ABSTRACT

Squamous metaplasia is a common abnormality of cellular development and differentiation in the epithelia of many organs, including the prostate gland in humans. Agents known to elevate the level of intracellular cyclic adenine nucleotide have been found to direct various kinds of epithelial cells onto a pathway of epidermoid (squamous) development and a differentiation marked by keratin production. We report here that the mixture of the cyclic adenine nucleotide; N\textsuperscript{6},O\textsuperscript{2}-dibutyryladenosine cyclic 3':5'-monophosphate (0.1 mM); prostaglandins E\textsubscript{1}, E\textsubscript{2}, and B\textsubscript{1} (each 5 µg/ml); and papaverine (1 µM) induces extensive squamous metaplasia and keratin production in the epithelial cells of mouse ventral prostate glands cultured for 3 weeks. The components of the mixture act synergistically. A 10-fold higher concentration of N\textsuperscript{6},O\textsuperscript{2}-dibutyryladenosine cyclic 3':5'-monophosphate (1 mM) alone is as effective as is the mixture. The presence of either the retinoid, retinylidene dimedone, or the phorbol ester, phorbol-12,13-didecanoate, at 1 µM completely prevents the induction of squamous metaplasia. The results suggest that cyclic adenine nucleotide and indirectly prostaglandins E\textsubscript{1} and E\textsubscript{2} may play important roles in the spontaneous and exogenously induced production of squamous metaplasia. These same inducing agents have also been found in other studies to bring about squamous metaplasia and keratinization in culture in the mammary glands of mice and in the breast tissue of humans, as well as to accelerate normal epidermization in chick embryo skin. The findings in prostate gland, mammary glands, and skin derived from embryonic or adult animals and humans, spanning three species and two classes of vertebrates, suggest that cyclic adenine nucleotide augmented by prostaglandins E\textsubscript{1} and E\textsubscript{2} may act generally on diverse types of epithelia to bring about normal and metaplastic squamous cell development and a differentiation marked by keratin production.

INTRODUCTION

Squamous metaplasia is a common aberration of cellular development and differentiation in the epithelia of many organs. The normal epithelia are replaced by cells that resemble those of epidermis. The metaplastic cells differentiate into flattened cells that may cornify into keratin. The lesion often results in decrease or loss of normal function. The metaplastic cells may regress, may remain benignly squamous, or they or related cells may progress to epidermoid carcinoma (1).

Squamous metaplasia is common in the prostate glands of animals and elderly human males (Refs. 8 and 29; see below). The abnormality is inducible in rodent prostate glands in vivo by vitamin A deficiency (48), estrogen (3, 4), carcinogen (5, 9, 24, 26, 32, 34), and estrogen with carcinogen (20, 21, 23, 25). Retinoids can prevent and reverse the metaplasias that arise as a result of these treatments (24–26, 32). However, these observations do not provide a basis for an understanding of the underlying physiological mechanism involved in the induction of this common lesion.

Agents that are known to elevate the level of intracellular cyclic AMP\textsuperscript{3} have recently been found to induce normal and abnormal epidermoid cell development in culture. The combination of dbcAMP, PGE\textsubscript{1}, PGE\textsubscript{2}, and PAP enhances the rate of the normal process of epidermoid cell development in chick embryo skin (43) and also induces the markedly abnormal condition of extensive squamous metaplasia and considerable keratinization in mouse mammary gland (42). In search of whether these agents may further act generally to mediate the production of the spontaneous as well as the exogenously induced squamous metaplasias, the present investigation examined the effects of these substances on mouse prostate gland in culture. The mouse prostate gland is especially suitable for such study, inasmuch as the findings may provide insight into the basis of the apparently analogous aberration in that organ in humans.

MATERIALS AND METHODS

Reagents. Culture Medium CMRL-1066 and penicillin were purchased from Grand Island Biological Co., Grand Island, N. Y. dbcAMP, PAP, dbcGMP, 5'-adenylic acid, sodium butyrate, streptomycin sulfate, and amphotericin B were obtained from Sigma Chemical Co., St. Louis, Mo. 3-Methylcholanthrene was purchased from Eastman Kodak Co., Rochester, N. Y., and further purified by crystallization. PGE\textsubscript{1}, PGE\textsubscript{2}, and PGB\textsubscript{1} were kindly donated by Dr. J. E. Pike of the Upjohn Co., Kalamazoo, Mich. The retinoid, 2-retinylidene-5,5-dimethyl-1,3-cyclohexanedione (retinylidene dimedone), was synthesized by Drs. Nancy Acton and Arnold Brossi and was kindly provided by Dr. Michael B. Sporn, all of the National Cancer Institute. Phorbol-12,13-didecanoate was purchased from CCR Inc., Eden Prairie, Minn.

Culturing and Processing of Prostate Glands. The ventral prostate glands of in-house-bred (special-pathogen-free) male BALB/c mice, 2 to 3 months old, were cut into 1-mm-cube fragments, spread on Dacron rafts, and floated in 1 ml of CMRL-1066 medium in 35-mm tissue culture dishes (4 to 8 fragments/dish). The basal medium contained 5% horse serum (inactivated at 56° for 30 min), 0.1 mg streptomycin sulfate per ml, 0.1 mg amphotericin B per ml, and 100 IU penicillin per ml (10). The cultures were treated with or without additional supplements at 37° in a water-saturated atmosphere of 50% O\textsubscript{2}, 45% N\textsubscript{2}, and 5% CO\textsubscript{2}.

\textsuperscript{3} The abbreviations used are: cyclic AMP, cyclic adenosine 3':5'-monophosphate; dbcAMP, N\textsuperscript{6},O\textsuperscript{2}-dibutyryladenosine cyclic 3':5'-monophosphate; PGE\textsubscript{1}, prostaglandin E\textsubscript{1}; PGE\textsubscript{2}, prostaglandin E\textsubscript{2}; PGB\textsubscript{1}, prostaglandin B\textsubscript{1}; PAP, papaverine; dbcGMP, N\textsuperscript{6},O\textsuperscript{2}-dibutyrylguanosine cyclic 3':5'-monophosphate.

\textsuperscript{1} Supported in part by USPHS Grants CA-30036, CA-05945, CA-06927, and RR-05539 from the National Cancer Institute and by an appropriation from the Commonwealth of Pennsylvania.

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and 5% CO₂ for various periods up to 4 weeks. All experimental and control cultures received the same amount of ethanol (0.3% total) that was used as vehicle for the additives under test. Media and supplements were replaced every 2 to 3 days. Samples of prostate glands that were used in individual experiments were derived from randomly mixed pools of dissected fragments of the glands of different mice. At the end of the culture period, the fragments were fixed in acetic acid:ethanol (1:3, v/v), stained with alum:carmine for detection during histological processing, dehydrated in ethanol, transferred to xylene, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin for histological study.

**Scoring of Squamous Metaplasia.** A system of scoring with a range from 0 to 4 was developed for evaluating the degree and extent of squamous metaplastic development and differentiation in the cultured prostate glands. Stage 0 denoted fragments of the glands with no squamous metaplasia. At Stage 1, a single site of definite but poorly developed metaplasia was evident. In Stages 2 and 3, less than one-half or more than one-half, respectively, of the epithelial cells in the fragments were squamoid without keratin flakes. In Stage 4, keratin production was present in a background of extensive squamous metaplasia. A random single section of each fragment was scored independently, in most cases without foreknowledge of its history. Fragments that were treated similarly were grouped to yield an average of the integral score values and a standard error of the mean.

**RESULTS**

**Induction of Squamous Metaplasia in Prostate Gland.** Fragments of freshly explanted ventral prostate glands typically contained 6 or more acini that were lined with single layers of columnar secretory epithelial cells supported by a fibromuscular stroma (Fig. 1A). Fragments of prostate glands can be cultured for at least 4 weeks on CMRL-1066 medium supplemented with 5% horse serum. In the absence of further additives (control cultures), the columnar epithelial cells lining the acini of the glands exfoliated and were replaced during the first week by a layer of mixed columnar and cuboidal cells. The exfoliation and replacement by increasing the proportion of cuboidal cells proceeded at a slow rate throughout the usual culture period of 3 weeks (Fig. 1B).

Exposure of the prostate glands to the combination of dbcAMP (0.1 mM), PAP (1 μM), and PGE₁, PGE₂, and PGB₁ (each 5 μg/ml) for 3 weeks induced an extensive squamous metaplasia in the acinar epithelium (Table 1). The components of the mixture acted synergistically. The dbcAMP (0.1 mM) by itself and the PAP alone were marginally active (Series 1 and 11), and their combination was slightly more so (Series 2). The mixture of the 3 prostaglandins by themselves was mildly inhibitory (Series 12). Further, each of the 3 prostaglandins, either at 5 μg/ml or even at 15 μg/ml (not listed), individually in the presence of the dbcAMP and PAP had little effect (Series 3 to 5). However, when the 3 prostaglandins were present together with the dbcAMP and PAP, an extensive squamous metaplasia resulted (Series 6).

The addition of a 10-fold higher concentration of dbcAMP (1 mM) virtually abrogated the requirement for the prostaglandins and PAP in the induction of extensive squamous metaplasia in the cultured prostate epithelia (Table 1; Fig. 1C). dbcAMP alone at 1 mM induced a high degree of squamous metaplasia (Series 7), while the dbcAMP with PAP were equally effective (Series 8). In contrast to the ability of the prostaglandins to enhance synergistically the activity of 0.1 mM dbcAMP, at 1 mM dbcAMP the prostaglandins were actually slightly inhibitory (Series 9).

The production of the squamous metaplasia in the prostate glands specifically required the cyclic adenine nucleotide. Sodium butyrate at 2 mM, dbcGMP at 1 mM, and 5'-AMP at 1 mM all could not replace the dbcAMP (Table 1, Series 13 to 15). A series of these reagents at 0.1 mM was also ineffective (not listed).

A near-maximal degree of squamous metaplasia was induced at 3 weeks in culture. The time courses of the process brought about by a mixture of dbcAMP (1 mM) and PAP (1 μM) or by the combination of dbcAMP (0.1 mM), PAP (1 μM), and PGE₁, PGE₂ and PGB₁ (each 5 μg/ml) are shown in Chart 1. The earliest indication of squamous development in the prostate gland fragments was detectable at 2 weeks. At 3 weeks, the squamous metaplasia was extensive with evident small foci of keratin. In contrast, the control glands (without inducers) at 3 weeks were only slightly metaplastic, with foci usually restricted

### Table 1

<table>
<thead>
<tr>
<th>Series</th>
<th>Cyclic adenine nucleotide</th>
<th>Supplements</th>
<th>No. of cultures</th>
<th>Stage of metaplasia</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>dbcAMP, 0.1 mM</td>
<td>None</td>
<td>46</td>
<td>1.0 ± 0.2⁻</td>
</tr>
<tr>
<td>2</td>
<td>PAP</td>
<td></td>
<td>62</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>3</td>
<td>PAP + PGE₁</td>
<td></td>
<td>24</td>
<td>1.7 ± 0.2</td>
</tr>
<tr>
<td>4</td>
<td>PAP + PGE₂</td>
<td></td>
<td>16</td>
<td>1.5 ± 0.2</td>
</tr>
<tr>
<td>5</td>
<td>PAP + PGB₁</td>
<td></td>
<td>20</td>
<td>1.1 ± 0.2</td>
</tr>
<tr>
<td>6</td>
<td>PAP + PGE₁, PGE₂, PGB₁</td>
<td></td>
<td>45</td>
<td>3.1 ± 0.2</td>
</tr>
<tr>
<td>7</td>
<td>dbcAMP, 1.0 mM</td>
<td>None</td>
<td>74</td>
<td>3.2 ± 0.2</td>
</tr>
<tr>
<td>8</td>
<td>PAP</td>
<td></td>
<td>101</td>
<td>3.2 ± 0.1</td>
</tr>
<tr>
<td>9</td>
<td>PAP + PGE₁, PGE₂, PGB₁</td>
<td></td>
<td>73</td>
<td>2.2 ± 0.1</td>
</tr>
<tr>
<td>10</td>
<td>None</td>
<td>None</td>
<td>142</td>
<td>0.6 ± 0.1</td>
</tr>
<tr>
<td>11</td>
<td>PAP</td>
<td></td>
<td>43</td>
<td>1.0 ± 0.1</td>
</tr>
<tr>
<td>12</td>
<td>PGE₁, PGE₂, PGB₁</td>
<td></td>
<td>36</td>
<td>0.4 ± 0.1</td>
</tr>
<tr>
<td>13</td>
<td>5'-AMP</td>
<td></td>
<td>27</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>14</td>
<td>Sodium butyrate</td>
<td></td>
<td>32</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>15</td>
<td>dbcGMP</td>
<td></td>
<td>29</td>
<td>0.8 ± 0.2</td>
</tr>
</tbody>
</table>

⁻ Average ± S.E.
to a single site of poorly developed but somewhat flattened cells in any fragment.

Prevention of Squamous Metaplasia in Prostate Gland.

The induction of squamous metaplasia in the cultured prostate glands was blocked by either retinoid or phorbol ester. The presence of the retinoid, retinylidene dimedone at 1 μM, or phorbol-12,13-didecanoate at 10 μM and 1 μM throughout the culture period of 3 weeks completely prevented the onset of squamous metaplasia either by dbcAMP at 1 mM or by the mixture of dbcAMP (0.1 mM), PAP, and PGE1, PGE2, and PGB1 [Series 2 and 6, versus Series 1 and 5; Series 3 and 4 and Series 7 and 8 versus Series 1 and 5 (Table 2); Fig. 1D versus Fig. 1C]. The retinoid and the phorbol ester each markedly lessened the degree of squamous metaplasia to that of the control cultures (Series 9). Retinylidene dimedone and phorbol didecanoate at 100-fold lower concentration (both 10 nM) were each only marginally preventive (not listed). Neither of the reagents by themselves at the higher concentrations caused any metaplasia (Series 10 and 11).

Additional experiments confirmed the ability of retinoid to prevent the induction of squamous metaplasia in prostate gland by the carcinogen, 3-methylcholanthrene (5, 9, 24, 26, 32, 34). Using the protocol described by Lasnitzki (26), cultured fragments of mouse prostate gland were incubated with the serum-supplemented medium and the carcinogen (5 μg/ml) for 2 weeks and then were maintained in the absence of the carcinogen for an additional week. Other glands were likewise treated with the carcinogen but were also exposed to retinylidene dimedone (1 μM) for the entire 3 weeks. In 28 control cultures that were given neither carcinogen nor retinoid, there was a complete absence of squamous metaplasia (score, 0). However, the 23 carcinogen-treated glands were significantly metaplastic (score, 1.2 ± 0.3). In contrast, the incubation of 15 glands with the retinoid totally prevented the induction of metaplasia by the carcinogen (score, 0).

Questions whether the continued presence of dbcAMP, PAP, and the prostaglandins is necessary for the squamous metaplastic state and whether retinoid can reverse the process were unresolved. The absence of the inducers or the presence of 1 μM retinylidene dimedone for 2.5 weeks after the production of the metaplasia for 3 weeks, caused acinar degeneration in some glands, making evaluations uncertain.

DISCUSSION

Cyclic adenine nucleotide and specific prostaglandins synergistically bring about an extensive squamous metaplasia in mouse prostate glands in culture. The finding opens up the possibility that these substances may be the physiological mediators of the analogous common lesion in the prostate glands of humans and animals (see "Introduction").

The 3 types of squamous metaplasia-inducing reagents, cyclic adenine nucleotide, specific prostaglandins and PAP, are known to be able to elevate the level of intracellular cyclic AMP (a) by the entry of exogenous dbcAMP, (b) as a result of the stimulation of adenyl cyclase by the prostaglandins (40, 41), and (c) through inhibition by PAP of the degradation of the nucleotide by phosphodiesterase (37). That the induction of squamous metaplasia in prostate gland may be the consequence of an increased level of intracellular cyclic AMP is

Table 2 Prevention of squamous metaplasia in cultured prostate glands of mice

<table>
<thead>
<tr>
<th>Series</th>
<th>Inducers</th>
<th>Inhibitors</th>
<th>No. of cultures</th>
<th>Stage of metaplasia</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>dbcAMP, 0.1 mM + PAP + PGE1, PGE2, PGB1</td>
<td>None</td>
<td>37</td>
<td>2.5 ± 0.1*</td>
</tr>
<tr>
<td>2</td>
<td>Retinoid, 1 μM</td>
<td>22</td>
<td>0.4 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Phorbol didecanoate, 1 μM</td>
<td>11</td>
<td>0.5 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Phorbol didecanoate, 10 μM</td>
<td>13</td>
<td>0.4 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>dbcAMP, 1 mM</td>
<td>None</td>
<td>38</td>
<td>3.2 ± 0.1</td>
</tr>
<tr>
<td>6</td>
<td>Retinoid, 1 μM</td>
<td>20</td>
<td>0.2 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Phorbol didecanoate, 1 μM</td>
<td>15</td>
<td>0.7 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Phorbol didecanoate, 10 μM</td>
<td>12</td>
<td>0.6 ± 0.2</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>None</td>
<td>None</td>
<td>25</td>
<td>0.3 ± 0.1</td>
</tr>
<tr>
<td>10</td>
<td>Retinoid, 1 μM</td>
<td>21</td>
<td>0.3 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>Phorbol didecanoate, 10 μM</td>
<td>11</td>
<td>0.5 ± 0.2</td>
<td></td>
</tr>
</tbody>
</table>

* Average ± S.E.
supported by the finding that 10-fold higher concentration of dbcAMP causes considerable squamous metaplasia in the absence of prostaglandins and PAP. The higher concentration is not cytotoxic in prostate gland, unlike the concentration in mouse mammary gland (42).

The induction of squamous metaplasia brought about by the cyclic adenine nucleotide-elevating reagents first becomes morphologically evident as a hyperplasia of preexisting epithelium and then as squamous cell proliferation in foci within the annular structure that outlines the acini of the prostate gland. Keratin production when present is sparse. In contrast, squamous metaplasia caused by the same reagents in cultured mammary gland is abundant and is not restricted to any one structure. Cornification is extensive, often resulting in numerous keratin “pearls” (42). The root of the difference may reside in the nature of the sites of squamous cell proliferation. The prostate acini are surrounded by a relatively thick fibromuscular stroma that may restrain the amount of squamous cell proliferation and keratin deposition. In contrast, the epithelium in mouse mammary gland is relatively unrestricted, being bound by only thin supportive fibrous layer and subjacent fat cells. Alterations in courses of differentiation as a result of a physical constraint on cell conformation have been reported with regard to epithelial cells of mammary gland and skin (7, 14, 16).

Retinoid suppresses the induction of squamous metaplasia in the prostate gland caused by the combination of dbcAMP, PGE₁, PGE₂, and PGB₁, and PAP, and also by a 10-fold higher concentration of dbcAMP alone. Retinoids have previously been found to prevent the squamous metaplasias that result from carcinogen, estrogen, or their combination in prostate gland (Refs. 24 to 26 and 32; this report) and also to prevent and even to reverse squamous metaplasias caused by vitamin A deficiency, mechanical injury, and chemical carcinogens in other organ systems (18, 19, 30, 33, 48). This prevention by retinoid in prostate gland is in contrast to the lack of similar activity thus far with the squamous metaplasia caused by the cyclic adenine nucleotide-elevating reagents in cultured mammary glands (42). Retinylidene dimedone was used because it previously prevented, suppressed, and apparently reversed the procarcinogen-mediated transformation of cultured mammary gland lobuloalveoli to a hormone-independent state (12, 13). The contrasting responses in the 2 glands point to the different susceptibilities of squamous metaplasias to retinoid in different organs.

Phorbol didecanoate also prevents the appearance of squamous metaplasia in the cultured mouse prostate gland. Inhibitions (11, 22, 45, 49), as well as inductions (27, 38, 39, 46), of specific patterns of cell differentiation have been previously elicited by phorbol esters. The mechanisms by which these apparently opposite actions occur are unknown. The reports of the effects of phorbol esters on the levels of intracellular cyclic AMP and prostaglandins provide no clue as to the probable mode of action involved in the prevention of the squamous metaplasia. Phorbol esters inconsistently affect the level of the nucleotide in different systems (15, 17, 31, 36), and they generally elevate, rather than depress, the content of cellular prostaglandins (2, 6, 28, 35, 47, 50). Nevertheless, the findings that both retinoid and phorbol ester can prevent the onset of squamous metaplasia point to the likelihood that the induction of metaplastic squamous differentiation can at least be blocked at the 2 levels at which these inhibitors act.

Cyclic adenine nucleotide, augmented by specific prostaglandins, may be the general physiological inducer of normal and metaplastic squamous cell development in diverse epithelia, followed by a differentiation marked by keratin production. The possibility arises that these agents may mediate the production of the squamous metaplasias that are apparently of spontaneous origin, as well as those brought about by carcinogens and vitamin A deficiency (see “Introduction”). Four supporting examples have now been established, as summarized in Fig. 2: (a) cyclic adenine nucleotide operating synergistically with prostaglandins accelerates normal squamous cell development in cultured embryonic chick skin (43); (b) the identical reagents bring about considerable squamous metaplasia and moderate amount of keratin formation in cultured prostate glands of mice, an aberrant condition in that organ (this report); (c) the same substances acting together are potent inducers of extensive squamous metaplasia and keratin production in cultured whole mammary glands of mice, a markedly abnormal state in that organ (42); (d) the same compounds induce squamous metaplasia and keratinization in human breast tissue (44). Cyclic adenine nucleotide and specific prostaglandins thus accelerate normal squamous cell development and induce squamous metaplasia followed by keratin production in 3 different organs that are derived from embryonic and adult animals and humans, spanning three species and 2 classes of vertebrates.

ACKNOWLEDGMENTS

We thank Gail Nussbaum and Grace Kroetz for technical assistance, Dr. J. Pike and the Upjohn Company for a gift of prostaglandins, and Dr. Michael B. Sporn for providing retinylidene dimedone, which was synthesized by Drs. Nancy Acton and Arnold Brossi, all of the National Cancer Institute.

REFERENCES


Fig. 1. Induction of squamous metaplasia in cultured prostate gland of the mouse. Glands were cultured for 3 weeks, fixed, dehydrated, embedded, sectioned at 5 μm, and stained with hematoxylin and eosin. The data provided in the text are from 6- to 8-week-old BALB/c mouse B6 control prostate gland cultured for 3 weeks in the absence of squamous metaplasia-inducing supplements; C, prostate gland cultured for 3 weeks in the presence of dbcAMP (1 mm) and retinylidene dimer-dione (1 μM).

Fig. 2. Induction of squamous metaplasia and keratin production in 4 organs by cyclic adenosine nucleotide, PGE_2, PGE_3, PGB_2, and PAP.
Fig. 1—A to D

- chick embryo skin
- mouse prostate gland
- mouse mammary gland
- human breast

accelerated normal development

squamous metaplasia

Fig. 2
General Process of Induction of Squamous Metaplasia by Cyclic Adenine Nucleotide and Prostaglandins: Mouse Prostate Glands

Frederick V. Schaefer, R. Philip Custer and Sam Sorof


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