, H&E, ×200. *C*, PIN in the DLP of an animal treated with T + E. Dysplastic cells form alveolar structures, which vary in structure and cell composition. Two types of dysplastic cell populations could be distinguished in the lesions: one of cubical cells that forms the glandular structures, and a second one in the center (*arrow*), which is composed of undifferentiated cells that apparently have lost their ability to form glands, which are overcrowded and with high mitotic activity (*arrows*). H&E, ×200. *D*, PIN in the AP of a T + E-treated animal. Note the dysplastic cells in PIN with intensely stained enlarged nucleus and reduced cytoplasm. The cells appear crowded together with reduced intercellular space. H&E, ×200. *E*, multiple PIN lesions in the DLP of an animal treated with 9cRA. Several ducts are occupied by dysplastic cells, which form alveolar or cystic structures. The ductal structures are surrounded by collagen and stroma components. H&E, ×200. *F*, PIN in the AP of an animal treated with 9cRA. Note the high number of desquamated and necrotic cells (*arrow*) among the lesions. H&E, ×200. *G*, CIS in a DLP of rat treated with 4-HPR for 36 weeks. The whole ductal lumen is occupied by tumor cells, which in certain areas disintegrate (*arrow*). Tumor cells do not penetrate the basal lamina. H&E, ×200. *H*, CIS in the AP of an animal treated with 4-HPR for 36 weeks. In addition to CIS there are lesions with characteristics of PIN. H&E, ×200.

in morphology to PIN of human prostate, and finally the sensitivity to specific retinoids make PIN in Noble rats a promising intermediate end point for prostate chemoprevention studies.

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