Squamous Metaplasia in Human Breast Culture: Induction by Cyclic Adenine Nucleotide and Prostaglandins, and Influence of Menstrual Cycle

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

The Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia, Pennsylvania 19111

ABSTRACT

Cultured mammary epithelium has been set on a course of abnormal cellular development leading to squamous metaplasia and subsequent differentiation marked by abundant production of keratin. In a study involving 29 women, the mixture of the cyclic adenine nucleotide, dibutyryl cyclic adenosine 3':5'-monophosphate (0.1 mM), prostaglandins E, E2, and B1 (each 5 μg/ml), and the phosphodiesterase inhibitor, papaverine (1 μM), induced an extensive squamous metaplasia and excessive keratin production, along with considerable hyperplasia of the normal epithelium, in nonmalignant breast tissues maintained in organ culture for 10 days. These groups of reagents are known to elevate the level of intracellular cyclic adenosine 3':5'-monophosphate in other systems. The three types of inducers acted synergistically. Individually at the above concentrations, they had little or no activity, but their combination was highly active. A 10-fold-higher concentration of dibutyryl cyclic adenosine 3':5'-monophosphate (1 mM) by itself brought about as great a degree of squamous metaplasia and keratinization with hyperplasia as did the complete mixture. Cyclic adenine nucleotide may therefore be the primary mediator of the induction, with the prostaglandins and papaverine operating indirectly through the nucleotide. The stage of the menstrual cycle at the time of the breast biopsy strongly influenced the responsiveness to the inducers. The mammary glands were most resistant during the first 10 days of the menstrual cycle, very susceptible during the final 10 days of the cycle, and intermediate-ly sensitive after menopause. The induction process was strongly inhibited by the presence of either the phorbol ester, phorbol 12,13-didecanoate, at 10 μM, or the retinoid, retinylidenedimethonate, at 1 μM, throughout the culture period of 10 days. The results are consistent with the possibility that cyclic adenosine nucleotide and, indirectly, certain prostaglandins may exert important roles in the spontaneous induction of squamous metaplasia in human breast. These same inducers have also been found recently to bring about squamous metaplasia and keratinization in cultures of mammary glands and prostate glands of mice following methylicholanthrene treatment (10, 22, 23, 52) or 7,12-dimethylbenz(a)anthracene feeding (31). Squamous metaplasia in human mammary tissue can occur in organ culture (2, 12, 55), notably as a result of high concentrations of insulin (4, 11). The lesion is also caused by chemical carcinogens in vitro in mammary glands (54) and prostate glands (25) of mice and in tracheas of hamsters (17).

We described recently the induction of extensive squamous metaplasia with accompanying keratinization in mammary glands and in prostate glands of mice (46, 49) and the enhancement of normal epidermoid development in the skin of chick embryos (47), all caused in organ culture by exposure to the combination of dbcAMP,4 PAP, and PGE1, PGE2, and PGB1. These groups of substances elevate the level of intracellular cyclic adenosine 3':5'-monophosphate in other systems (see 'Discussion'). The present study therefore set out to determine whether these reagents, separately or in combination, can induce aberrant epithelial developmental and differentiation leading to squamous metaplasia and abundant keratin production in mammary tissues obtained from women in different menstrual states and also to examine agents that prevent these abnormal conditions.

INTRODUCTION

Squamous metaplasia is a common abnormality of cellular development and differentiation in which various epithelia are replaced by cells resembling those of the epidermis. The metaplastic cells differentiate into flattened cells that frequently form keratotic flakes and whorls. The lesion often results in a reduction or loss of normal functions. The cells may regress, remain benignly squamous, or progress to epidermoid carcinoma (1).

Squamous metaplasia in the human breast occurs in ‘‘cystic mastitis complex,’’ fibroadenoma, and other states (8, 18, 43). In addition, squamous differentiation in mammary ductal carcinoma, first described in 1838 (37), has been reported to be ‘‘the most common metaplastic variant in human breast cancer’’ (29). The incidence ranges from 0.5 to 2.0% (13). Pure squamous carcinoma of the human breast is rare (3). However, squamous metaplasia with keratinization is prevalent in preneoplastic and neoplastic mammary glands of mice following methylicholanthrene treatment (10, 22, 23, 52) or 7,12-dimethylbenz(a)anthracene feeding (31). Squamous metaplasia in human mammary tissue can occur in organ culture (2, 12, 55), notably as a result of high concentrations of insulin (4, 11). The lesion is also caused by chemical carcinogens in vitro in mammary glands (54) and prostate glands (25) of mice and in tracheas of hamsters (17).

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The abbreviations used are: dbcAMP, N6,O4'-dibutyryl cyclic adenosine 3':5'-monophosphate; PAP, papaverine; PGE1, prostaglandin E1; PGE2, prostaglandin E2; PGB1, prostaglandin B1; dbcGMP, N6,O4'-dibutyryl cyclic guanosine 3':5'-monophosphate; PDD, phorbol-12,13-didecanoate; PGE, prostaglandin E.
Reagents. Waymouth MB752/1 culture medium and potassium Penicillin G were purchased from Grand Island Biological Co., Grand Island, N. Y. DbcAMP, PAP, dbcGMP, 5'-adenylic acid, sodium butyrate, streptomycin sulfate, β-estradiol, and progesterone were obtained from Sigma Chemical Co., St. Louis, Mo. PDD was purchased from CCR, Inc., Eden Prairie, Minn. PGE1, PGE2, and PGB2 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 dimerdone), was synthesized by Dr. Nancy Acton and Dr. Arnold Brosi and was kindly provided by Dr. Michael B. Sporn, all of the National Cancer Institute.

Culturing and Staining of Breast Tissue. Samples of nonmalignant breast tissues (0.5 to 1 cm in each dimension) were obtained from regions adjacent to benign lumps being excised. The specimens were placed in sterile serum-free Waymouth MB752/1 medium and transferred to the laboratory. The time interval between excision and culturing was generally less than 30 min. In order to reduce the possibility of local tissue variation, the samples were divided so that adjacent slices (5 to 10 x 2 x 2 mm) were used in matching control and test cultures. Individual slices were floated on Dacron rafts in 2 ml of serum-free Waymouth MB752/1 medium containing insulin (5 μg/ml), L-glutamine (350 μg/ml), potassium penicillin G (35 μg/ml), and indicated supplements in separate 35-mm plastic tissue culture dishes. All cultures, including those without additives (controls), contained 0.3% ethanol that was solvent for the prostaglandins, PAP, retinoid, and PDD. The tissue slices were incubated at 37° in a water-saturated atmosphere of 95% O2 and 5% CO2. Media were changed every 2 or 3 days. Cultures were terminated at the end of 10 days. The fragments were then fixed in acetic acid:ethanol (1:3, v/v), stained with alun-carmine for visualization during processing in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin for histological analysis and scoring of squamous metaplasia and keratinization.

Scoring of Squamous Metaplasia and Keratinization. The histological sections of cultured mammary tissues contained lobules of up to 50 small acini and occasional intervening ducts. The extent of squamous metaplasia with or without keratinization was scored on the following scale of 0 to 4, according to criteria typified in Fig. 1 and its legend. A score of 0 denoted no detectable squamous metaplasia and keratinization. A value of 1 indicated very limited squamous metaplasia at one to 3 sites. Tissues with numerous foci of metaplasia without morphologically visible keratinization were scored as 2. Squamous metaplasia with numerous regions of early but morphologically visible keratinization were scored as 3. Considerable squamous metaplasia and widespread occurrence of keratinization was scored as 4. The presence of excessive keratin in representative sections was verified histochemically by use of the Masson trichrome stain (bright red color). The scores of tissue slices that were subjected to identical protocols were expressed as averages of their integral values with standard errors of the means. Each tissue section was evaluated independently by the same investigator, almost always without foreknowledge of the donors and previous treatments of the cultures.

RESULTS

Breast Tissue Donors. The following types of information were obtained from each donor of mammary tissue: age; pregnancies; number of children that were breast fed; status with regard to menopause; duration of usual menstrual cycle; interval between breast surgery and onset of last menstrual period; history of drug and hormonal treatments; and history of breast disease. The information was obtained and recorded by an intermediary, who had no knowledge of the experimental protocols and results. The interviews occurred almost always within 2 days after the breast lumpectomies and never more than 10 days thereafter. Interviewing of patients at least prior to surgery has been reported to be "a reasonable means of obtaining menstrual dates based on comparison" (56). In support of the accuracy of the information obtained at our interviews, we found unequivocal agreement between the menstrual states thus determined and the histological characters of the breast tissues of 6 premenopausal women whose samples provided adequate mammary lobules for evaluation of their menstrual phases according to reported criteria (56).

Of the 29 women in the present study, 18 were premenopausal, with menstrual cycles ranging from 26 to 31 days in duration, 3 were perimenopausal, and 8 were postmenopausal. In the data presented below, no significant correlation (Mann-Whitney and Spearman tests) was found between the extent of induction of squamous metaplasia and various combinations of numbers of pregnancies and breast-fed children. There was insufficient group size of similar drug or hormonal usage and family history to allow statistical analysis.

Breast Diagnoses. The mammary tissues of 27 of the 29 women were either normal or fibrocystic on the basis of histological examinations. Benign fibroadenomas were present in the surgical samples of 2 premenopausal women. However, all samples that were cultured were diagnosed as normal or fibrocystic. Limited hyperplasia was evident in certain of the fibrocystic tissues.

Induction of Squamous Metaplasia and Keratinization in Culture at Different Menstrual States of Women. The degree to which squamous metaplasia and keratinization were induced in culture was markedly dependent on the menstrual states of the women at the time of the surgery. The breast tissues were evaluated after 10 days of culturing. The samples were then viable and morphologically intact, as attested to by Figs. 1 and 2, and by their differential responsiveness to specific inducers of squamous metaplasia (below). Even after 3.5 weeks, there was only minimal morphological cell degradation in the control cultures that contained the basal serum-free medium of Waymouth MB752/1, insulin, L-glutamine, potassium penicillin G, and ethanol as solvent (not shown). After 10 days in culture, such control tissues from all women were completely free of squamous metaplasia and keratinization. All yielded scores of 0 (Table 1, Series 1, 3, 5, 7, and 9). The normal ductular structure in a cultured control tissue is shown in Fig. 2A. However, when the breast tissues were incubated in the presence of dbcAMP at 0.1 mM, PAP at 1 μM, and PGE1, PGE2, and PGB2, each at 5 μg/ml, there was an intermediate amount of squamous metaplasia (score, 2.1 ± 0.3) when the 29 women were considered collectively (Series 2). There was no significant difference in this regard between the averages of all of the 18 premenopausal and all of the 8 postmenopausal women (Series 4 and 10). Their respective ratings of 2.2 ± 0.3 and 1.9 ± 0.5 indicate that their breast tissues on average were intermediate in their responses. However, when the data on the premenopausal women were segregated according to their stages in the menstrual cycle, there were marked differences. The breast tissues of the women in the first 10 days of the cycle were resistant to the induction process. Their average score was only 0.8 ± 0.2 (Series 6). In contrast, the mammary tissues from women in the last 10 days of their cycle were induced to near maximal squamous metaplasia and keratinization.
Squamous Metaplasia in Human Breast Cultures

Slices of breast tissues from women in indicated menstrual states were incubated in Waymouth MB752/1 medium containing insulin (5 μg/ml), l-glutamine (350 μg/ml), and potassium Penicillin G (35 μg/ml), with or without the indicated supplements of dbcAMP at 0.1 mM, PAP at 1 μM, and PGE1, PGE2, and PGB1, each at 5 μg/ml. All cultures contained 0.3% ethanol that was solvent for the prostaglandins and PAP. At the end of 10 days in culture, the tissues were processed and scored for squamous metaplasia and keratinization on a scale of 0 to 4. Details are in the text. The group consisted of 18 premenopausal, 3 perimenopausal, and 8 postmenopausal women.

<table>
<thead>
<tr>
<th>Series</th>
<th>Menstrual states</th>
<th>Culture supplements</th>
<th>No. of women</th>
<th>State of metaplasia</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>All</td>
<td>None</td>
<td>29</td>
<td>0±0.1</td>
</tr>
<tr>
<td>2</td>
<td>Premenopausal, all</td>
<td>dbcAMP + PAP + PGE1, PGE2, PGB1</td>
<td>29</td>
<td>2.1 ± 0.30</td>
</tr>
<tr>
<td>3</td>
<td>Premenopausal, Days 1–10</td>
<td>None</td>
<td>18</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>Premenopausal, final 10 days</td>
<td>dbcAMP + PAP + PGE1, PGE2, PGB1</td>
<td>18</td>
<td>2.2 ± 0.30</td>
</tr>
<tr>
<td>5</td>
<td>Premenopausal, Days 1–10</td>
<td>None</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>Premenopausal, final 10 days</td>
<td>dbcAMP + PAP + PGE1, PGE2, PGB1</td>
<td>8</td>
<td>0.8 ± 0.2</td>
</tr>
<tr>
<td>7</td>
<td>Premenopausal, final 10 days</td>
<td>None</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>Premenopausal, final 10 days</td>
<td>dbcAMP + PAP + PGE1, PGE2, PGB1</td>
<td>10</td>
<td>3.4 ± 0.1</td>
</tr>
<tr>
<td>9</td>
<td>Postmenopausal</td>
<td>None</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>Postmenopausal</td>
<td>dbcAMP + PAP + PGE1, PGE2, PGB1</td>
<td>8</td>
<td>1.9 ± 0.5</td>
</tr>
</tbody>
</table>

* Average of the integral scores.  
* Mean ± S.E.

zation, yielding an average score of 3.4 ± 0.1 (Series 8). The last 2 groups were clearly different according to the Mann-Whitney 2-sided test (p = 1.2 × 10⁻⁵) and the Spearman test (p = 0.01). Fig. 2B shows a nodule of squamous metaplasia (Grade 4) with associated excessive keratin in maximally induced breast tissue that had been excised late in the menstrual cycle. Thus, the breast tissues of women in the last 10 days of the menstrual cycle are markedly resistant to induction of squamous metaplasia and keratinization by the combination of dbcAMP, PAP, and PGE1, PGE2, and PGB1. In contrast, the mammary glands of women in the last 10 days of the menstrual cycle are especially susceptible to the process.

Synergy of the Chemical Inducers of Squamous Metaplasia and Keratinization. The 3 types of inducers acted synergistically to induce squamous metaplasia and keratinization in the cultured mammary tissues. The individual groups at their standard concentrations (above) had little or no activity. How ever, their combination was highly active. The prostaglandins and the PAP also had little or no activity, as indicated by a score of 0.5 ± 0.2 (Series 6). Thus, it is evident that the presence of PGE1, PGE2, PGB1, and PAP considerably enhances the induction of squamous metaplasia and production of keratin in the cultured breast tissues when dbcAMP is at the standard concentration of 0.1 mM.

Primary Role of Cyclic Adenine Nucleotide in the Induction of Squamous Metaplasia and Keratinization. Cyclic adenosine nucleotide may be the primary mediator of the induction. Tenfold-concentrated dbcAMP at 1 mM by itself was sufficient to bring about as high a degree of metaplasia (score, 3.0 ± 0.2) as was achieved by the complete inducing mixture (Table 2, Series 4 versus Series 2; Fig. 1, B to F). The data thus indicate that the prostaglandins and the PAP may mediate their effects indirectly through the cyclic adenosine nucleotide. The specificity of the effect of the cyclic adenosine nucleotide was indicated by the lack of response with dbcGMP, AMP, and sodium butyrate at concentrations equivalent to that of the dbcAMP in the standard mixture (Series 8 to 10). In addition, dbcGMP at 1 mM has thus far had no significant activity (score, 0, 2 women; not in Table 2).

Epithelial Cell Proliferation and Squamous Metaplasia. The induction of squamous metaplasia appears to be associated with normal epithelial cell proliferation. The cultured mammary tissues induced breast tissue that had been excised late in the menstrual cycle. At the end of 10 days in culture, the tissues were processed and scored for squamous metaplasia and keratinization on a scale of 0 to 4. Details are in the text. The group consisted of 18 premenopausal, 3 perimenopausal, and 8 postmenopausal women.

Table 1
Effect of menstrual state in the induction of squamous metaplasia in cultured breast tissues of women

<table>
<thead>
<tr>
<th>Series</th>
<th>Menstrual states</th>
<th>Culture supplements</th>
<th>No. of women</th>
<th>State of metaplasia</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>All</td>
<td>None</td>
<td>29</td>
<td>0±0.1</td>
</tr>
<tr>
<td>2</td>
<td>Premenopausal, all</td>
<td>dbcAMP + PAP + PGE1, PGE2, PGB1</td>
<td>29</td>
<td>2.1 ± 0.30</td>
</tr>
<tr>
<td>3</td>
<td>Premenopausal, Days 1–10</td>
<td>None</td>
<td>18</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>Premenopausal, final 10 days</td>
<td>dbcAMP + PAP + PGE1, PGE2, PGB1</td>
<td>18</td>
<td>2.2 ± 0.30</td>
</tr>
<tr>
<td>5</td>
<td>Premenopausal, Days 1–10</td>
<td>None</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>6</td>
<td>Premenopausal, final 10 days</td>
<td>dbcAMP + PAP + PGE1, PGE2, PGB1</td>
<td>8</td>
<td>0.8 ± 0.2</td>
</tr>
<tr>
<td>7</td>
<td>Premenopausal, final 10 days</td>
<td>None</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>8</td>
<td>Premenopausal, final 10 days</td>
<td>dbcAMP + PAP + PGE1, PGE2, PGB1</td>
<td>10</td>
<td>3.4 ± 0.1</td>
</tr>
<tr>
<td>9</td>
<td>Postmenopausal</td>
<td>None</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>Postmenopausal</td>
<td>dbcAMP + PAP + PGE1, PGE2, PGB1</td>
<td>8</td>
<td>1.9 ± 0.5</td>
</tr>
</tbody>
</table>

* Average of the integral scores.  
* Mean ± S.E.

Table 2
Synergy of chemical inducers and prevention of squamous metaplasia in cultured breast tissues of women

<table>
<thead>
<tr>
<th>Series</th>
<th>Culture supplements</th>
<th>No. of women</th>
<th>Stage of metaplasia</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>None</td>
<td>12</td>
<td>0±0.1</td>
</tr>
<tr>
<td>2</td>
<td>dbcAMP + PAP + PGE1, PGE2, PGB1</td>
<td>12</td>
<td>3.1 ± 0.20</td>
</tr>
<tr>
<td>3</td>
<td>dbcAMP</td>
<td>6</td>
<td>0.8 ± 0.2</td>
</tr>
<tr>
<td>4</td>
<td>dbcAMP, 1 mM (10x)</td>
<td>8</td>
<td>3.0 ± 0.2</td>
</tr>
<tr>
<td>5</td>
<td>PAP</td>
<td>5</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>6</td>
<td>dbcAMP + PAP</td>
<td>6</td>
<td>0.5 ± 0.2</td>
</tr>
<tr>
<td>7</td>
<td>PGE1, PGE2, PGB1</td>
<td>7</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>8</td>
<td>dbcGMP</td>
<td>7</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>9</td>
<td>AMP</td>
<td>8</td>
<td>0.1 ± 0.1</td>
</tr>
<tr>
<td>10</td>
<td>Sodium butyrate</td>
<td>6</td>
<td>0.2 ± 0.1</td>
</tr>
<tr>
<td>11</td>
<td>dbcAMP + PAP + PGE1, PGE2, PGB1 + PDD</td>
<td>6</td>
<td>0.8 ± 0.2</td>
</tr>
<tr>
<td>12</td>
<td>dbcAMP + PAP + PGE1, PGE2, PGB1 + retinoid</td>
<td>6</td>
<td>0.9 ± 0.5</td>
</tr>
</tbody>
</table>

* Average of the integral scores.  
* Mean ± S.E.
tissues from 11 women were selected for their near maximal responses to inducers of squamous metaplasia with keratinization (scores, 3 and 4). The group consisted of both premenopausal women in early and late stages of their menstrual cycles and postmenopausal women. At the end of the 10 days in culture, the epithelia were examined for manifestations of ductal and/or alveolar hyperplasia and cells in mitosis. The control cultures, which had been exposed to insulin (5 μg/ml) as the sole supplement, showed the consequences of only slight cell proliferation (Figs. 1A and 2A). In contrast, the normal epithelium in the tissues treated with either dbcAMP at 10^{-3} M or the mixture of dbcAMP (10^{-4} M), PAP, and PGE_{1}, PGE_{2}, and PGB_{1}, was considerably hyperplastic (Fig. 1, B, C, and F). However, virtually no mitoses were present in either the control or the induced epithelia at the end of the 10-day period.

**Prevention of Squamous Metaplasia and Keratinization.**

Even though the mammary tissues were exposed to the complete inducing mixture during the 10 days in culture, the presence of either PDD at 10 μM or retinylidene dimedone at 1 μM throughout that period strongly inhibited the abnormal pathway of development and differentiation. Whereas the induced cultures had an average score of 3.1 ± 0.2, the inhibited cultures had scores of 0.8 ± 0.2 and 0.9 ± 0.5 (Table 2, Series 1 and 2 versus Series 2). The differences are clearly significant according to the 2-sided Mann-Whitney test (p = 0.0006 and 0.0023, respectively). Stained histological sections of retinoid-inhibited and PDD-inhibited breast tissues are shown in Fig. 2, C and D. Thus, the induction of squamous metaplasia and keratinization can be blocked by either phorbol ester or retinoid.

**DISCUSSION**

Human mammary epithelium can be set on a course of abnormal cellular development leading to squamous metaplasia and subsequent aberrant differentiation marked by excessive production of keratin. These lesions are brought about in organ culture by the cyclic adenine nucleotide, dbcAMP, aided by PGE_{1}, PGE_{2}, PGB_{1}, and PAP. The sensitivity of the mammary tissue to the induction process is governed by the menstrual cycle. Unknown factors also appear to play a role, as is indicated by the considerable individual variations and greater average susceptibility of the mammary epithelium of the postmenopausal women than of the premenopausal women in the first 10 days of their menstrual cycles. Thus far, we have not observed a direct enhancing effect of progesterone and/or estradiol when added to cultures of breast tissues from 4 women that were early in their menstrual cycles (data not presented), permitting the possibility that in vivo factors may be additionally required. The cyclic variations in the serum levels of follicle-stimulating hormone, luteinizing hormone, thyroid-stimulating hormone, growth hormone, and adenocorticotrophic hormone do not appear to correlate in time (9, 24, 50) with the observed differences in susceptibility to the induction of squamous metaplasia. Further, the concentration of PGE in serum is unaltered during progression of the normal menstrual cycle (21, 40).

The induction of squamous metaplasia in human mammary epithelium was accompanied by hyperplasia of the normal columnar type. The mammary tissues that were induced to squamous metaplasia and keratinization by either cyclic adenine nucleotide alone or by cyclic adenosine nucleotide, prostaglandins, and PAP displayed considerable ductal and alveolar hyperplasia in the presence of a low concentration of insulin (5 μg/ml) over the 10-day period in culture. In contrast, the control cultures that were exposed solely to the insulin underwent little cell proliferation. Cell mitoses were virtually absent in both at the end of the 10 days. In agreement, thymidine is incorporated maximally between Days 1 and 2, and essentially none at Day 10, in mouse mammary glands cultured with the inducers and insulin; control cultures incorporate much less throughout the entire period. Further, the present observed lack of cell proliferation and squamous metaplasia in the control human mammary cultures is in accord with previous reports of the inactivity of low concentrations of insulin in this regard. High concentrations of insulin (>10 μg/ml) were required to bring about hyperplasia, squamous metaplasia, and keratinization (11). Worthy of study is the question whether high concentrations of insulin cause these reported effects by means of an elevation in the level of intracellular cyclic adenosine 3':5'-monophosphate.

Retinoid blocked the induction of squamous metaplasia and marked keratinization by the combination of the dbcAMP, PGE_{1}, PGE_{2}, PGB_{1}, and PAP in the human mammary tissues. Retinoids have been demonstrated previously to inhibit the production of squamous metaplasias that result from carcinogen, estrogen, or their combination in prostate glands of mice (46). In addition, estradiol promotes the induction of squamous metaplasia in prostate tissue of rats (5). Mammary cell proliferation, which is brought about by these steroid hormones (19), may perhaps facilitate the induction process. Unknown factors also appear to play a role, as is indicated by the considerable individual variations and greater average susceptibility of the mammary epithelium of the postmenopausal women than of the premenopausal women in the first 10 days of their menstrual cycles. Thus far, we have not observed a direct enhancing effect of progesterone and/or estradiol when added to cultures of breast tissues from 4 women that were early in their menstrual cycles (data not presented), permitting the possibility that in vivo factors may be additionally required. The cyclic variations in the serum levels of follicle-stimulating hormone, luteinizing hormone, thyroid-stimulating hormone, growth hormone, and adenocorticotrophic hormone do not appear to correlate in time (9, 24, 50) with the observed differences in susceptibility to the induction of squamous metaplasia. Further, the concentration of PGE in serum is unaltered during progression of the normal menstrual cycle (21, 40).

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*F. V. Schaefer, R. P. Custer, and S. Sorof, unpublished studies.*
metaplasias caused by vitamin A deficiency, mechanical injury, and chemical carcinogens in other organ and animal systems (16, 17, 33, 36, 57). Retinoid also blocks the induction of squamous metaplasia and keratinization by the cyclic adenosine nucleotide-elevating reagents in cultured prostate glands of mice (49) but surprisingly is not preventive in mammary glands of mice (48). These contrasting responses, especially in the mammary glands of mice and women, point to the different susceptibilities of squamous metaplasias to retinoid in diverse organs and species.

The phorbol ester, PDD, also prevented the induction of squamous metaplasia and keratinization in the cultured breast tissues. Similar prevention has been achieved in mouse prostate glands (49). Inhibitions (7, 20, 51, 58) as well as inductions (28, 41, 42, 53) of cell differentiation have been elicited by phorbol esters by mechanisms that are unknown. Phorbol esters consistently alter the level of intracellular cyclic adenosine 3':5'-monophosphate in different tissues (14, 15, 34, 38) and hence provide no clue as to their action in the present study. Nevertheless, as with prostate glands of mice (49), the findings that both retinoid and phorbol ester block the induction of squamous metaplasia and keratinization indicate that the process can be interrupted at the presumed 2 different levels at which these inhibitors act.

The 3 types of squamous metaplasia-inducing reagents (cyclic adenosine nucleotide, specific prostaglandins, and PAP) are able in certain systems to elevate the level of intracellular cyclic adenosine 3':5'-monophosphate (a) by the entry of exogenous dbcAMP, (b) through the stimulation of adenyl cyclase by the prostaglandins (44, 45), and (c) through the inhibition by PAP of the degradation of the nucleotide by phosphodiesterases (39). The possibility that the induction of squamous metaplasia in human mammary gland may be the result of an increased level of intracellular cyclic adenosine 3':5'-monophosphate is supported by the finding that the 10-fold-higher concentration of dbcAMP alone caused as much squamous metaplasia and keratinization as did the complete mixture. This higher concentration of dbcAMP alone is also very active in induction of squamous metaplasia in mouse prostate glands (49) but is cytotoxic in mouse mammary glands (46). It is noteworthy that elevated levels of intracellular cyclic adenosine 3':5'-monophosphate and cyclic guanosine 3':5'-monophosphate resulting from dietary methylxanthines have also been associated with the development of fibrocystic disease in breasts of humans (32).

Cyclic adenosine nucleotide, acting in concert with specific prostaglandins (probably excluding PGB1), may be a physiological inducer of normal and metaplastic squamous cell development and accompanying differentiation marked by excessive keratinization in various types of epithelia. It seems reasonable to speculate that these agents may mediate the production of squamous metaplasia that are apparently of spontaneous origin in many organs, as well as such lesions brought about by carcinogens (6, 17, 27, 35, 54), vitamin A deficiency (57), estrogens (5, 8), and chronic mechanical injury (30, 33). Evidence in 4 systems now supports a general role of cyclic adenosine nucleotide and prostaglandins in the induction of squamous metaplasia and excessive keratin production in different organs. (a) Cyclic adenosine nucleotide acting synergistically with the prostaglandins accelerates normal squamous cell development in cultured embryonic chick skin (47). (b) The same reagents cause considerable squamous metaplasia and a moderate production of keratin in cultured prostate glands of mice (49). (c) These substances are also potent inducers of extensive squamous metaplasia and keratinization in cultured whole mammary glands of mice (46). (d) The same compounds bring about squamous metaplasia and formation of keratin squames in the cultured breast tissues of women (this report). Thus, cyclic adenosine nucleotide, assisted by specific prostaglandins, accelerates normal squamous cell development and also induces squamous metaplasia and excessive production of keratin in different organs of embryonic and adult animals, including humans.

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Fig. 1. Histological grades of squamous metaplasia and keratinization in human mammary epithelium. All breast tissues were cultured for 10 days in the presence of insulin (5 µg/ml) as the only supplement (control) (A) or with insulin and dbcAMP at 10^{-3} M. Ethanol (0.3%) as solvent was present in all cultures. Squamous metaplasia and keratinization induced by the nucleotide are demonstrated (B to F). The corresponding control cultures of the same breast samples displayed none of these characteristics, as in A. The original columnar epithelium lining the lumina in B, C, and F is hyperplastic. Paraffin sections, 5 µm. A to D and F, × 400; E, × 80. Details are provided in the text. A, Grade 0. Normal mammary architecture with typical columnar stratified epithelium arranged in numerous alveoli and supported by loose intralobular stroma. No evidence of squamous metaplasia, keratinization, or proliferation is detectable (premenopausal 48-year-old woman biopsied at 13 days into menstrual cycle). B, Grade 1. Mammary epithelium containing limited squamous metaplasia throughout the entire histological section. No keratinization is detectable. Two foci of squamous metaplasia are shown at the basilar level (arrows) (premenopausal 35-year-old woman biopsied at 10 days into menstrual cycle). C, Grade 2. Mammary epithelium containing numerous regions of squamous metaplastic cells without evident keratinization throughout the histological section. Most cells in this panel are metaplastic squamous type (premenopausal 42-year-old woman biopsied at 16 days into menstrual cycle). D, Grade 3. Mammary epithelium containing numerous regions of squamous metaplastic cells and extracellular keratin flakes (arrows) (postmenopausal 52-year-old woman). E and F, Grade 4. Mammary duct lined by columnar epithelium with a well-defined substratum of squamous metaplasia. E and an enlargement (F) of the outlined portion demonstrate extensive squamous metaplasia with intracellular keratosis in the basal level. Similar alterations in alveolar epithelium could also be shown in other fields (postmenopausal 62-year-old woman).
Fig. 2. Induction and prevention of squamous metaplasia and of excessive production of keratin in cultured mammary epithelium of women. The specimens of A and B were from a 32-year-old woman in the 19th day of a 28-day cycle. The tissues of C and D were from a 38-year-old woman in the 22nd day of a 30-day cycle. The normal epithelium in all panels is slightly hyperplastic. The tissues were cultured for 10 days, fixed, dehydrated, embedded in paraffin, and sectioned at 5 μm. H & E, ×320. Details are provided in the text. A, control mammary tissue cultured in medium containing insulin (5 μg/ml) in the absence of squamous metaplasia-inducing reagents. The tissue is essentially normal in appearance. B, breast tissue cultured in medium containing dbcAMP (0.1 mM), PAP (1 μM), and PGE1, PGE2, PGB2, and insulin (each 5 μg/ml). Squamous metaplasia and excessive keratin are evident, with a score of 4. The lesion intrudes into the lumen of a ductule, surface columnar epithelium having exfoliated. C, mammary tissue cultured in medium containing the squamous metaplasia-inducing reagents (as for B) and retinylidene dinedone at 1 μM. Squamous metaplasia and keratinization are absent, as in A. D, breast tissue cultured in medium containing the squamous metaplasia-inducing reagents (as for B) and PDD at 10 μM. The tissue is normal in appearance, as in A and C.
Squamous Metaplasia in Human Breast Culture: Induction by Cyclic Adenine Nucleotide and Prostaglandins, and Influence of Menstrual Cycle

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


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