Nononcogenic Hormone-independent Alveoli Produced by Carcinogens in Cultured Mouse Mammary Glands

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ABSTRACT

Treatment of mouse mammary glands with a high concentration of 7,12-dimethylbenzo(a)anthracene in whole organ culture was reported by Banerjee et al. to transform foci of lobuloalveolar to a hormone-independent state, and to give rise to mammary hyperplastic outgrowths and adenocarcinomas in vivo. In the present study using the identical system, mammary glands of BALB/c mice were exposed to 7,12-dimethylbenzo(a)anthracene or N-2-fluorenylacetamide at low concentrations that bring about maximal incidences of the hormone-independent hyperplastic lobuloalveolar lesions with minimal cytotoxicity. After morphological development of the lobuloalveolar in culture, the glands were enzymatically dissociated into cells and inoculated into gland-free inguinal mammary fat pads of syngeneic mice bearing pituitary gland implants during the initial 8 weeks. After 11 months, fragments of the resultant mammary outgrowths from each mouse were implanted into the gland-free inguinal mammary fat pads of 3 syngeneic mice (not bearing pituitary gland supplements) and were permitted to grow for another 11 months. Mammary outgrowths from the primary and secondary implants were neither neoplastic, anaplastic, nor dysplastic. Also, no hyperplasia in any mammary outgrowth could be attributed to the action of either carcinogen, especially when outgrowths were compared with contralateral outgrowths that arose from the control glands exposed to dimethyl sulfoxide (solvent of the carcinogens) in culture and/or with untreated thoracic mammary glands of the same hosts. One interpretation of these findings is that the hormone-independent, hyperplastic alveolar lesions may not be an appropriate in vitro marker of oncogenic transformation by chemical carcinogens in culture. The great variety of procarcinogens and activated carcinogens that bring about this lesion in vitro and its morphological similarity to presumptive mammary preneoplastic lesions in vivo weigh against this interpretation. A second hypothesis is that high concentrations of procarcinogens, despite their considerable cytotoxicity, complete a multistep process of oncogenic transformation in surviving mammary epithelium, whereas low concentrations optimized to produce the lesions in maximal number do not.

INTRODUCTION

Chemical carcinogens induce a phenotypic alteration (transformation) in which there is a focal loss of hormonal dependency of the growth of lobuloalveolar epithelium of mouse mammary glands in whole organ culture (2, 7, 8, 12, 19). The carcinogen-altered foci are manifest as fully developed alveolar nodules in selective hormone-deficient medium in which the transformed mammary gland epithelium was further indicated in previous studies with retinoids. Retinoids antipromotionally prevented, suppressed, and apparently reversed the appearance of the lesions brought about by relatively low concentrations of procarcinogens, e.g., 10 nM DMBA and 1 μM FAA (7, 8). In contrast, retinoids did not prevent the otherwise similar transformations caused by 100-fold higher concentrations of these same procarcinogens, or by low concentrations of activated and direct-acting carcinogens (8). Whether this inability is a reflection of a more advanced state of the carcinogen-induced phenotypic alteration in the neoplastic process is not known. Therefore, the present investigation involving approximately 1300 mice was undertaken to determine whether mammary gland transformation under conditions of minimal cytotoxicity in vitro gives rise to mammary hyperplasia and tumors after implantation into mice.

MATERIALS AND METHODS

Carcinogens. Commercially obtained DMBA (Eastman Kodak Co., Rochester, N. Y.) was further purified by crystallization from absolute ethanol. The product was assayed using a high-pressure liquid chromatographic apparatus (Waters Associates, Inc., Milford, Mass.) and a μporasil C18 column that was pre-equilibrated with 50% methanol and high-purity water and eluted with a 50 to 100% methanol gradient. The DMBA was found to be >99% pure, and it chromatographically matched a standard DMBA sample obtained from the Chemical Repository of the National Cancer Institute Carcinogenesis Research Program.

FAA was indicated by the vendor (Fluka Chemical Co., Buchs, Switzerland) to be >99% pure. In our laboratory, it melted at 191–192° and migrated as a single spot in thin-layer chromatography on activated silica gel containing fluorescent indicator (Eastman Kodak Co.) in chloroform:methanol (97:3) and benzene:ethyl acetate (1:1).

Reagents were purchased from sources indicated previously (7, 19). to as the production of nodular alveolar lesions that result from the escape from normal hormonal controls of alveolar development in mouse mammary gland in whole organ culture (7, 8, 19).
Culturing and Phenotypic Conversion of Whole Mammary Glands. Details of the procedure of culturing whole mammary glands of mice have been described by Banerjee et al. (1) and by us (7, 19). Briefly, both second thoracic mammary glands of in-house bred, virgin female, estrogen- and progesterone-primed BALB/c mice were floated on Dacron rafts in the same tissue culture plastic dish containing 2 ml of medium without serum. The medium consisted of Waymouth MB752/1 supplemented with L-glutamine (350 µg/ml), potassium penicillin G (35 µg/ml), and appropriate steroid and polypeptide hormones (below). Cultures were maintained at 37°C in a water-saturated atmosphere of 95% oxygen and 5% carbon dioxide.

Culturing of the second thoracic mammary gland in the above medium with insulin, prolactin, aldosterone, and hydrocortisone, each at 5 µg/ml (development medium), for 10 days (Days 1 to 10) results in full lobuloalveolar development and functional differentiation (9, 17, 19, 20, 22). On the third day, the culture fluid was replaced with medium containing 0.1% DMSO (v/v) with or without (control) the carcinogen, DMBA or FAA, at various concentrations in experiments that scored for mammary gland conversion (below). All solutions of the carcinogens were freshly prepared. All manipulations with solutions and cultures involving DMSO were carried out under dim or yellow lighting. The mammary glands that were tested for the formation of hyperplastic outgrowths and tumors were similarly treated in cultures with 10 nm DMBA or 1.0 nM FAA (below). The medium was renewed without carcinogen or DMSO 24 hr later (Day 4) and routinely every 2 or 3 days thereafter. At the end of the 10 days in the development medium, cultured glands that were tested for their abilities to generate hyperplastic outgrowths and tumors were divided into the following 2 groups:

- For the scoring of the carcinogen-induced hormone-independent foci and cytotoxicity of mammary glandular epithelium, approximately one-half (approximately 20) of the number of the developed glands in 4 individual experiments were exposed to regression medium containing insulin (5 µg/ml) without other added hormones for an additional 15 days (Days 10 to 25). On the 25th day, the glands were fixed with acetic acid:ethanol (1:3, v/v), stained with alum carmine, and scored for the altered foci and cytotoxicity. Unaltered alveoli in the cultured glands involuted in the regression medium. In contrast, glands that had undergone the focal conversion caused by chemical carcinogens contained alveolar nodules that had not regressed (2, 7, 8, 12, 19). Relative cytotoxicity was rated on the basis of the number of the regressed glands in which there was an absence or paucity (<50%) of alveolar buds (19) (see below).

- Dissociation of Cultured Mammary Cells. The remaining one-half of the number of glands in the 4 individual experiments were examined for their abilities to form hyperplastic outgrowths and mammary tumors after serial implantation into gland-free mammary fat pads. Following the first 10 days in culture (above), each of 4 experiments, 20 mammary glands were harvested, weighed, and minced in a dissociation medium that consisted of 20 ml of Waymouth MB752/1 medium per g of tissue, 0.1% collagenase, and 0.1% hyaluronidase. Using the procedure of DeOme et al. (6), as also used by Lyer and Banerjee (10) and Telang et al. (16), the clumps of tissue were treated with Pronase, and the cells were isolated. The cells were immediately suspended in the Waymouth medium at a concentration of 10⁶ cells/ml, kept on ice, and implanted into the gland-free mammary fat pads of 20 mice in each of 4 experiments (first-generation mice) over approximately a 2-hr period (see below).

Inoculation of Cultured Mammary Cells into First-Generation Mice. Prior to the first (primary) implantation, both inguinal mammary gland fat pads of in-house bred, 21-day-old BALB/c mice were cleared of glandular parenchyma by cauteryization (5). Immediately thereafter, the mice were entered retroperitoneally to implant under the right kidney capsules 2 pituitary glands from 4- to 5-week-old BALB/c mice removed immediately beforehand. Two weeks later, the gland-free inguinal fat pads were reexpanded, and 10 µl of the above suspension containing 10⁶ dissociated cells from the cultured glands were injected into the inguinal mammary fat pads of 20 mice in each of 4 experiments. Cells from the control glands exposed to the DMSO were injected into the right inguinal fat pads. Cells from carcinogen-treated glands were inoculated into the left inguinal fat pads. Eight weeks thereafter (10 weeks after passage of pituitary glands), the implanted pituitary glands were cauterized. Thereafter, the inoculated mammary inguinal fat pads were palpated periodically in search of nodules or tumors. At the end of 11 months, every inguinal mammary fat pad (whether or not containing outgrowths from the implants) was removed. Portions thereof were used for repassaging, and the remainders were examined microscopically at the whole-mount and tissue levels, all as described directly below.

Passage of Mammary Outgrowths into Second-Generation Mice. Two fragments (each <1 mm in diameter) of the outgrowths from each first generation mouse were inoculated into the inguinal gland-free mammary fat pads of 3 or 4 21-day-old BALB/c mice immediately following cauteryization to destroy those mammary glands. As in the first passage, control outgrowths were placed in right inguinal fat pads; outgrowths derived from carcinogen-treated glands were placed in the left pads. (The recipient mice had no implanted pituitary glands.) Eleven months later, the resulting second-generation (secondary) outgrowths were harvested and scored (below).

Whole-Mount and Histological Examinations of Mammary Glands and Outgrowths. Mammary glands and outgrowths were fixed and stained with alum carmine (19) and then were evaluated at the whole-mount level (x10) for the presence of cytotoxicity and nodular alveolar lesions (see above). Similarly, the mammary outgrowths and host mammary glands of the first- and second-generation mice were examined for ductal and alveolar changes. Thereafter, the tissues were transferred to xylene, embedded in paraffin, sectioned at 5 µm, stained with hematoxylin and eosin, and examined histologically in search of the presence and degree of hyperplasia, dysplasia, anaplasia, and neoplasia (compare Refs. 7, 10, 16, and 19). Tissues were grouped, coded, and scored without knowledge of the previous treatment of the glands. Each experiment included almost equal numbers of experimental and control glands.

RESULTS

Determination of Optimal Concentrations of Carcinogens That Transform Cultured Mammary Glands. Initial experiments sought the concentrations of the carcinogens DMBA and FAA that cause the maximal incidence of the hormone-independent nodular alveolar lesions of the cultured mammary glands with least cytotoxic effects. DMBA at 1 and 10 ng/ml yielded the highest incidence of transformation (64 and 50%, respectively) and the minimal cytotoxicity (22%) (Table 1). Increasing the concentration to 1.0 µg/ml lowered the frequency of transformation to 25% and markedly elevated the cytotoxicity to 97%, so that there was a >50% loss of alveolar buds in virtually all glands. On the basis of these data, 10 nm (2.56 ng/ml) was chosen as the optimum concentration of DMBA to cause transformation in culture.

Likewise, the optimum concentration of FAA was 1 µM FAA (223 ng/ml) on the basis of our previous study (Ref. 19, Table 1). This concentration is between 100 ng/ml, which yielded a 30% incidence of glands with lesions and no cytotoxicity, and 500 ng/ml, which resulted in a 70% incidence of carcinogen-altered foci and a 14% frequency of cytotoxicity.

Attempted Oncogenic Transformation of Mammary Glands by Optimal Concentrations of DMBA and FAA in Culture. The experiments that tested for the oncogenic transformation of mouse mammary glands in culture by DMBA and FAA used the optimum concentrations of these carcinogens. The protocol matched that used by Lyer and Banerjee (10) and Telang et al. (16), except with regard to the concentration of DMBA. It is noteworthy that the previously reported oncogenic transformation of mouse mammary glands by DMBA in the same culture system was achieved using 2.0 µg/ml (7.8 µM) (10, 16), a...
Table 1
Transformation of mouse mammary glands in whole-organ culture

Mouse mammary glands were treated with the listed concentrations of carcinogen, DMBA or FAA, in the solvent, DMSO (0.1% v/v), for 24 hr on Days 3 to 4 in whole-organ culture. Control glands were similarly exposed to only DMSO in matching experiments. After 10 days in development medium (Days 1 to 10), the glands were incubated in regeneration medium for 15 days (Days 10 to 25), stained, and scored at the whole-mount level (x 10 magnification) for cytotoxicity and hormone-independent alveolar nodules (transformation). Details are provided in the text and in previous reports (7, 8, 19).

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Concentration (ng/ml)</th>
<th>No. of glands</th>
<th>Cyto-</th>
<th>Incidence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>53</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>DMBA</td>
<td>1</td>
<td>36</td>
<td>22</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>22</td>
<td>22</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>43</td>
<td>43</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>500</td>
<td>38</td>
<td>57</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>1000 (3.9 μM)</td>
<td>36</td>
<td>97</td>
<td>9</td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>40</td>
<td>38</td>
<td>4</td>
</tr>
<tr>
<td>FAA</td>
<td>2.56 (10 nm)</td>
<td>40</td>
<td>48</td>
<td>19</td>
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<td>Control</td>
<td>0</td>
<td>29</td>
<td>21</td>
<td>0</td>
</tr>
<tr>
<td>FAA</td>
<td>223 (1 μM)</td>
<td>33</td>
<td>33</td>
<td>11</td>
</tr>
</tbody>
</table>

a Percentage of the number of regressed glands with absence or paucity (<50%) of alveolar buds (7, 8, 19).

b Mammary glands with transformed alveolar nodules which did not involute in regeneration medium in culture (2, 7, 8, 12, 19).

c Concentration approximately 1000-fold greater than required for maximal production of the hormone-independent foci of alveoli, and twice more than that which caused an extensive cytotoxicity in all glands in the present study.

d The optimum concentration of FAA for test of its ability to bring about oncogenic transformation was chosen to be 1 μM FAA (see above). Oncogenic transformation of mammary glands by FAA has not been reported previously.

Forty mammary glands in culture were treated with 10 nm DMBA and permitted to fully develop their alveoli in each of 2 experiments. Of these glands, 20 in each experiment were regressed for analyses of transformation and cytotoxicity, while the other 20 glands were processed for implantation of their dissociated cells into gland-free mammary fat pads of pituitary gland-supplemented mice. Equal numbers of glands were treated, with only DMSO, as culture solvent-controls. Of the total of 40 DMBA-treated glands, 48% were found to have hormone-independent alveolar foci and, also, 48% had evidence of cytotoxicity (Table 1). By comparison, 4 of the 40 DMSO-treated glands had unregressed alveolar nodules (10% incidence of transformation) with cytotoxicity in 38% of the glands. Likewise, a total of 73 mammary glands in 2 experiments were treated with FAA at 1 μM (0.223 μg/ml). Of these, 33 glands were fully developed and then regressed and assayed for hormone-independent alveolar foci and cytotoxicity. The remaining glands were dissociated into cells and inoculated into gland-free mammary fat pads, as above. Similar numbers of glands were treated with DMSO as controls. Of a total of 33 FAA-treated glands, 11 (33%) were found to contain transformed alveoli, and a same number of glands (33%) showed effects of cytotoxicity (Table 1). In comparison, none of the 29 DMSO-control glands had transformed foci, and 21% had evidence of cytotoxicity. One may conclude that, with the low concentrations of DMBA and FAA, the cytotoxicity was mainly related to exposure to the DMSO.

First-Generation Mammary Outgrowths. No neoplasia, anaplasia, or dysplasia was detected in any primary outgrowth that resulted from the implantation of mammary glands treated with either DMBA or FAA in culture. Nor were there such lesions in the outgrowths that originated from the DMSO-treated glands (culture controls) or in the thoracic mammary glands of the recipient mice (host controls). Table 2 lists the numbers of the recipient mice, mammary outgrowths, and thoracic glands that were thus evaluated. Fig. 1, a to c, demonstrates the typical histological morphology of these tissues, namely, the branching ductal system, the tiny alveolar buds, and the absence of cellular aberrations.

Further, no hyperplasia that can be attributed to the action of either carcinogen was detected in any first-generation outgrowth. Although hyperplasia was present in 6 of 25 outgrowths and in 5 of 20 outgrowths that arose from mammary glands treated with DMBA and FAA, respectively, the same mice also exhibited at least as much hyperplasia in their untreated thoracic mammary glands (host controls) and/or their outgrowths that arose from the DMSO-treated control glands (Table 2; Fig. 1). It is assumed that the hyperplasia in the outgrowths in these few mice likely resulted from hormonal hyperstimulation by functioning residues of the implanted pituitary glands that had escaped complete regression in all glands in the present study.
The conclusion is that the DMBA and FAA did not act on the mammary glands in culture to bring about oncogenic transformation, anaplasia, dysplasia, or hyperplasia in the primary mammary outgrowths.

Second-Generation Mammary Outgrowths. Likewise, no neoplasia, anaplasia, or dysplasia was discernible in any secondary outgrowth that arose indirectly by transplantation of the mammary glands that contained the hormone-independent alveoli as a result of exposure to either DMBA or FAA in culture. Nor were there such lesions in the DMSO-control outgrowths in the host control thoracic glands (Table 2; Fig. 1, d to f).

In addition, no hyperplasia that could be attributed to the actions of DMBA or FAA was present in any secondary mammary outgrowth. The same glandular pattern observed in the first generation was found (Fig. 1, f and i). Although hyperplasia was evident in 6 of the 60 outgrowths and in 3 of the 44 outgrowths that arose secondarily from mammary glands exposed in culture to DMBA and FAA, respectively, the same mice also had as much or more hyperplasia in their host control thoracic mammary glands or in their DMSO-control secondary outgrowths (Table 2; Fig. 1f). One secondary outgrowth from the mammary glands treated with DMBA was more hyperplastic than was its DMSO-control secondary outgrowth (Fig. 1h). However, in the same mouse, its control thoracic mammary gland was equally hyperplastic (Fig. 1g).

Only one hyperplastic primary DMBA-outgrowth gave rise to a hyperplastic outgrowth in one second-generation mouse (one of 2 surviving mice from 3 inoculated mice; see “Materials and Methods”). However, in that same mouse, the DMBA outgrowth, the control DMSO outgrowth, and the host control thoracic gland were all equally hyperplastic.

The conclusion is, therefore, that no neoplasia, anaplasia, dysplasia, or hyperplasia results from exposure of mouse mammary glands to concentrations of DMBA and FAA that bring about maximal incidences of hormone-independent nodular alveolar foci and minimal cytotoxicity in culture.

**DISCUSSION**

Mouse mammary glands that are transformed in whole-organ culture by DMBA or FAA under conditions that lead to maximal numbers of hormone-independent nodular alveolar lesions are not oncogenic in mice. Nor does this phenotypic alteration give rise to mammary anaplasia, dysplasia, or hyperplasia. This absence of cellular aberration occurred despite the use of 3 protocols that increased the growth and neoplastic potentials of altered mammary gland epithelium: (a) The cells of the carcinogen-treated glands were dissociated before implantation in vivo (6, 10, 16). (b) The first-generation mice were hyperstimulated with additional pituitary glands (16). (c) The primary mammary outgrowths were increased 3-fold in number, and they were allowed to progress by serial passage into second-generation mice. This absence of cellular aberration indicates that the presence of carcinogen-induced hormone-independent alveolar nodules in mouse mammary glands in culture is not itself sufficient for oncogenesis.

Mouse mammary glands that are phenotypically altered in whole-organ culture by low and high concentrations of DMBA apparently have different oncogenic potentials. Oncogenic transformation did not result from exposure to the low concentrations of DMBA and FAA that bring about maximal incidences of hormone-independent nodular alveoli and minimal cytotoxicity. In contrast, Telang et al. (16) and Iyer and Banerjee (10) obtained hyperplastic outgrowths and adenocarcinomas by the use of an approximately 1000-fold greater concentration of DMBA in the same protocol. However, that finding is complicated by associated high cytotoxicity (Ref. 19 and present report). In addition, the cultured glands that were treated with the high concentration of the carcinogen may have had a higher level of residual hydrocarbon dissolved in their fat pads, despite the change of medium at 24 hr, and were, therefore, exposed to the carcinogen for a longer duration.

The divergent findings of oncogenicity of mammary glands transformed by a high concentration of a procarcinogen (10, 16), and the nononcogenicity of glands containing maximal numbers of hormone-independent nodular alveolar lesions produced by low concentrations of procarcinogens (present report), permit at least 2 interpretations. In the first, neoplasia may originate from mammary gland epithelium other than that of the hormone-independent alveolar nodules. In that case, sufficient unknown target epithelium presumably survives the toxic actions of a high concentration of the DMBA. The previously studied hormone-independent alveolar nodules would then be inappropriate early markers of a step in the oncogenic process. In accordance, previous in vivo and in vitro studies suggested that carcinogen-induced hyperplastic nodules are not preneoplastic lesions in mammary carcinogenesis in rat (14). On the other hand, a considerable variety of procarcinogens and activated and direct-acting carcinogens bring about the formation of the hormone-independent alveolar nodules in cultured mouse mammary glands, and chemically close and unrelated noncarcinogens have little or no activity. A compilation of these chemicals is presented in Table 3. In addition, the transformed alveolar nodules in culture are morphologically similar to the hyperplastic alveolar nodules and the atypical lobules, which have been implicated as preneoplastic lesions in the mammary glands of mice (13) and women (21), respectively.

A second interpretation of the observed difference in oncogenic potential of the carcinogen-altered mammary glands allows for the existence of early and late stages of transformed alveolar nodules in the oncogenic sequence. The early stage of transformation may be manifest as the nodular alveolar lesion that arises under the selective condition of hormone deficiency in culture.

**Table 3**

<table>
<thead>
<tr>
<th>Chemical carcinogens that transform mammary glands in whole-organ culture</th>
<th>Transforming activity</th>
<th>No weak transforming activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMBA (7, 8, 19)</td>
<td>Naphthalene (19)</td>
<td>Fluorene (19)</td>
</tr>
<tr>
<td>3-Methylcholanthrene (2)</td>
<td>Anthracene (11)</td>
<td>2-Nitrofluorene (19)</td>
</tr>
<tr>
<td>Benz(a)pyrene (8)</td>
<td>Benzanthracene*</td>
<td>2-Aminofluorene (19)</td>
</tr>
<tr>
<td>Benz(a)pyrene-trans-7,8-dihydrodiol (8)</td>
<td>Rotinyl acetate (5)</td>
<td>2-Naphthylamine (19)</td>
</tr>
<tr>
<td>Benz(a)pyrene-diol-9,10 epoxy (ante) (8)</td>
<td>5,6-Benzoflavone*</td>
<td>1-Naphthylamine (19)</td>
</tr>
<tr>
<td>FFA (8, 19)</td>
<td>7,8-Benzoflavone*</td>
<td>1-Methyl-1-nitrosourea (8)</td>
</tr>
<tr>
<td>N-Hydroxy-N-2-fluorenylacetamide (8)</td>
<td>Retinylidenedimadone (7, 8)</td>
<td></td>
</tr>
<tr>
<td>N-Acetoxy-N-2-fluorenylacetamide (8)</td>
<td>Retinyl acetate (8)</td>
<td>N-(4-Hydroxyphenyl)-aflatoxin (8)</td>
</tr>
<tr>
<td>2-Aminofluorene (19)</td>
<td>DMSO (7, 8, 19)</td>
<td>Ethanol (19)</td>
</tr>
<tr>
<td>2-Nitrofluorene (19)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-Naphthylamine (19)</td>
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<td></td>
</tr>
<tr>
<td>1-Naphthylamine (19)</td>
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</tr>
<tr>
<td>1-Methyl-1-nitrosourea (8)</td>
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<tr>
<td>N-Methyl-N'-Nitro-N'-nitrosoguanidine (8)</td>
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<td></td>
</tr>
<tr>
<td>Dimethyl Nitrosamine*</td>
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<td></td>
</tr>
<tr>
<td>Diethylnitrosoamine (4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alloxan B*</td>
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</tr>
</tbody>
</table>

* M. S. Dickens, Q. J. Tonelli, and S. Sorof, unpublished observations.
This level of deregulation may arise from a molecular event brought about in greatest frequency by low concentrations of procarcinogen, e.g., DMBA, in the present study. The late state of transformation may come about from at least 2 molecular events resulting from high concentrations of the procarcinogens, or low concentrations of activated or direct-acting carcinogens. Although toxic to most mammary alveoli, these latter conditions may cause a sufficient number of molecular alterations in the relatively few surviving alveoli which become sufficiently directed to progress to frank tumor. Previous findings with retinoids are consistent with such a sequence of mouse mammary gland transformation by chemical carcinogens in culture. Retinoids are effective antipromoters in prevention of mammary gland transformations by low concentrations of the procarcinogens, DMBA, benzo(a)pyrene, and FAA in cultured mammary glands (7, 8). In contrast, retinoids do not prevent the otherwise apparently similar alterations produced by high concentrations of these procarcinogens or by low concentrations of activated and direct-acting carcinogens (8). Accordingly, retinoids may block the expression of the early state of the transformation, but not of the late state. A related situation apparently exists in the transformation of cultured keratinocytes of mice by DMBA and N-methyl-N'-nitrosourea. Transformed keratinocytes proliferate under conditions that favor the terminal differentiation and death of normal keratinocytes (24), a selection system not unlike that with the hormone-independent alveoli in cultured mouse mammary glands (see “Introduction”). Further, as with the carcinogen-altered mammary glands, the transformed keratinocytes are nontumorigenic (23). In addition, fibroblasts that are transformed in culture by chemical carcinogens survive and grow under conditions that are nonpermissive for untransformed fibroblasts, namely, low concentration of serum and absence of substrate for cell anchorage. In these cases, the carcinogen-induced alterations exist in a multistep sequence leading to tumorigenicity (reviewed in Ref. 3; Refs. 15 and 18). These and other findings indicate that acquisition of certain carcinogen-induced states of phenotypic alterations in various epithelial cells and fibroblasts may not of itself be sufficient to trigger the neoplastic process to proceed to oncogenicity. Additional studies seem warranted to examine the validity of a multistep mechanism of epithelial transformation in chemical oncogenesis of the mouse mammary gland.

ACKNOWLEDGMENTS

We are grateful to Dr. Mihir Banerjee for helpful discussion at the start of this study. We also thank Gail Nussbaum, John J. Churey, and Grace Kroetz for competent technical assistance. The valuable donation of a high-purity sample of DMBA from the Chemical Repository of the National Cancer Institute Carcinogen Research Program is acknowledged.

REFERENCES


Fig. 1. Histological comparison between normal second thoracic mammary glands (a, d, g) (host controls), and outgrowths of implants from DMSO-treated whole-mammary-gland cultures (b, e, h) (culture-solvent controls) and from DMBA-treated whole-mammary-gland cultures (c, f, i) (carcinogen-transformed). Mouse mammary glands were treated with either DMBA or DMSO (solvent control) in whole-organ culture and then were inoculated into gland-free fat pads of siogenic mice. The resultant first generation (primary) mammary outgrowths were transplanted into inguinal gland-free fat pads of mice giving rise to second-generation (secondary) mammary outgrowths. Both outgrowths were compared histologically with the second thoracic mammary glands of the same mice (host controls). All outgrowths and glands have well-developed ductal networks and small lobuloalveolar buds, the epithelial components being comparable, with no evidence of neoplasia, anaplasia, or dysplasia. Each transverse row represents a single mouse; a, b, c, d, e, and f (second generation implant) degree of growth generally occurred in the outgrowths from the transformed and solvent control glands (e, f) closely matching the host control thoracic gland (d); g, h, and i, maximal activity found in the second implant generation of the glands treated with DMBA in culture (i), exceeding that in the DMSO-treated control culture (h). However, since the hyperplasia is still greater in the host control (g), no influence of the carcinogen (DMBA) can be implied. Hematoxylin-eosin stain, x 90. Details are provided in the text.
Thoracic Mammary Glands (Host Controls)

Outgrowths from DMSO-treated Glands (Culture Controls)

Outgrowths from DMBA-treated Glands

Top: FIRST IMPLANT GENERATION MICE

Middle and Bottom: SECOND IMPLANT GENERATION MICE
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