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1-Dehydro-melengestrol acetate inhibits the growth and protein kinase C activity of androgen-independent Dunning rat prostatic tumors*

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Summary. Androgen-independent Dunning R3327-AT3 rat prostate tumors are considered an appropriate model of advanced prostate cancer in humans. We recently reported that the progestational steroid melengestrol acetate (MGA) inhibited growth of these tumors on oral administration but also induced a marked involution of adrenals and androgen target organs (prostate, seminal vesicles, and testes). We report herein that the 1-dehydro derivative of melengestrol acetate (dMGA) fed to rats for 21 days also inhibited the growth of Dunning AT3 tumors by ~55% without causing a significant regression of adrenals or androgen-dependent tissues. Thus, tumor-growth inhibition was induced by dMGA in the absence of glucocorticoid activity. Cytosolic AT3 tumor fractions obtained by diethylaminoethyl (DEAE)-Sephacel batch chromatography were assayed for lipid- and Ca^{2+}-dependent (PKC) and -independent protein kinase activities. Prostatic cytosols had equivalent activity levels of both types of kinases (~2 nmol γ-[^{32}P]-adenosine 5′-triphosphate (ATP) incorporated mg protein^{-1} min^{-1}. The PKC activity recovered from the cytosol of untreated AT3 tumors was ~4 times higher. Oral administration of dMGA reduced this activity by >95%. The relationship between protein-kinase activity levels and dMGA-induced growth inhibition of androgen-independent tumors in this animal model is discussed.

Introduction

Adenocarcinoma of the prostate remains one of the leading causes of death in men. Each year, approximately 100,000 new cases of prostate cancer are diagnosed – the true incidence of the disease is thought to be even greater, as occult localized tumors are often found during autopsies of patients who have died of other causes. The significance of testosterone as a trophic hormone for this neoplasm was recognized as early as in 1941, with the introduction of surgical castration [13] to reduce serum testosterone levels. Administration of estrogens [17] to inhibit testosterone production by suppressing follicle-stimulating hormone, thereby producing a "chemical castration," was a subsequent modification of this treatment [17]. In most instances, early detection of prostatic neoplasms followed by androgen deprivation therapy has provided significant increases in survival and quality of life. In other cases, however, suppression of serum testosterone levels has had little effect, if any, on the course of the disease [27]. Most notably, recurrence has taken the form of androgen-independent neoplasms that have rendered conventional hormonal therapy largely ineffective [15].

The Dunning R3327 transplantable, rat prostatic tumor line was established from a spontaneous papillary adenocarcinoma that originated from the ventral prostate of a mature Copenhagen rat [10]. Isaacs et al. [14] developed a variety of Dunning sublines through androgen deprivation of rats bearing the original Dunning R3327-PAP neoplasm (previously known as R3327-H). These early investigations as well as recent ones employing molecular probes have led to the concept that progression from androgen dependence to independence in the Dunning tumor sublines is correlated with a loss of androgen receptors [26] in a manner analogous to the human situation. Recently, Padilla and co-workers [25] reported that the orally active progesterone derivatives megestrol acetate (MA) and melengestrol acetate (MGA) inhibited the growth of both androgen-dependent (R3327-PAP) and androgen-autonomous (R3327-AT3) Dunning tumors but had variable inhibitory effects on protein kinase activities derived from these tumors [1]. MGA has glucocorticoid activity, as evidenced by its effect on adrenal weight. In contrast, its 1-dehydro analogue (dMGA, Fig. 1) is virtually devoid of such glucocorticoid activity. It was therefore of interest to determine its effects on Dunning AT3 tumor growth so as to evaluate the contribution of glucocorticoid activity to

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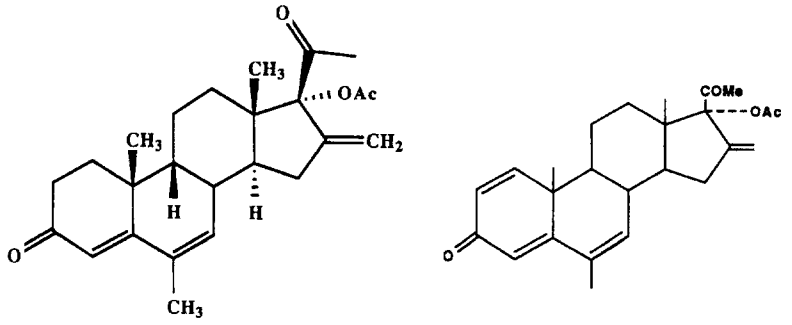

MELENGESTROL ACETATE 1-DEHYDRO-MELENGESTROL ACETATE

Fig. 1. Structural formulae of melengestrol acetate (MGA) and 1-dehydro-melengestrol acetate (dMGA)

tumor inhibition. We report that oral dMGA has no effect on the adrenals but does inhibit the growth and activities of calcium/lipid-independent and -dependent protein kinases recovered from androgen-independent Dunning AT3 tumors.

Calcium/lipid-dependent protein kinases (PKC) are members of a family of enzymes that play a role in the hormonal signal-transduction cascade [21]. They have also been implicated in a variety of cellular transformation processes [19] and as participants in malignancies such as breast cancer [3]. Recent studies indicate that PKC levels in human breast-adenocarcinoma samples were significantly higher than those found in normal breast tissue [24], and the relationship of PKC in colon cancer is also being examined [22].

Materials and methods

Tumor growth and drug treatment. Male rats (150–200 g, Copenhagen x Fisher F1) were implanted s. c. in each flank with 0.2 ml culture medium containing ~10^6 Dunning R3327-AT3 tumor cells as described elsewhere [25]. For the next 21 days, rats were given dMGA mixed in their laboratory food (Purina Lab Chow) to yield a daily dose of ~20 mg kg^{-1} [25]. Food consumption was monitored daily. At animal sacrifice, relevant androgen target tissues and tumors were removed, weighed, snap-frozen, and preserved at –80° C for subsequent analyses.

Tissue preparation and protein-kinase activity assays. Cryopreserved samples were suspended in 6 vol. ice-cold homogenization buffer [20 mM TRIS-HCl (pH 7.5), 2 mM ethylenediaminetetraacetic acid (EDTA), 10 mM ethylene glycol tetraacetic acid (EGTA), 250 mM sucrose, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol, and 0.02% (w/v) leupeptin] and disrupted over ice with a Polytron homogenizer (30-s pulses × 4, at 15-s intervals). Homogenates were centrifuged at 10,000 *g* for 20 min and then at 100,000 *g* for 60 min. The 100 k supernatants (cytosols) were adjusted to pH 7.6 with 2 M TRIS and subjected to batch diethylaminoethyl (DEAE) chromatography (Personal communication, J. Blumenthal, Biochemistry Department, Duke University Medical Center). Sephacel gel was activated by sequential washing with 4 vol. distilled water, 6 vol. 100 mM TRIS (pH 7.5), and 6 vol. buffer A [20 mM TRIS-HCl (pH 7.5), 0.5 mM EDTA, 0.5 mM EGTA; and 10 mM β-mercaptoethanol]. Cytosol samples were mixed at 4° C into a slurry with 0.5 vol. ice-cold "activated" DEAE-Sephacel gel for 30 min. The Sephacel/cytosol slurries were then washed with 8 vol. 30 mM NaCl in buffer A, resuspended in 0.75 vol. 30 mM NaCl in buffer A, transferred to a 3- x 10-cm column, and pre-eluted with 2 vol. 30 mM NaCl in buffer A. The eluant was discarded. The column was then eluted at 4° C with 4 vol. 100 mM NaCl in buffer A, the optical density of the effluent was monitored at 280 nm using a single-path flow cell (Pharmacia, UV-1), and 10-ml fractions were collected. The absorbance of the chromatographic fractions at 280 nm was verified on a spectrophotometer (Beckman, Acta II). In addition, the protein content of the fractions analyzed for PKC activity was determined by the method of Bradford [7].

Fractions were analyzed for calcium/lipid-independent and PKC activity in duplicate using the standard assay of Bell and co-workers [2, 12]. The reaction mixture (0.25 ml) contained 5 µmol TRIS-HCl (pH 7.4), 250 nmol $CaCl_2$ or EGTA, 50 µg histone (type III-S), 2.5 µmol $MgCl_2$, 2.5 nmol [γ-^{32}P]-adenosine 5′-triphosphate (ATP; 3–5 × 10^5 cpm/nmol). Lipids [1 µg phosphatidyl serine and 75 ng diacylglycerol (1,2-Diolene)] were dispersed in water by sonication and added to the reaction mixture (25 µl). The reaction was carried out at room temperature (20°–22° C) for 15 min and was terminated by the addition of 1 ml ice-cold 25% trichloroacetic acid and 1 ml ice-cold bovine serum albumin (500 µg/ml). Precipitates were collected on 2.4-cm GF/C filters (Whatman Ltd.,) and the radioactivity was counted with a scintillation spectrometer (Beckman, LS 5000TD).

Chemicals. TRIS-HCl, PMSF, EDTA, EGTA, ATP, histone (type III-S), Coomassie brilliant blue G, and leupeptin were purchased from Sigma Chemical Co. (St. Louis, Mo.). [γ-^{32}P]-ATP, triethylammonium salt, was obtained from Amersham Corp. (Arlington Heights, Ill.). DEAE-Sephacel was purchased from Pharmacia LKB (Piscataway, N.J.). Phosphatidyl serine and L-α-dioleoyl lecithin were purchased from Avanti Polar-Lipids Inc. (Pelham, Ala.). The lecithin was converted into 1,2-diacylglycerol by enzymatic digestion with phospholipase C. β-Mercaptoethanol was obtained from Malinckrodt Inc. (Paris, Ky.). Dithiothreitol was obtained from P-L Biochemicals (Milwaukee, Wis.).

Results

Table 1 summarizes the effects of dMGA given orally for 21 days to rats implanted with Dunning R3327-AT3 tumors. dMGA inhibited AT3 tumor growth by ~56% as compared with controls (column 2). Adrenal weights were not affected, showing a reduction of <3% (column 3). Weights of ventral prostates and seminal vesicles, on the other hand, were reduced by about 18% and 13%, respectively (columns 4, 5). These changes were not statistically significant according to Student's *t*-test ($P \geq 0.05$). The testes were not affected by dMGA, indicating a lack of androgenic activity. During the course of treatment, the body weight of control rats increased by ~36%, whereas the dMGA-treated rats showed a 2.7% loss of body weight. Thus, dMGA appears to exert a substantial catabolic effect. In a separate experiment, dMGA given at this dose did not appear to be toxic; in fact, it increased the survival of AT3

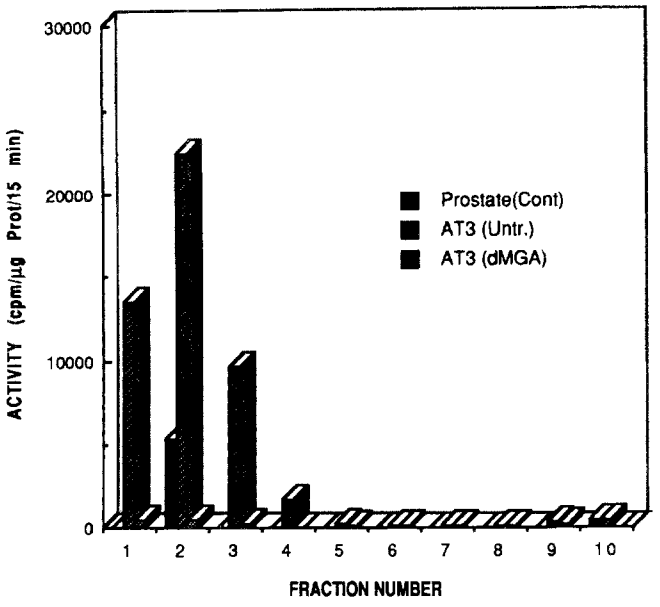

Fig. 2. PKC activity profiles of DEAE-Sephacel chromatographic fractions obtained by batch elution with 0.1 M NaCl from cytosol of untreated rat prostates (*Cont*) and of untreated [*AT3 (Untr.)*] and dMGA-treated [*AT3 (dMGA)*] Dunning AT3 tumors. PKC activity is expressed as cpm γ-[^{32}P]-ATP incorporated into histone (Ca^{2+}/lipid-dependent – cpm Ca^{2+}/lipid-independent) µg protein^{-1} 15 min^{-1}

tumor-bearing rats by 7–10 days in comparison with that of controls.

Figure 2 shows the PKC activity profiles of the first ten cytosolic fractions from control prostates and from dMGA-treated and -untreated AT3 tumors following DEAE-Sephacel batch chromatography. Cytosol samples were prepared from approximately equal quantities of untreated rat prostates (5.15 g), control AT3 tumors (5.42 g), and dMGA-treated AT3 tumors (5.14 g) as described in Materials and methods. The homogenization was carried out in a buffer that included EDTA and EGTA at concentrations designed to prevent the activation of Ca^{2+}-dependent proteases and to allow release of PKC into the cytosol [2, 12]. This was found to be the case in experiments comparing the distribution of PKC activities in Dunning R3327-AT3 and R3327-PAP tumors. A 3-fold higher PKC activity was recovered in AT3 100,000-*g* supernatant than in the pellet (15.07 vs 5.03 pmol ATP incorporated mg protein^{-1} min −1; McDonald et al., unpublished data). Tumor samples were obtained from rats used in the experiment summarized in Table 1. Untreated prostates were collected and cryopreserved from control adult rats (Copenhagen x Fisher) used in other studies. Insufficient quantities of dMGA-treated prostates prevented similar analyses of PKC activity. As shown in Fig. 2, PKC activity (defined as Ca^{2+}/lipid-dependent PKC activity – Ca^{2+}/lipid-independent protein kinase activity) was primarily recovered in the first four DEAE fractions from untreated AT3 tumors. Levels of PKC activity were markedly reduced in the fractions derived from dMGA-treated tumors. Only fraction 2 from untreated prostates showed substantial PKC activity.

Table 2 details the relative distribution of Ca^{2+}/lipid-dependent and -independent protein kinase activities recovered in the first four chromatographic fractions. As they represent fractions containing partially purified enzymes, activity levels were normalized on the basis of protein content and were combined only for purposes of comparison. First, the Ca^{2+}/lipid-dependent PKC recovered from untreated prostatic cytosolic fractions was much lower than that recovered from untreated AT3 tumors (column 3). Second, dMGA reduced this activity in AT3 tumors by >95%. Third, prostatic and untreated AT3 cytosol samples had similar levels of Ca^{2+}/lipid-independent protein kinase activity (column 4), which were substantially reduced by dMGA. These preliminary data indicate that dMGA given in vivo inhibited both types of protein kinase activity in AT3 tumors to levels lower than those in prostatic cytosol, having a greater effect on the PKC activity.

Discussion

In an earlier study, Padilla and co-workers [25] reported that two progesterone analogues, megestrol acetate (MA) and melengestrol acetate (MGA), inhibited the growth of Dunning R3327-AT3 tumors but also induced regression in the adrenals. The results of the present study show that dMGA inhibited AT3 tumor growth without affecting the adrenals (Table 1). The relevant point of these two studies is that unlike MA and MGA, dMGA has an inhibitory effect on AT3 tumor growth in spite of a lack of glucocor-

Table 1. Effect of dMGA on Dunning AT3 tumors, adrenals, androgen target organs, and body weight in a 21-day study of oral treatment

Group	Mean values ± SE					Body wt. change (%)[a]
	Tumors (g)	Adrenals (mg)	Ventral prostate (mg)	Seminal vesicles (mg)	Testes (g)	
			(X ± SE)			
Control ($n = 4$)	34.8 ± 2.1	10.9 ± 0.9	121 ± 1	178 ± 7	1.05 ± 0.02	+36
dMGA ($n = 5$)	15.5 ± 3.0* (55.5)	10.6 ± 0.9 (2.8)	99 ± 0.9 (18.2)	155 ± 3 (12.9)	1.05 ± 0.01 (0)	–2.7**

Numbers shown in parentheses for dMGA treatment indicate decreases expressed as a percentage of control values

[a] Percentage of initial body wt.

* $P \leq 0.0072$, ** $P \leq 0.0029$ according to Student's *t*-test (statistically significantly different from control values)

Table 2. Ca^{2+}/lipid-dependent and -independent protein kinase activity of DEAE fractions from rat prostate and from untreated and dMGA-treated Dunning AT3 tumor cytosols

Tissue	Fraction number	Ca^{2+}/lipid-dependent PK activity[a]	Ca^{2+}/lipid-independent PK activity[a]
Prostate	1	1.32	2.23
	2	0.87	0.12
	3	0.02	0.01
	4	0.02	0.02
		2.23	2.38
Untreated tumors	1	2.31	1.09
	2	3.92	0.73
	3	1.37	–
	4	0.24	–[b]
		7.84	1.82
dMGA treated tumors	1	0.10	0.04
	2	0.11	0.05
	3	0.06	0.03
	4	0.02	0.02
		0.290	0.140

[a] Protein-kinase (PK) activity units = nmol γ-[^{32}P]-ATP incorporated into histone mg protein^{-1} min^{-1}

[b] Activity recovered: ~0.002 units

ticoid activity (i.e., no induction of adrenal involution). Although the mechanism of tumor-growth inhibition by these steroids is unknown, recent evidence indicates that progesterone antagonists may induce receptor-mediated increases in terminal cellular differentiation, resulting in cell death [18]. The level of tumor-growth inhibition found in the present study suggests that approximately 55% of the AT3 tumor cells were sensitive to these steroids, raising the possibility of the existence of specific dMGA receptors in the sensitive clones or of the presence of an overlap in the affinity of dMGA for progesterone receptors. The partial growth inhibition induced by these steroids also suggests that androgen-independent tumors are composed of heterogeneous cell populations with a spectrum of hormonal sensitivities. There is no experimental evidence to exclude any of these possibilities. It would be reasonable to suggest that dMGA may be exerting a growth-inhibitory effect on androgen-independent tumors by disrupting signal-transduction pathways dependent on a given level of PKC activity [21].

The relevance of PKC enzymes in tumor biology is a subject of current interest [19, 20, 22]. Evidence from several laboratories has established a role for this family of enzymes in cellular signaling and regulation of molecular processes leading to mitosis and cell proliferation [11, 16, 19, 20, 25]. The observations that PKC is stimulated by known tumor promoters (e.g., phorbol esters) and that oncogenes are key PKC substrates lend support to the hypothesis that PKC enzymes play a critical role in the progression of neoplastic diseases [6, 9]. Recent work suggest a possible involvement of PKC in human colon adenocarcinoma [22]. PKC was shown to be stimulated by various bile acids. A high-fat diet, which leads to increased levels of bile secretion, may induce a diseased state through this mechanism. Increased levels of PKC activity were also found in samples of human breast adenocarcinoma as compared with normal breast tissue [24]. This observation was made in both estrogen- and progesterone-receptor-positive and -negative cell lines [3], and the possibility of using PKC activity levels as a tumor marker was suggested [22, 24].

In the present study, we found higher PKC activity levels in Dunning AT3 tumor cytosols relative to PKC levels in the prostate. These findings are similar to those observed in breast cancer [24], suggesting the existence of common regulatory mechanism(s) of tumor growth and proliferation in which PKC enzymes most likely play a role. Our studies also suggest that a possible correlation exists between dMGA-induced tumor-growth inhibition and decreases in PKC activity. Since calcium/lipid-independent protein kinase activities were also present in the prostate and AT3 tumors and were similarly sensitive to dMGA, a unique link cannot be assigned solely to the dMGA-induced attenuation of PKC activity and tumor-growth inhibition until the PKC substrates in these tumors have been identified and their phosphorylation status has been delineated with respect to their regulatory role on tumor-cell proliferation. Furthermore, protein kinase activity studies should be carried out using primary or established human prostatic tumor lines with differing hormonal (androgen) dependence in conjunction with treatment with a combination of other hormones [28] such as somatostatin [5], tamoxifen [23], antiestrogens [4], and luteinizing hormone-releasing hormone (LH-RH). A correlation of PKC activity changes with acid phosphatase and prostate-specific antigen levels in a clinical setting could ultimately lead to the development of sensitive tumor markers that might be of more prognostic value. With regard to chemotherapy, dMGA may prove to be a valuable adjunct by virtue of its oral activity and its lack of glucocorticoid activity. Megestrol acetate, a related progestational steroid, is currently in use in the clinic [8].

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