Effect of Coumarin on the Normal Rat Prostate and on the R-3327H Prostatic Adenocarcinoma

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ABSTRACT

Coumarin, the parent compound of warfarin, has been observed to stimulate macrophages, increase phagocytosis, and induce changes in lymphocyte-mitogen responsiveness in cancer patients. Coumarin has been reported to have antitumor activity in human melanomas and renal cancer when used in conjunction with the H-2 antagonist, climetidine.

We have observed that coumarin has antiprostatic activity in rats. When coumarin was given to mature rats at a dose of 40 mg/kg, a significant decrease in the size of the prostate, seminal vesicles, and testes was observed. Testosterone levels were unchanged or slightly elevated, consistent with an antiandrogenic-like activity. Similarly, coumarin significantly inhibited the androgen-induced increase in prostatic size when administered to castrated rats receiving testosterone. Coumarin given to rats bearing the R-3327H androgen-sensitive, prostate-derived tumor decreased the size of the primary tumor. The effect was greater than that produced by castration. Coumarin is worthy of further consideration as an agent for use in controlling the normal and abnormal growth of the prostate.

INTRODUCTION

Coumarin (2H-1-benzopyran-2-one, also 1,2-benzopyrone), the parent compound of warfarin, has been widely used in the past as a flavoring agent in foods and drugs (1, 2) (Fig. 1). Originally obtained as a natural product from tonka beans, commercial coumarin is now prepared synthetically. Coumarin, unlike warfarin, has no antithrombin activity. Multiple studies on the physiology, pharmacology, and toxicology of coumarin have been reported in the early literature, but it was not until recently that significant immunological and antitumor effects have been reported (2–8). Coumarin has been observed to induce changes in lymphocyte-mitogen responsiveness in cancer patients, and to increase the number and activation state of macrophages (3, 4). Such activities are thought to be of importance in the control of metastasis and destruction of tumor cells (9). The mechanism of the above findings is unknown.

Coumarin as a single agent or in combination with other immune modulators has demonstrated antitumor activity against human malignant melanoma and metastatic renal cell carcinoma (6–8). Our interest in coumarin originated following multiple experiments which showed that coumarin had significant antiprostatic activity. In preliminary observations published in abstract forms, we reported the results of coumarin treatment in the Mat-LyLu metastatic, anaplastic, androgen-dependent prostate cancer rat model (10, 11). Coumarin-treated groups showed statistically significant decreases in the number and size of metastatic lesions in their lungs. During these studies we observed that coumarin produced a remarkable decrease in size and weight of the male rat testes and sex accessory organs (11). This antiprostatic activity of coumarin has not been previously described nor characterized. This report further characterizes the effect of coumarin in non-tumor-bearing male rats and rats bearing the Dunning R-3327H prostatic adenocarcinoma.

MATERIALS AND METHODS

Animals

Sprague-Dawley rats were obtained from Sprague-Dawley (Indianapolis, IN), and Copenhagen rats were obtained from the National Cancer Institute. All rats were maintained in the animal facility with a 12-h cycle of light and dark. Rat chow and water were given ad libitum.

Chemicals

Coumarin was purchased from Sigma Chemical Co. (St. Louis, MO). Coumarin was dissolved in a vehicle containing 10% ethanol, 10% propylene glycol, and 80% normal saline. The control animals received injections of vehicle only, 2.0 ml/animal/day. All injections were given i.p. between 10:00 and 11:00 a.m. Testosterone as the propionate (4-androstene-17β-ol-3-one propionate) was purchased from Steraloids, Inc. (Manchester, NH), dissolved in sesame oil (Fisher Chemical Co., Fairlawn, NJ), and given 1.0 ml/kg/s.c. in the flank. Serum testosterone concentrations were measured using an i251-testosterone radioimmunoassay kit from Radioassay Systems Laboratories, Inc. (Carson, CA) (12).

Tumors

The R-3327H tumor was provided by Dr. John Isaacs of the Brady Urological Research Institute, John Hopkins Hospital, Baltimore, MD. A single cell suspension containing 1.5 × 10⁶ of the R-3327H tumor cells was injected in the flank, each tumor was measured weekly with microcalipers, and tumor volumes were calculated by the formula (l × w × h × 0.524) as previously described (13).

Treatment Regimens

Non-Tumoring-bearing Animals. The initial experiments were designed to study the dose effect of coumarin on sexually mature, non-tumor-bearing male Sprague-Dawley rats. The animals were randomly assigned to four groups of six rats per group. All rats were of the same age and weighed between 350 g and 370 g. The first group received coumarin, 80 mg/kg/day, i.p.; the second group received 40 mg/kg/day; the third group received 20 mg/kg/day; the fourth group, 10 mg/kg/day; and the fifth group received i.p. injections of vehicle only, 20% ethanol and 80% saline, 2.0 ml/animal/day, i.p., and was considered as the control group. The animals were treated for 21 days. On Day 21 all animals were euthanized. None of the rats in any of the treatment groups expired during the experiment. In other protocols 10% of the ethanol was replaced with 10% of propylene glycol. Animal weight, ventral prostate, and testes weight were recorded. A blood sample was obtained via cardiac puncture for determination of serum testosterone levels. Tissue specimens were preserved in formalin for histopathological studies.

The second treatment protocol was performed on castrated rats which received androgen replacement therapy. All rats were castrated...
COUMARIN

Fig. 1. The chemical structure of coumarin.

via the scrotal route using pentobarbital, 60 mg/kg, i.p. as the anesthetic. The castrated Sprague-Dawley rats were randomly assigned to 8 groups. Ten days following castration, the first group (n = 7) (control group) received vehicle for both the testosterone propionate (VTP-sesame oil, 1.0 mg/kg/day, s.c. in the flank) and coumarin (VC-10% ethanol-10% propylene glycol-80% normal saline, 2.0 ml/animal/day, i.p.), and the treatment groups were as follows: (a) VC + VTP (control) (n = 7); (b) VC + 100 µg of TP (n = 7); (c) VC + 300 µg of TP (n = 7); (d) VC + 2000 µg of TP (n = 7); (e) 40 mg/kg of coumarin + VTP (n = 7); (f) 40 mg/kg of coumarin + 100 µg of TP (n = 7); (g) 40 mg/kg of coumarin + 100 µg of TP (n = 7); (h) 40 mg/kg of coumarin + 300 µg of TP (n = 7); (i) 40 mg/kg of coumarin + 2000 µg of TP (n = 7). The injections were given between 9:00 a.m. and 10:00 a.m. for 11 days. The 40-mg/kg dose of coumarin was chosen because of the results in the dose-response experiment, which demonstrates that 80 mg were more toxic in terms of body weight loss without substantial additional decreased prostatic size.

Tumor-bearing animals. This experiment was designed to study the effect of coumarin on the growth of the Dunning R-3327H prostatic carcinoma when compared to the effect of no treatment or castration. Sexually mature Copenhagen rats were implanted with 1.5 × 10⁶ androgen-responsive viable R-3327H prostatic cancer cells as previously described (13). Six mo later these tumor-bearing animals were randomly assigned to three treatment groups: control (vehicle only, VC); coumarin, 40 mg/kg/day; or castration (vehicle only, VC). Each group had six animals. The tumor volume on the day the treatment began ranged between 2.5 and 3.2 cm³. Castration was performed via the scrotal route. Injections of 20% alcohol vehicle only (control, castrate) or coumarin were started 20 days postcastration in the castrated group.

The injections were continued for 56 days. The animals were then euthanized. Blood was obtained by cardiac puncture, and the serum obtained was assayed for its concentration of testosterone by radioimmunooassay. The tumors and sex accessory tissues were surgically excised, freed of fat and connective tissue, and weighed to the nearest milligram, and aliquots were taken for histological and biochemical evaluation. DNA was extracted by differential base and acid hydrolysis (0.4 M final perchloric acid, 20 min, 70°C), and the DNA content was approximated as 32 µg of hydrolyzed DNA in 0.4 M perchloric acid = l.OOA at 260 nm based on similarly treated calf thymus DNA (13, 14).

Statistical analysis was performed by the two-tailed t test using the statistical package of the Memorial Sloan-Kettering Cancer Center's clinical computer system.

RESULTS

Table 1 summarizes the results of the first experiment. Coumarin treatment significantly reduced the size of the prostate by 30, 40, and 44% in the 20-, 40-, and 80-mg/kg treatment groups, respectively (P < 0.05). There was no statistical difference between the control and 10-mg/kg dose of coumarin in terms of prostate weight.

The average weight of the testes was significantly reduced from 3.29 g in the control untreated rats to 1.69, 1.71, and 1.47 g in rats treated with 80, 40, and 20 mg/kg of coumarin, respectively (P < 0.05). There was no statistical difference between the groups treated with 20, 40, or 80 mg/kg in terms of testicular weight. Treatment with coumarin at 10 mg/kg did not cause the testes' weight to differ significantly from control.

The mean of the serum testosterone levels of the intact male rats was slightly, but not significantly, elevated in the coumarin-treated groups versus the controls, i.e., 1.92 ng/ml versus 2.31 ng/ml in the group treated with 80 mg/kg of coumarin.

Pathological examination of histological sections of the coumarin-treated prostate revealed evidence of a decrease in the volume of the cytoplasm of epithelial cells lining the acini. A change in the morphology of these cells was noticed from columnar to cuboidal with minimal stromal changes. H & E, × 400. A, photomicrograph of a normal prostate from the control group. Note the columnar shape of the cells and morphology of the acini. H & E, × 400. B, photomicrograph of a treated prostate following coumarin treatment, 40 mg/kg, i.p., for 21 days. Note the effect on the epithelial cell lining in the acini and the cuboidal shape of the cells with minimal stromal changes. H & E, × 400.

In the second experiment, exogenous testosterone restored the size of the ventral prostate following castration to different degrees, depending on the amount of replacement androgen.
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Fig. 3. A, photomicrograph of normal testes from the control group. Note the presence of Leydig cells, and all stages of spermatogenesis, with mature sperm in the center. H & E, × 400. B, photomicrograph of testicular tissue from a rat treated with coumarin, 40 mg/kg, i.p., for 21 days. Note the remarkable decrease in all stages of spermatogenesis with preservation of Leydig cells. H & E, × 400.

Fig. 4. The effect of coumarin treatment on castrated rats receiving testosterone. The addition of coumarin significantly inhibited the growth of the androgen-induced castrated prostate (P < 0.001). Bars, SD. * P < 0.05.

The third experiment explored the effect of coumarin on the growth of the R-3327H tumor. As seen in Fig. 5, coumarin treatment produced a continuing slight decrease in tumor volume with the castrate group exhibiting a slight increasing volume, both of which are dramatically suppressed relative to the control. Table 2 summarizes the results of coumarin treatment on the hormone-sensitive R-3327H tumor relative to the effect of castration. The growth of the primary tumor was inhibited 90% relative to the control group and exceeded the effect of castration. The mean tumor weight in the untreated control rats was 26.78 g at the end of the experiment. The coumarin-treated group had a mean tumor weight of 2.77 g, and the castrated group was 8.70 g. The tumor weight in the coumarin-treated group was significantly less than that observed in the castrated group. In contrast, the castrate rat ventral prostate was more reduced in weight than the coumarin-treated group, averaging 100 mg in the castrated group versus 201 mg in the coumarin-treated group relative to 420 mg in the control group.

Testosterone serum levels were unchanged in the coumarin-treated group despite the significant reduction in weight of the tumor and ventral prostate.

We measured the amount of DNA present as a reflection of the cellularity of treated and untreated tumors (Table 3). There was no statistical difference between coumarin-treated, non-treated, or castrated animals in the DNA content in microgram per gram of tumor tissue even though the coumarin-treated group had the lowest, and the castrate group had the highest, content. As expected, therefore, the total DNA per tumor was significantly different (P < 0.001) among all of the groups.

In Fig. 6 it can be observed that there was no major histological difference between the treatment groups except that the coumarin group appeared to exhibit the highest abundance of acini filled with secretions, and the castrated group the least.

DISCUSSION

Coumarin treatment of sexually mature male rats demonstrated a dose-dependent decrease in the weight of the male rat significantly suppressed the androgen-induced increase in prostatic size relative to the untreated castrate by 28% for 100 µg of TP, 42% for 300 µg of TP, and by 36% for the 2000-µg dose of TP.

Average serum testosterone levels increased from 0.03 ng/ml to 0.45, 1.47, and 8.1 ng/ml with 0, 100, 300, and 2000 µg of testosterone replacement therapy, respectively. These levels of testosterone were not significantly affected by the addition of coumarin averaging 0.03, 0.611, 1.1, and 6.15 ng/ml with coumarin combined with 0, 100, 300, or 2000 µg of testosterone, respectively.

administered (Fig. 4). The average weight of the ventral prostate in the control castrated rats was 80 mg, while the average weight of the ventral prostate in the castrated group receiving 40 mg/kg of coumarin was significantly less at 40 mg. This weight of the castrate prostate was significantly increased between all groups on an average to 190 mg, 391 mg, and 520 mg with 100, 300, and 2000 µg of testosterone per rat per day, respectively. These increases were lessened to 160, 261, and 361 mg when 100, 300, and 2000 µg of testosterone were combined with coumarin, 40 mg/kg. The presence of coumarin

Fig. 5. The growth of the R-3327H tumor in normal, castrated and coumarin-treated rats. All means of each group are significantly different from each other at the conclusion of the experiment on Day 56 (P < 0.005). Bars, SD.
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Table 2 Effect of castration or coumarin treatment on the growth of the R-3327H prostate-derived tumor

Six mo following implantation of the R-3327H tumor, the tumor-bearing animals were randomly assigned to three groups, intact, castrate, or coumarin. Ten days following castration of the castrate group, animals were given injections of vehicle only (intact, castrate) or coumarin, 40 mg/kg, for 56 days. Following the 56th injection, the animals were euthanized, blood was drawn, and tumors were removed and weighed.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Animal wt (g)</th>
<th>Tumor wt (g)</th>
<th>Tumor wt (g/100 g body wt)</th>
<th>Ventral prostate (mg)</th>
<th>Testosterone (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intact</td>
<td>368 ± 8.4°</td>
<td>26.78 ± 3.42°</td>
<td>7.18 ± 1.67</td>
<td>420 ± 34°</td>
<td>0.710 ± 0.3</td>
</tr>
<tr>
<td>Castrated</td>
<td>357 ± 28.6</td>
<td>8.70 ± 1.81°</td>
<td>2.49 ± 0.64°</td>
<td>100 ± 7°</td>
<td>0.021 ± 0.02°</td>
</tr>
<tr>
<td>Coumarin</td>
<td>322 ± 15.43</td>
<td>2.77 ± 1.46°</td>
<td>0.86 ± 0.30°</td>
<td>201 ± 20°</td>
<td>0.635 ± 0.3</td>
</tr>
</tbody>
</table>

* Mean ± SE.
° Values significantly different from castrated (P < 0.05).
* Values significantly different from control (P < 0.05).

Sexual organs. The reduction in the size of the ventral prostate and testes at doses greater than 20 mg/kg was dramatic and statistically significant. The histological changes in the prostate and testes were consistent with the possibility that coumarin was acting like an estrogen or an antiandrogen. With coumarin treatment, testosterone levels were found to be unchanged despite the significant reduction in the size of the testes and prostate.

If coumarin were acting like an estrogen, then the decrease in weight of the prostate could be due to an estrogen-like inhibition of pituitary gonadotropins, which would result in decreased serum levels of luteinizing and follicle-stimulating hormone. This would reduce the testicular production of testosterone, decrease sperm production, and decrease the size of the testes as well (15–17). This is not the case here, as coumarin administered in the manner described did not reduce serum testosterone. These results suggest that coumarin is behaving more like an antiandrogen.

A standard biological assay for antiandrogenic activity is to induce restoration of the castrated rat prostate with androgen and to determine whether the test compound will block that androgen-induced restoration (16). Coumarin did diminish the androgen-induced restoration of the prostate in non-tumor-bearing rats. Coumarin had no androgenic activity of its own as it tended to further reduce the weight of the castrate prostate. This is consistent with antiandrogen-like activity. Unlike an antiandrogen, however, the percentage of decrease in prostatic wet weight of the testosterone-induced increase of the castrate prostate remained about 30 to 40% regardless of the amount of androgen administered. A pure antiandrogen would be expected to be most effective against a lower concentration and less effective against a higher concentration of androgen. In this respect coumarin did not behave like a pure antiandrogen.

In previous reports, coumarin was shown to alter the activity of drug-metabolizing enzymes. It is possible that this antiandrogenic activity is due to an increase in the rate of degradation of the administered testosterone (5). We observed that coumarin did not affect the concentration of testosterone found in the serum, whether the testosterone was produced by the testes or whether exogenous testosterone was administered. More studies are needed to further define the mechanism of coumarin action.

Because coumarin was active in decreasing the size of the normal rat prostate, we proceeded to examine the activity of coumarin against the R-3327H prostate cancer tumor model to determine if it would affect cancerous as well as normal prostate tissue. The R-3327H subline is a well-differentiated adenocarcinoma composed of multiple tumor acini. The tumor is approximately 95% epithelium, and the remaining 5% is a fine

Fig. 6. Photomicrographs of the R-3327H tumor from the three treatment groups: control (A); castrated (B); and coumarin (C). Note that the coumarin group appeared to exhibit the highest abundance of acini filled with secretions, and the castrated group the least. H & E, × 400.
fibrous stroma which surrounds each tumor acinus (18). The R-3327H tumor has been reported to be composed of approximately 70% androgen-sensitive and 30% insensitive cells to possess 5α-reductase activity and both androgen and estrogen receptors (13, 18, 19). In these experiments, coumarin was active against the R-3327H tumor. In our study the coumarin-treated group had a mean tumor volume less than that observed for the castrated group. It is unusual to find any hormone therapy that will achieve greater antiprostatic tumor effects than castration (13). Estrogens administered to animals with small tumor burdens may achieve a greater reduction in tumor volume that castration, but this is always accompanied by substantial loss in body weight, which was not observed with coumarin in this study (12).

The mechanism(s) of coumarin’s antiprostatic activity remains to be determined. It is not acting like an estrogen and may not be acting like an antiandrogen. It remains to be determined whether it inhibits 5α-reductase and prevents the conversion of testosterone to the more active prostatic androgens. Coumarin has been reported to activate macrophages. This could result in an endogenous increase in a variety of cytokines, such as interleukin 1, tumor necrosis factor, interferon, and other biological response modifiers (9, 20). If coumarin does stimulate macrophage production of tumor necrosis factor, the recent observation by Sherwood et al. is of interest (21). They reported that tumor necrosis factor α was cytotoxic to prostatic carcinoma cells and inhibited the growth of normal prostatic epithelial cells.

At this time, we are conducting more studies to examine coumarin’s mechanism of action on the normal and cancerous prostate. Coumarin is extensively metabolized, and that metabolism may be affected by other agents, such as alcohol (21, 22). Bearing in mind that it has been reported that the human and the rat differ significantly in the manner in which they metabolize coumarin (22, 23), and that the dose-response studies in the rat and the low bioavailability in humans suggest a higher dose requirement of coumarin than that used so far in human trials (8, 24–26), we believe that treatment with coumarin or perhaps a coumarin derivative is worthy of further investigations as therapy in the treatment of abnormal prostatic growths, such as prostatic cancer.

ACKNOWLEDGMENTS

The authors express their thanks to Rose Vecchiolla and Cynthia Gonzalez for typing this manuscript. The authors wish to acknowledge the excellent skillful technical assistance of Robert Huryk.

REFERENCES

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