Prevention of mouse skin tumor promotion by dietary energy restriction requires an intact adrenal gland and glucocorticoid supplementation restores inhibition

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Our laboratory has demonstrated in the previous studies that dietary energy restriction (DER) inhibited the promotion of skin tumorigenesis and others have found that adrenalectomy may reverse that inhibition. The purpose of the research reported here was to determine if circulating corticosterone (CCS) may be the adrenal hormone responsible for DER prevention of skin carcinogenesis. Female SENCAR mice were initiated with 7,12-dimethylbenzanthracene (DMBA) and promoted with 12-O-tetradecanoylphorbol-13-acetate (TPA) in either sham-operated or adrenalectomized (ADX) mice fed ad libitum (AL) or energy restricted diets. DER was 60% of the AL calorie intake with the removal of energy from fat and carbohydrates. CCS, the main glucocorticoid hormone secreted by the murine adrenal gland, was added to the drinking water of AL/ADX and DER/ADX groups to determine the role of CCS in the DER inhibition of tumor development. In sham-operated groups, DER compared with AL-fed mice experienced significantly decreased papilloma incidence and multiplicity (P < 0.0001). ADX did not alter papilloma incidence or multiplicity in AL-fed mice but ADX partially reversed the inhibition of papilloma multiplicity and incidence in DER mice. CCS supplementation to both DER/ADX and AL/ADX mice resulted in reduced papilloma incidence and multiplicity. In DER/ADX mice, CCS dramatically reduced papilloma rates while in AL/ADX mice CCS reduced the papilloma rates to those seen in the DER sham group. DER significantly reduced carcinoma multiplicity mean counts per effective animal (P < 0.0001) compared with AL-fed groups in sham and ADX/CCS groups. DER/ADX mice lost the carcinoma multiplicity protection seen in sham/DER mice. CCS treatment of ADX mice significantly decreased total carcinoma (in situ and invasive) incidence rates per effective animal (P < 0.0003). ADX followed by CCS treatment in the DER mice resulted in the lowest carcinoma incidence and multiplicity. Thus, DER-inhibition of skin tumorigenesis was mediated at least in part through CCS. However, CCS was more effective in preventing papillomas and carcinomas in DER/ADX mice than in AL/ADX mice, suggesting that other factors may also be involved in the DER prevention of tumor formation.

Introduction

With the growing evidence that obesity is a risk factor for human cancer (1) and the increasing rate of obesity throughout the world (2), determining the mechanisms whereby dietary intake modulates cancer has taken on a new significance. There has long been evidence that restricting dietary intake could significantly reduce cancer rates. In particular, studies demonstrated that when mice were fed a calorie restricted (60% of control calories) diet instead of an ad libitum (AL) diet the mice exhibited ~40% decreased tumor incidence in skin and mammary tissues (3,4). In addition, a more moderate restriction (80% of control intake) was also effective in skin cancer prevention (5). Since these early findings, studies have demonstrated dietary or calorie restriction are effective in the prevention of liver, lung, colon, breast and skin cancers (6–9).

Previous research in our laboratory that is relevant to assessing the mechanism of dietary energy restriction (DER) prevention of skin cancer, compared dietary restriction (reduction of all dietary components) and DER (reduction of calories from fat and/or carbohydrates while maintaining other nutrient intakes). The study showed that both dietary restriction and DER reduced papilloma and carcinoma incidence, and multiplicity and also delayed papilloma appearance but that DER was the most effective strategy (7). Furthermore, our laboratory has shown that the DER inhibition of tumorigenesis occurs during the promotion stage of 7,12-dimethylbenzanthracene (DMBA) initiated and 12-O-tetradecanoylphorbol-13-acetate (TPA) promoted mouse skin tumorigenesis (10). Research in other laboratories showed that the adrenal gland was required for diet restriction prevention of skin and lung tumorigenesis (11,12). The studies of skin cancer determined that feeding less of the control diet reduced DMBA-initiated and TPA-promoted mouse skin carcinogenesis in intact mice but not in adrenalectomized (ADX) mice. These studies fed less of all dietary components (diet restriction), they did not perform DER as was used in the present investigation. Furthermore, they did not assess the key hormone from the adrenal gland that was responsible for the dependence.

The adrenal gland cortex produces three major classes of lipid soluble hormones derived from cholesterol: mineralocorticoids (controls levels of sodium and potassium), glucocorticoids (controls general cell function and especially maintains glucose homeostasis during food deprivation) and androgens (similar hormonal function as the male sex hormones) (12,13). The adrenal medulla produces the catecholamines: epinephrine and norepinephrine (13). Studies reported by Pashko and Schwartz demonstrated that adrenalectomy reverses the

Abbreviations: AP-1, activator protein-1; AL, ad libitum; ADX, adrenalectomized; CCS, circulating corticosterone; DER, dietary energy restriction; DHEA, dehydroepiandrosterone; DMBA, 7,12-dimethylbenzanthracene; ERK, extracellular signal regulated kinase; IGF-1, insulin growth factor-1; TPA, 12-O-tetradecanoylphorbol-13-acetate.
dietary-restriction-inhibition of TPA promotion of skin papillomas (11,12). In these studies dietary restriction increased the circulating corticosterone (CCS) but there was no difference in the levels of dehydroepiandrosterone (DHEA) or aldosterone in cases where these hormones were measured (11,12,14). In our earlier studies of DER, with reduction of fat and carbohydrate calories, we observed elevated levels of corticosterone in DER mice but found no evidence of activated glucocorticoid receptors in the skin of DER mice (15).

Several lines of evidence suggest that glucocorticoid hormone may be involved in the prevention of skin carcinogenesis in DER animals. First, it is known that energy restriction elevated circulating glucocorticoid hormones as noted above (15). Second, glucocorticoid hormones were demonstrated in a number of laboratories to be potent inhibitors of mouse skin tumorigenesis (16,17). Finally, the importance of the glucocorticoid receptor in interfering with a key transcription factor, activator protein 1 (API), in mouse skin carcinogenesis suggests potential mechanisms for DER elevated corticosterone in preventing mouse skin tumorigenesis (18,19). Studies focused on DER prevention of mammary carcinogenesis suggested that changes in CCS metabolism contributed to, but did not explain, cancer prevention in this model (20).

Our objectives in this research were to determine if the DER (restriction of energy from fat and carbohydrate) inhibition of skin tumorigenesis in intact mice, would be lost in ADX/DER mice, whether supplementation of the ADX mice with corticosterone would restore the inhibition of skin tumorigenesis and whether the corticosterone would inhibit skin carcinogenesis when administered to ADX mice fed the control diet.

Materials and methods

Animals and diets

The Iowa State University Committee on Animal Care approved the procedures used for the two-stage tumorigenesis model in mice. Six-week-old SENCAR female animals were obtained from the National Institute of Health (NIH) in Frederick, MD. Animals were allowed a 1-week acclimatization period on an AL-fed, control diet (AIN-76/93) (21) as seen in Table I (control diet). The mice were housed individually in humidity and temperature controlled rooms with a 12 h light/dark cycle. The mice were allowed a 2-week recovery period before treatment began. A dorsal skin sample was collected from the back of each animal and whether the corticosterone would inhibit skin tumorigenesis when administered to ADX mice fed the control diet.

<table>
<thead>
<tr>
<th>Diet components</th>
<th>Control (AL)</th>
<th>40% DER</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>As formulated</td>
<td>As given</td>
</tr>
<tr>
<td>Corn oil</td>
<td>5.0</td>
<td>3.8</td>
</tr>
<tr>
<td>Casein</td>
<td>20.0</td>
<td>31.1</td>
</tr>
<tr>
<td>nt.-Methionine</td>
<td>0.3</td>
<td>0.5</td>
</tr>
<tr>
<td>Dextrose</td>
<td>15.0</td>
<td>11.5</td>
</tr>
<tr>
<td>Dextrin</td>
<td>49.9</td>
<td>38.0</td>
</tr>
<tr>
<td>Fiber</td>
<td>5.0</td>
<td>7.8</td>
</tr>
<tr>
<td>AIN-93 mineral mix</td>
<td>3.5</td>
<td>5.4</td>
</tr>
<tr>
<td>AIN-93 vitamin mix</td>
<td>1.0</td>
<td>1.5</td>
</tr>
<tr>
<td>Choline bitartrate</td>
<td>0.3</td>
<td>0.4</td>
</tr>
<tr>
<td>Total amount of food</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>
g fed. g control consumed | 1.0 | 0.64 |

Pelleted diets were custom made by Harlan Teklad, Madison, WI. The catalogue numbers were TD99433 for the control (AL) diet and TD99467 for the DER diet. The amount of DER diet that was offered was calculated weekly as 64% of the weight consumed by the control mice which provided the DER mice a 40% reduction of calories from fat and carbohydrate.

Statistical analysis

The weekly food consumption levels of all the AL mice were analyzed by ANOVA using the MIXED procedure in SAS with multiple comparisons by least squares means for the first 3 weeks prior to treatment with the initiator of carcinogenesis.
and 5-week blocks during the rest of the experiment. All the animals on the AL diet were included in the data set until they were terminated from the experiment owing to ill health or the completion of the experimental period. Log-rank and Wilcoxon tests were used to compare Kaplan-Meier estimates of survivor curves for the six groups of DMBA/TPA treated mice (24). In this analysis, survival times for animals that completed the entire 31 weeks of the experiment were treated as censored at 31 weeks.

Body weights were analyzed with repeated ANOVA measures using the MIXED procedure in SAS with multiple comparisons by least squares means at each time point. The variable of surgery had three possibilities: sham/ADX or sham/ACETONE procedure in SAS with multiple comparisons by least squares means. Effective mice for the carcinoma analysis were defined as the mice sampled with both the Kaplan-Meier procedure and the MIXED procedure that showed no significant difference in body weight between the AL-fed mice versus the DER-fed mice. Data for mice that survived <23 weeks were excluded from the carcinoma analysis. Papilloma multiplicity at specific time points with acetone and DMBA/TPA were also analyzed with the GENMOD procedure, using all the mice that survived to the specified time point, but greater variability necessitated the use of the negative binomial distribution (variance > mean). Fisher’s exact test was used as an overall test for treatment effects on final carcinoma incidence or papilloma incidence at each time point. If the overall test showed significant differences at the 0.05 level, Fisher’s exact test was used to compare each pair of treatments. Plasma corticosterone and IGF were analyzed in the surviving mice at week 31 by ANOVA, using the GLM procedure in SAS to compare each pair of treatments. Plasma corticosterone and IGF were analyzed in the surviving mice at week 31 by ANOVA, using the GLM procedure in SAS to compare each pair of treatments.

Table II. Food consumption of ad libitum (AL) fed mice

<table>
<thead>
<tr>
<th>Weeks</th>
<th>DMBA/TPA</th>
<th>Acetone</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N AL/sham</td>
<td>N AL/ADX/saline</td>
</tr>
<tr>
<td>−3 to −1</td>
<td>29</td>
<td>3.8 ± 0.1a</td>
</tr>
<tr>
<td>6 to 10</td>
<td>29</td>
<td>4.3 ± 0.1b</td>
</tr>
<tr>
<td>16 to 20</td>
<td>29</td>
<td>4.9 ± 0.1a</td>
</tr>
<tr>
<td>26 to 30</td>
<td>17</td>
<td>4.7 ± 0.2a</td>
</tr>
</tbody>
</table>

Values are shown as mean g/day ± SEM. Food consumption was statistically analyzed from −3 to 30 weeks of the experiment using 5-week averages. Data for weeks 0–5, 11–15 and 21–25 are similar but not shown. The DMBA/TPA effect on food consumption was significant, P < 0.0069, 0.0001 and 0.0236 for weeks 6–10, 16–20 and 26–30, respectively. Individual means across rows were analyzed by ANOVA-Mixed and then compared with multiple comparisons by least squares means. Row means with different superscript letters are significantly different at the P < 0.05 level and values with more than one letter are not significantly different from means sharing either of the letters. Analysis by Anova-Mixed revealed that there was a significant interaction between DMBA/TPA and corticosterone treatment at weeks 26–30 for DMBA/TPA and acetone treated AL/ADX/CCS mice.

Fig. 1. Experimental design.

Results

Food consumption
Consumption of diet by AL-fed mice is shown in Table II. Within the DMBA/TPA treated mice the AL/sham and AL/ADX/saline groups consumed significantly more control diet from 16 to 30 weeks than the AL/ADX/CCS group. In comparison with acetone treated mice, DMBA/TPA treated AL/sham and AL/ADX/saline groups consumed significantly more control diet from 6 to 30 weeks. In contrast, within the AL/ADX/CCS group, the acetone treated mice consumed more control diet after week 26 than the DMBA/TPA treated mice.

Body weight
The mice on DER showed a significant loss in body weight during the first 4 weeks following the switch to this diet but maintained the reduced body weight for the rest of the experimental period (Figure 2). The AL-fed mice showed increase in body weight as the experiment progressed (Figure 2). At week 31 the DER-fed mice weighed significantly less than the AL-fed mice (P < 0.0001) as shown in Figure 2. Animals treated with acetone exhibited the same significant difference in body weights between the AL-fed mice versus the DER-fed mice (data not shown).

The changes in the body weight at the beginning of the experiment and weeks 4, 8 or 31 are provided in Table III. It is to be noted that the maximum body weight gains were in the AL/ADX/CCS treated groups in both the carcinogen (DMBA/TPA) and vehicle (acetone) administrated groups. In contrast, among the DER treated groups, body weight changes did not differ between the sham, ADX/saline or ADX/CCS treated groups.

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Mice in the AL/sham group exhibited a significant increase in papilloma multiplicity compared with the other DER and AL/ADX/saline treated mice. The mice receiving the DER/ADX/CCS protocol exhibited the most pronounced inhibition of papilloma incidence consistently throughout the study (Figure 3A). At week 31 DER significantly reduced papilloma incidence and CCS reduced papilloma incidence in both AL/ADX and DER/ADX groups (P < 0.0001).

Papilloma multiplicity: mean count per animal

Papilloma multiplicity was defined as the number of papillomas per animal. Mice in the AL/sham and AL/ADX/saline groups exhibited a significantly higher papilloma incidence at 8 weeks (P < 0.0001) compared with other mice (Figure 3A). By week 10 the DER/ADX/saline treated mice were also exhibiting a significantly increased papilloma incidence compared with the mice fed the other DER diets and the AL/ADX/CCS. The mice receiving the DER/ADX/CCS protocol exhibited the most pronounced inhibition of papilloma incidence consistently throughout the study (Figure 3A). At week 31 DER significantly reduced papilloma incidence and CCS reduced papilloma incidence in both AL/ADX and DER/ADX groups (P < 0.001).

Fig. 3. Papilloma incidence (A) and multiplicity; mean count/mouse (B). Data shown are for mice treated with DMBA/TPA. Values represent mean ± SEM, N = 8–38. (A) Incidence analyzed by Fisher’s exact test. (B) Analyzed by GENMOD with multiple comparisons by least squares means. P < 0.0001 for differences by diet at each time point ≥8 weeks. Means at each time point with different superscript letters are significantly different at the P < 0.05 level and values with more than one letter are not significantly different from means sharing either of the letters. Only one papilloma was seen in one acetone treated mice fed the AL/ADX/saline diet while 75% (40/53) of all of the acetone treated mice survived up to the end of the experiment.
DER/ADX/CCS significantly reduced papilloma multiplicity compared with all other groups \((P < 0.0001)\).

**Carcinoma incidence rates and multiplicity**

Total carcinoma incidence rates include \textit{in situ} and invasive carcinomas. The response to diet and CCS was similar for the total carcinoma incidence rates and the papilloma incidence rates (Table IV). There were significant differences among total carcinoma incidence rates \((P < 0.0003)\) and also among \textit{in situ} \((P < 0.0001)\) and invasive \((P < 0.0001)\) carcinoma incidence rates. Total carcinoma incidence rates were lowest in the DER/ADX/CCS group and highest in the AL/sham group. \textit{In situ} carcinoma incidence rates were lowest in the DER/ADX/CCS group and highest in the AL/ADX/saline group. Invasive carcinoma incidence rate was significantly increased in the AL/sham diet compared with the other five groups \((P < 0.0001)\) (Table IV). Carcinoma multiplicity was defined as the number of carcinomas per carcinoma bearing animal. Total carcinoma multiplicity was significantly lower for the DER/sham than the AL/sham group \((P < 0.05)\) and it was lowest in the mice in the DER/ADX/CCS group \((P < 0.0001)\). \textit{In situ} carcinoma multiplicity was lowest in the DER/ADX/CCS group compared with the other five groups \((P < 0.0002)\). Invasive carcinoma multiplicity also varied significantly across treatment groups \((P < 0.0001)\) and was lowest in the DER/ADX/CCS group and highest in the AL/sham mice (Table IV).

**Other pathology**

There was a significant increase in the appearance of lymphosarcoma in the spleens of the AL/ADX/saline mice compared with the DER/sham and DER/ADX/CCS groups \((P < 0.0350)\) (Table IV).

**Gross observations**

Skin ulceration in DMBA/TPA treated mice that survived at least 23 weeks differed significantly between groups \((P < 0.00005)\). This lesion was observed in 27% (8/30) of the mice in the AL/sham, 47% (8/17) of the mice in the AL/ADX/saline and 6% (2/32) of the mice in the DER/sham groups. Skin ulcerations were not observed in other groups (0/56 mice). Casual observation of intense scratching directly correlated with the observations on skin ulcerations.

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### Table IV. Epidermal carcinoma and lymphosarcoma

<table>
<thead>
<tr>
<th>N</th>
<th>Incidence (%)</th>
<th>Multiplicity</th>
<th>Incidence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>In situ</td>
<td>Invasive</td>
</tr>
<tr>
<td>AL/sham</td>
<td>30</td>
<td>83a</td>
<td>67b,c</td>
</tr>
<tr>
<td>AL/ADX/saline</td>
<td>17</td>
<td>77b</td>
<td>71a,b</td>
</tr>
<tr>
<td>AL/ADX/CCS</td>
<td>13</td>
<td>59c</td>
<td>54b,c</td>
</tr>
<tr>
<td>DER/sham</td>
<td>32</td>
<td>69b</td>
<td>59b</td>
</tr>
<tr>
<td>DER/ADX/saline</td>
<td>14</td>
<td>50b,c</td>
<td>50b,c</td>
</tr>
<tr>
<td>DER/ADX/CCS</td>
<td>19</td>
<td>21a</td>
<td>16a</td>
</tr>
<tr>
<td>P &lt;</td>
<td>0.0003</td>
<td>0.0001</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

\(N = \) Effective mice treated with DMBA/TPA that survived at least 23 weeks. Carcinoma multiplicity is the number of carcinomas per carcinoma bearing animal that survived at least 23 weeks or longer.

Values represent mean ± SEM. \((P < 0.0003)\) with multiple comparisons by Fisher’s exact test for incidence rates and \(P < 0.0001\) GENMOD with multiple comparisons by least square means for multiplicity rates.) Column means with different superscript letters are significantly different at the \(P < 0.05\) level and values with more than one letter are not significantly different from means sharing either of the letters.

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### Table V. Plasma corticosterone concentration (ng/ml) in mice surviving to 31 weeks

<table>
<thead>
<tr>
<th>N</th>
<th>Sham</th>
<th>ADX</th>
</tr>
</thead>
<tbody>
<tr>
<td>AL/sham</td>
<td>33</td>
<td>143 ± 20a</td>
</tr>
<tr>
<td>AL/ADX/saline</td>
<td>34</td>
<td>75 ± 20a</td>
</tr>
<tr>
<td>AL/ADX/CCS</td>
<td>15</td>
<td>75 ± 20a</td>
</tr>
<tr>
<td>DER/sham</td>
<td>36</td>
<td>151 ± 19a</td>
</tr>
<tr>
<td>DER/ADX/saline</td>
<td>22</td>
<td>101 ± 25a</td>
</tr>
<tr>
<td>DER/ADX/CCS</td>
<td>23</td>
<td>282 ± 24b</td>
</tr>
<tr>
<td>P &lt;</td>
<td>NS</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

There was no significant difference between animals treated with DMBA/TPA or acetone for any dietary-surgery treatment. So DMBA/TPA and acetone treatment groups were combined to obtain treatment group mean concentrations. Mice were killed when maximal plasma corticosterone levels were expected. Thus sham-operated mice were killed in the morning while ADX mice were killed in the evening. Therefore, these groups are presented in separate columns and analyzed separately. Values represent mean ± SEM. Individual means within the column were analyzed by ANOVA-GLM and then compared with multiple comparisons by least squares means. Means within a column with different superscript letters were significantly different at the \(P < 0.05\) level. DER/ADX/CCS corticosterone concentration was significantly higher than the other adrenalized treatment groups, \(P < 0.0001\) (ANOVA-GLM).

**Plasma corticosterone**

There was no statistical difference in plasma corticosterone concentrations between mice treated with DMBA-TPA or acetone for any dietary-surgery treatment. So these groups were pooled and are presented as dietary-surgery treatment group in Table V. Since the sham-operated and ADX mice were killed in the morning and evening, respectively, these groups are presented in separate columns in Table V and analyzed separately. Mice in the DER/ADX/CCS group exhibited significantly higher plasma corticosterone concentrations than any of the other ADX groups \((P < 0.0001)\).

**Plasma insulin growth factor-1 (IGF-1)**

In the ADX mice there was no significant difference in plasma IGF-1 concentration between the animals receiving corticosterone acetone for any dietary-therapy groups were combined to obtain treatment group mean concentrations. Mice were killed when maximal plasma corticosterone levels were expected. Thus sham-operated mice were killed in the morning while ADX mice were killed in the evening. Therefore, these groups are presented in separate columns and analyzed separately. Values represent mean ± SEM. Individual means within the column were analyzed by ANOVA-GLM and then compared with multiple comparisons by least squares means. Means within a column with different superscript letters were significantly different at the \(P < 0.05\) level. DER/ADX/CCS corticosterone concentration was significantly higher than the other adrenalized treatment groups, \(P < 0.0001\) (ANOVA-GLM).

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**Effect of ADX and CCS on DER inhibited skin tumorigenesis**

Downloaded from http://carcin.oxfordjournals.org/ at Pennsylvania State University on March 6, 2014
ADX/DER mice resulted in papilloma and carcinoma rates the adrenal gland may play a very different role in the con-
papillomas, total carcinomas and invasive carcinomas are not
in control-fed mice. The reasons for the different patterns of
carcinoma rates, invasive carcinomas were inhibited by ADX
the invasive carcinomas. In contrast to the general absence of
potent inhibitor of both the total number of carcinomas and
mice more than in the DER/sham mice but this difference was
Total carcinoma multiplicity was higher in the DER/ADX
rates largely paralleled the effects observed on papillomas.
sterone may have contributed to the higher tumor rate in the
DER mice (15) resulted in cancer prevention in both AL/ADX
achieve peak corticosterone concentrations in the range of the
in our laboratory (26) and by others (28). In
there is a cell survival factor that inhibits apoptosis and increases
IGF-1, were also modulated by diet restriction in a
cells. Indeed, in mice that were fed these diets and treated
only with a single treatment of TPA or acetone 1 or 4 h before
killing, corticosterone was generally equally elevated in both
the sham-operated DER mice and in the corticosterone sup-
plemented control-fed, and the corticosterone-supplemented
DER-fed groups as described below (17,26). An alternative
interpretation for the low rate of papilloma and carcinoma in
the DER/ADX/CCS group is that the adrenal gland might
provide both cancer-preventative (presumably elevated cor-
icosterone in DER mice) and cancer-enhancing impacts.
However, we are not aware of any data in support of this
alternative hypothesis.

In previous short term studies we assessed CCS in the diet
and hormonal protocols used in the experiment reported in
the present paper. In both studies after 10-12 weeks of treatment,
the plasma corticosterone concentration in 17- to 22-week-old
mice was elevated (3- to 5-fold) in sham/DER mice compared
with sham/AL mice (17,26). ADX mice had lower cortico-
sterone and did not differ by diet. An elevation in corticoste-
one in hormone supplemented groups to the level in the sham/
DER mice was observed in blood collected late in the day
(26). When the blood was collected in the morning, the sup-
plemented groups had only a minor increase in corticosterone
(17). However, we would not anticipate observing an eleva-
tion in CCS in the corticosterone-supplemented groups in the
morning because mice consumed more drinking water later in
the day when they had food available. Administration of
corticosterone in the drinking water was done in the current
experiment instead of implanting corticosterone pellets
because drinking water administration mimicked the CCS
levels of DER mice in earlier studies (21) more closely.
ADX and corticosterone supplementation did not change the
expression of IGF-1 in our earlier work (15).

Another hormone that merits being studied for contributing
to the DER prevention of skin carcinogenesis is IGF-1. IGF-1
is a cell survival factor that inhibits apoptosis and increases
cell proliferation (27). Diet restriction was shown to reduce
circulating IGF-1 in our laboratory (26) and by others (28). In
addition, IGF-binding proteins, which appear to control the
release of IGF-1, were also modulated by diet restriction in a
manner that would be expected to reduce IGF-1 activation
(29). The most compelling study to suggest that reduction in
circulating IGF-1 plays a key role in DER prevention of cancer
was conducted by Dunn et al. (28). Urinary bladder cancer was

### Table VI. Plasma insulin growth factor (IGF-1) concentration (ng/ml) in mice surviving to 31 weeks

<table>
<thead>
<tr>
<th>Carcinogen/vehicle</th>
<th>Sham</th>
<th>ADX</th>
</tr>
</thead>
<tbody>
<tr>
<td>AL/sham 4 DMBA/TPA</td>
<td>169 ± 31(^a)</td>
<td></td>
</tr>
<tr>
<td>AL/sham 2 Acetone</td>
<td>255 ± 44(^a)</td>
<td></td>
</tr>
<tr>
<td>AL/ADX 7 DMBA/TPA</td>
<td>201 ± 31(^c)</td>
<td></td>
</tr>
<tr>
<td>AL/ADX 2 Acetone</td>
<td>323 ± 58(^c)</td>
<td></td>
</tr>
<tr>
<td>DER/sham 9 DMBA/TPA</td>
<td>56 ± 21(^b)</td>
<td></td>
</tr>
<tr>
<td>DER/sham 2 Acetone</td>
<td>69 ± 44(^b)</td>
<td></td>
</tr>
<tr>
<td>DER/ADX 8 DMBA/TPA</td>
<td>42 ± 29(^d)</td>
<td></td>
</tr>
<tr>
<td>DER/ADX 2 Acetone</td>
<td>88 ± 58(^d)</td>
<td></td>
</tr>
<tr>
<td>(P &lt;)</td>
<td>0.0042</td>
<td>0.0015</td>
</tr>
</tbody>
</table>

\(^1\)The effect of corticosterone supplementation in the ADX mice was not
significant; therefore, the IGF data for ADX/saline or ADX/CCS drinking
water were combined in the analysis of differences between DMBA/TPA and
acetone. Mice were killed when maximal plasma corticosterone levels were
expected. Thus sham-operated mice were killed in the morning while ADX
mice were killed in the evening. These groups are presented in separate
columns and analyzed separately. Values represent mean ± SEM. Individual
means within the column were analyzed by ANOVA/GLM and then
compared with multiple comparisons by least squares means. Means within a
column with different superscript letters were significantly different at the
\(P < 0.05\) level. DER/sham was significantly lower than AL/sham and
DER/ADX was significantly lower than AL/ADX, \(P < 0.005\)
(ANOVA-GLM).

reduced to approximately one-third and one-fourth of the values
in mice that received the AL diet in the sham-operated groups
(\(P < 0.0042\)) and the ADX-operated groups (\(P < 0.0015\)).

### Discussion

A striking inhibition of DMBA initiated and TPA promoted
papilloma development was observed in sham-operated DER
mice, particularly with respect to papilloma number. However,
this inhibition was partially blocked in ADX-operated DER
mice. In comparison, ADX did not appreciably alter papilloma
development in control mice. These observations demonstrate
the dependence of DER, in the prevention of mouse skin
papillomas, on an intact adrenal gland. Furthermore, daily
supplementation with corticosterone in the drinking water to
achieve peak corticosterone concentrations in the range of the
DER mice (15) resulted in cancer prevention in both AL/ADX
and DER/ADX mice. This suggests that the loss of corticoste-
ron may have contributed to the higher tumor rate in the
ADX operated DER mice.

The impact of DER and ADX on mouse skin carcinoma
rates largely paralleled the effects observed on papillomas.
Total carcinoma multiplicity was higher in the DER/ADX
mice than in the DER/sham mice but this difference was
not statistically significant. However, corticosterone was a
potent inhibitor of both the total number of carcinomas and
the invasive carcinomas. In contrast to the general absence of
impact of ADX in the control-fed mice on papilloma and total
carcinoma rates, invasive carcinomas were inhibited by ADX
in control-fed mice. The reasons for the different patterns of
papillomas, total carcinomas and invasive carcinomas are not
clear, but these contrasts suggest that CCS in the absence of
the adrenal gland may play a very different role in the con-
version to invasive carcinomas than it plays in papilloma
development.

It is interesting that administering corticosterone to the
ADX/DER mice resulted in papilloma and carcinoma rates
that were lower than those observed in the sham-operated
DER mice. It is possible that the corticosterone administered
in the drinking water resulted in a circulating level that was
higher than was observed in the sham/DER mice. Currently, it
is not possible to perfectly mimic the CCS of intact mice in
ADX animals. Indeed, there are extra-adrenal sites near the
thoracic and abdominal sympathetic ganglia that may produce
glucocorticoid hormone (25) and these sites were not removed
in our ADX mice. The data presented in this paper in Table V
on plasma corticosterone in the mice surviving until the final
sacrifice show that the DER/ADX/CCS group had the highest
CCS of all treatment groups. Sham and ADX mice were killed
at different times to allow for comparison between AL and
DER mice at the times when the greatest differences in CCS
would be expected. Thus, we did not compare CCS concentra-
tion in sham and ADX mice in this experiment. While our
observations on CCS at the termination of the experiment are
intriguing, it is important to note that data from surviving
animals may be biased by uncontrolled differential survival
factors. Indeed, in mice that were fed these diets and treated
only with a single treatment of TPA or acetone 1 or 4 h before
killing, corticosterone was generally equally elevated in both
the sham-operated DER mice and in the corticosterone sup-
plemented control-fed, and the corticosterone-supplemented
DER-fed groups as described below (17,26). An alternative
interpretation for the low rate of papilloma and carcinoma in
the DER/ADX/CCS group is that the adrenal gland might
provide both cancer-preventative (presumably elevated cor-
icosterone in DER mice) and cancer-enhancing impacts.
However, we are not aware of any data in support of this
alternative hypothesis.

In previous short term studies we assessed CCS in the diet
and hormonal protocols used in the experiment reported in
the present paper. In both studies after 10-12 weeks of treatment,
the plasma corticosterone concentration in 17- to 22-week-old
mice was elevated (3- to 5-fold) in sham/DER mice compared
with sham/AL mice (17,26). ADX mice had lower cortico-
sterone and did not differ by diet. An elevation in corticoste-
one in hormone supplemented groups to the level in the sham/
DER mice was observed in blood collected late in the day
(26). When the blood was collected in the morning, the sup-
plemented groups had only a minor increase in corticosterone
(17). However, we would not anticipate observing an eleva-
tion in CCS in the corticosterone-supplemented groups in the
morning because mice consumed more drinking water later in
the day when they had food available. Administration of
corticosterone in the drinking water was done in the current
experiment instead of implanting corticosterone pellets
because drinking water administration mimicked the CCS
levels of DER mice in earlier studies (21) more closely.
ADX and corticosterone supplementation did not change the
expression of IGF-1 in our earlier work (15).

Another hormone that merits being studied for contributing
to the DER prevention of skin carcinogenesis is IGF-1. IGF-1
is a cell survival factor that inhibits apoptosis and increases
cell proliferation (27). Diet restriction was shown to reduce
circulating IGF-1 in our laboratory (26) and by others (28). In
addition, IGF-binding proteins, which appear to control the
release of IGF-1, were also modulated by diet restriction in a
manner that would be expected to reduce IGF-1 activation
(29). The most compelling study to suggest that reduction in
circulating IGF-1 plays a key role in DER prevention of cancer
was conducted by Dunn et al. (28). Urinary bladder cancer was
induced by p-cressidine and after pre-neoplasia was confirmed, mice were fed 20% DER with or without recombinant IGF-1 administered with mini-pumps. Mice fed ad libitum were included in a parallel group. The circulating IGF-1 was reduced by 24% after 5 weeks of feeding DER diet and the levels were restored to the AL values in the DER plus IGF-1 group. The progression of tumors was inhibited in the DER group but it was not reduced in the DER plus IGF-1 group (28). Studies by Zhu et al. (20) used dietary corticosterone supplements to assess the role of CCS in DER prevention of mammary cancer. In the Sprague-Dawley rat model used for these studies they observed elevated CCS and decreased IGF-1 in response to the dietary corticosterone supplement.

IGF-1 was measured in the mice in our tumor study is presented in this paper and in previous 12 week, short term studies of SENCAR mice (26). In the short term studies DER decreased circulating IGF-1 (231 ± 14 ng/ml, mean ± SEM) (P < 0.05) in comparison with AL mice (402 ± 18 ng/ml), but TPA treatment, ADX and corticosterone supplementation did not alter these values (26). In the present tumor study circulating IGF-1 was reduced in the DER mice irrespective of the other experimental treatments. These were important observations since considerable research has suggested that reduced circulating IGF-I or elevated IGF–BPs in the DER mice contributes to cancer prevention by DER (30). Thus, since the hormonal treatments that we used to modulate CCS did not mediate circulating IGF-1, the effects that we attribute to CCS were probably not owing to IGF-1 in the present investigation.

It is unlikely that aldosterone, epinephrine or norepinephrine were responsible for the adrenal dependence of DER inhibition of mouse skin carcinogenesis. Earlier studies in our laboratory indicated that there was no difference in aldosterone levels between the AL-fed mice or the mice on DER diet (15). Furthermore, we did not find reports on the role of epinephrine or norepinephrine in skin carcinogenesis.

Our tumor study determined that DER inhibition of skin tumor promotion was in large measure mediated through increasing CCS. However, it is noteworthy that CCS did not explain all the difference between the AL and DER mice since supplementation with corticosterone was more effective in preventing papillomas and carcinomas in DER/ADX mice than in AL/ADX mice. These results support the hypothesis that, while CCS contributes to cancer prevention by DER, other factors are also involved. It is important to note that changes in circulating IGF-I did not appear to contribute to the DER effects that were attributed to CCS in our study as noted above. However, the elevated glucocorticoid hormone may interact with other hormonal effects, such as the lowered IFG-1 to prevent skin tumorigenesis.

The changes in the body weight over time showed that the DMBA/TPA initiated, AL/ADX/CCS treated mice actually gained considerably more weight (more or less double) than the AL/sham operated mice (Table III). This observation was paralleled by data showing a lower food intake in these mice in comparison with the AL/sham and AL/ADX/saline groups from weeks 10–30 (Table II). These results suggest that the dose of corticosterone in these mice may have caused salt and water retention, known impacts of excessive mineralocorticoid in humans (25). This may have been owing to changes in potassium balance in these mice since there is evidence in humans that excessive glucocorticoid therapy can cause potassium depletion and associated water retention, and body weight gain with prolonged mineralocorticoid treatment (25). Intracellular potassium is replaced with sodium ions and water is retained with the sodium ions (25). Interestingly, the DMBA/TPA initiated, AL/ADX/CCS mice generally did not show elevated food intake in comparison with all of the acetone initiated groups (Table II).

An intriguing observation was the elevated lymphosarcoma incidence in the AL/ADX mice in comparison with the DER/sham and DER/ADX/CCS groups. This lesion appears to have been reduced by both DER and CCS treatments, and elevated by ADX in mice not supplemented with CCS. There is no evidence that this lesion interfered with skin tumor development in the current experiment since the results did not correlate clearly with any of the skin lesions. The other lesion observed was the high rate of skin ulceration in the AL/sham and AL/ADX/saline groups and a low rate in the DER/sham group. Skin ulceration was reduced either by CCS treatment or by DER. These lesions may have reduced survival in the two AL groups and thus may have blunted the papilloma and carcinoma rates in these groups.

In separate studies we are assessing the mechanism for DER and corticosterone prevention of mouse skin carcinogenesis. These studies suggest that DER and corticosteroid hormone prevention of mouse carcinogenesis occurs through inhibiting signaling down the extracellular signal regulated kinase (ERK)1,2, reducing activator protein 1 (AP1): DNA binding and inhibiting transcriptional regulation through AP1 (26,31). This inhibition appeared to be downstream from the inhibition of specific isoforms of protein kinase C—PKC α and PKC γ (32,33). These isoforms were also reduced by corticosterone supplementation in ADX mice in short-term studies (21). The inhibition of ERK1,2 (17) and the reduction in AP1:DNA (26) binding by DER were eliminated in ADX mice demonstrating the dependence of these DER effects on an intact adrenal gland. Supplementation with CCS in the ADX animals restored the inhibition of TPA-induced ERK1,2 and AP1:DNA binding (17,26). Studies of AP1 regulated genes indicate that DER inhibits TPA-induced transcriptional activation of an AP1-luciferase reporter gene in a transgenic mouse model (34). The known importance of TPA-induced ERK1,2 signaling and AP1 transcriptional regulation in mouse skin carcinogenesis suggests that DER blockage of these events causally contributes to DER prevention of skin carcinogenesis. The data presented in this paper show that skin tumor development in mice in protocols with DER, ADX and CCS treatment can be explained, at least in part, by these molecular effects of DER and CCS.

In summary, DER significantly inhibited tumorigenesis in intact mice as was previously observed. This inhibition was dependent upon an intact adrenal gland and corticosterone supplementation to DER/ADX mice restored the tumorigenesis inhibition. Corticosterone supplementation itself inhibited tumor progression when given to ADX mice fed the control diet and had an additive inhibitory impact when given to ADX mice fed the DER diet.

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References


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