SUMMARY

The immature mammary glands of BALB/c female mice were treated with 7,12-dimethylbenz[a]anthracene (DMBA), 2 μg/ml, or 3-methylcholanthrene (10 μg/ml) for a 24-hr period at different times during the initial six days of lobuloalveolar growth in hormone-supplemented organ culture. Nodule-like alveolar lesions (NLAL) were detectable in 80% of the glands treated with DMBA (40% in 3-methylcholanthrene-treated glands) in the presence of insulin + prolactin + aldosterone + cortisol in the medium. No NLAL were present in dimethyl sulfoxide-treated control glands cultivated with the same hormones. The hormone combination insulin + prolactin + cortisol was unfavorable for NLAL induction by DMBA, and the combination of aldosterone + insulin + prolactin was only moderately conducive. Thus, the presence of cortisol with insulin + prolactin + aldosterone enhances NLAL incidence of mammary cells by DMBA. The highest incidence was found in glands that were treated with DMBA for 24 hr between the third and fourth day of culture, the period corresponding to the onset of the second wave of DNA synthesis in the gland. Cytotoxicity of DMBA was pronounced between 24 and 48 hr, when a high frequency of cells were in DNA synthesis, and survival of the cells after the cytotoxic effect of DMBA appeared to play a role in NLAL incidence. This suggests that DMBA-induction of NLAL in mammary glands in organ culture involves a complex carcinogen-hormone-cell cycle interaction. We emphasize that, although NLAL morphologically resembles the hyperplastic alveolar nodules of mouse mammary gland in vivo, the ability of NLAL to produce typical hyperactive alveolar outgrowth and mammary tumor after transplantation in vivo remains to be determined.

INTRODUCTION

It is well established that HAN of the mouse mammary gland constitute an essential precursor in viral or chemical carcinogenesis of the breast tissue (7, 14, 18, 19). The induction of HAN in the mammary gland in vivo by MuMTV or chemical carcinogens depends greatly on hormonal stimulation of the mammary parenchyma (8, 18), and similar hormone treatment also activates DNA synthesis and cell proliferation in the mammary epithelium (2). It is well documented that increased frequency of cells in the proliferative pool at treatment enhances the susceptibility of target tissues, including the mammary cells, to the action of carcinogenic agents (1, 2, 5, 13). The development of an organ culture method for the entire mammary gland in a chemically defined medium now permits hormonal induction of epithelial proliferation in the culture medium (3, 12, 16, 20). Thus it is feasible to study the responses of the proliferative mammary epithelial cells after treatment of the entire gland in organ culture.

Previously, we reported that alveolar lesions that are morphologically similar to HAN can be induced by DMBA or MCA in MuMTV-free, nodule-inducing virus-positive C3Hf mouse mammary glands cultivated in organ culture (4). In order to distinguish them from HAN, the structures found in DMBA- or MCA-treated glands in organ culture will be called NLAL. This report presents the results of studies concerning: (a) induction of NLAL in organ culture of mammary gland of MuMTV- and nodule-inducing virus-free BALB/c mice, (b) relation between cell cycle and carcinogen treatment on mammary NLAL induction in culture, (c) favorable hormone environment for increased frequency of NLAL in organ culture, and (d) relation between cytotoxic effect of the carcinogens and NLAL incidence.

MATERIALS AND METHODS

Animals. MuMTV- and nodule-inducing virus-free BALB/c female mice (3 to 4 weeks old) obtained from the National Cancer Institute were used. As a prerequisite for the whole mammary gland organ culture procedure, mice were given 1 μg 17β-estradiol and 1 mg progesterone by s.c. injections daily for 9 days, according to the procedure previously described (12). One day after the last injection, mice were...
24). The 2nd thoracic mammary glands were cultivated in excised and cultivated in organ culture. The procedure for organ culture of whole mammary gland was essentially similar, as described (4, 12, 24). The 2nd thoracic mammary glands were cultivated in Falcon plastic culture dishes (60 x 15 mm) containing Waymouth's Medium MB 752/1 supplemented with L-glutamine (35 μg/ml), penicillin (3.5 μg/ml), and appropriate hormone combinations. Three glands were cultivated in each dish containing 3.5 ml medium, and incubation was carried out at 37° in an atmosphere of 95% O2 + 5% CO2. Glands were initially cultivated for 9 days in medium containing a hormone combination promoting alveolar growth. Subsequently, incubation was continued in a hormone-deficient medium for 12 to 15 days, which led to degeneration of normal alveolar structures.

**Hormone Supplementation.** Medium was supplemented with 4 different combinations of hormones and these were IPA, IPF, IPAF, and IA. The concentration of each hormone (insulin, prolactin, aldosterone, and cortisol) in medium was 5 μg/ml. Medium containing the hormone combination IA has been referred to as the "regression medium."

The carcinogenic hydrocarbons DMBA and MCA were dissolved in DMSO at a concentration of 1 and 5 mg/ml, respectively. The glands were treated with 2 μg DMBA or 10 μg MCA per ml of medium for a 24-hr period at different times of cultivation. Control glands were treated with DMSO. The final concentration of DMSO in the medium was 0.2% for both control and the carcinogen-treated gland. After carcinogen treatment, the incubation was continued in carcinogen- and DMSO-free (control cultures) fresh medium containing the same combination of hormones for a total period of 9 days. The glands were then incubated in the regression medium for another 12 to 15 days. Medium was regularly changed at 3-day intervals. Glands were then fixed in glacial acetic acid:ethanol (1:3, v/v), stained with carmine, and analyzed for NLAL as described (4).

**Cell Cycle Studies.** Cell cycle was estimated by determining the waves of DNA synthesis during a 6-day culture period. DNA synthesis in mammary glands cultivated in IPA-, IPF-, or IPAF-containing medium was monitored by [3H]thymidine pulse labeling of the mammary cells. The glands were transferred to fresh medium containing 5 μCi [methyl-3H]thymidine per ml (specific activity, 20 Ci/m mole; New England Nuclear, Boston, Mass.) and incubated for 60 min before termination of the culture at each 24-hr interval. At the end of the labeling period, the medium containing [3H]thymidine was immediately removed by suction, and the glands were washed 3 times with cold 0.9% NaCl solution. Glands used for chemical analysis were frozen in liquid nitrogen and stored at −20°. The glands used for histology were fixed in glacial acetic acid:ethanol (1:3). For chemical analysis of [3H]thymidine radioactivity, the tissue was defatted with 2 extractions, each with ethanol:ether (1:1), and ether; it was then homogenized in cold 5% TCA. The acid-insoluble precipitate was washed 3 times with cold 5% TCA to remove acid-soluble materials. Nucleic acids in the precipitate were hydrolyzed with 10% TCA for 20 min at 70°. Aliquots of the hydrolysate were used for estimation of DNA by the indole method (11) and for determination of radioactivity in toluene-based scintillation solution containing 4% NCS and Omnifluor. The samples were counted in a Beckman LS350 liquid scintillation spectrometer.

Hormones and other chemicals were obtained from the following suppliers: insulin, penicillin, and L-glutamine were from Calbiochem, LaJolla, Calif.; cortisol, aldosterone, prolactin, and DMBA were from Sigma Chemical Co., St. Louis, Mo.; and Waymouth's Medium MB752/1 was from Microbiological Associates, Bethesda, Md.

**Autoradiography.** Autoradiographs of histological sections were prepared by dipping the slides in Kodak NTB emulsion (Eastman Kodak Co., Rochester, N. Y.). The coated slides were then placed in Coplin jars containing Omnifluor:liquid scintillation solution. The slides were exposed in this solution in the dark for 48 hr at −20° and then developed according to the standard procedure (2).

**RESULTS**

**Alveolar Development and NLAL Induction.** Fig. 1 shows the mammary gland before onset of organ culture, and the parenchyma at this time was primarily ductal, with some terminal end buds. A pronounced lobuloalveolar development was observed when the cultures were terminated after 9 days cultivation in IPA- or IPAF-containing medium, whereas, lobuloalveolar structures in the gland cultivated in IPF-containing medium were not as extensive (Figs. 2 to 4). The alveolar structures induced in the growth-promoting medium containing IPA or IPAF regressed during subsequent 15-day cultivation in IA medium (Fig. 5), when the ductal parenchyma resembled the involuted mammary gland in vivo.

In experiments with the carcinogenic chemicals, the glands were treated with DMBA or MCA for 24-hr intervals between the 1st 5 days of lobuloalveolar growth in culture (Table 1). After treatment, cultivation was continued in carcinogen-free fresh medium for a total of 9 days, and this was followed by 15 days of cultivation in the regression medium containing the hormones IA. In many instances, nonregressed lobuloalveolar areas of variable sizes were present in the carcinogen-treated glands (Fig. 6), and the incidence of these NLAL was highest in glands treated between 3rd and 4th day of culture in the presence of the hormones IPAF. The lobuloalveolar histology (Fig. 7) and altered hormone responses of the lesions found in organ culture were similar to that of preneoplastic HAN induced by DMBA in mouse mammary gland in vivo (15). The control glands treated with DMBA failed to show any alveolar lesions.

**Cell Cycle.** The cell cycle was estimated by the waves of DNA synthesis monitored by [3H]thymidine incorporation during the initial 6-day period of lobuloalveolar development in IPF-, IPA-, or IPAF-containing medium (Chart 1). With these 3 different hormone combinations, the 1st wave of DNA synthesis in the mammary cell reached a peak around the 2nd day of cultivation, and DNA synthetic activity was more active in glands incubated in the medium with IPAF. A 2nd wave of DNA synthesis around the 4th day...
Table 1
Hormonal influence on frequency of nodules induced by DMBA in BALB/c mouse mammary gland in organ culture

Glands were treated with 2 μg DMBA per ml for 24 hr at different intervals of the culture, as indicated.

<table>
<thead>
<tr>
<th>Time of DMBA treatment (hr)</th>
<th>Glands with nodules/total no. of glands studied</th>
<th>Glands with nodules/total no. of glands studied</th>
<th>Glands with nodules/total no. of glands studied</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO control</td>
<td>0/12</td>
<td>0/29</td>
<td>0/30</td>
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<tr>
<td>0–24</td>
<td>0/11</td>
<td>0/20</td>
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<td>24–48</td>
<td>0/15</td>
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<td>48–72</td>
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<td>72–96</td>
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<td>96–120</td>
<td>0/15</td>
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on the 2nd day of cultivation, representing about 35% of the total epithelial cell population in S phase; a 2nd wave occurred on the 4th day, representing about 10% of the epithelial cells in S phase. By the 5th day, the number of cells in S phase was as low as that on the 6th day. The observation that DNA synthesis, as measured by chemical analysis, corresponds with that measured by [3H]thymidine-labeling index of the epithelium is consistent with earlier findings that only the mammary epithelial cells respond to

was present in glands cultivated in IPA and IPAF medium, but this was absent in glands cultivated with IPF. These results indicate that in IPA- or IPAF-containing medium, mammary cells undergo 2 division cycles but, in medium with IPF, only 1 cycle is present.

The DNA synthesis of mammary gland cultivated in IPAF-containing medium was also monitored by autoradiographic determination of the [3H]thymidine labeling index of mammary epithelial cells. As shown in Chart 2, the pattern of the waves of labeled cells was similar to that determined by TCA-insoluble radioactivity (Chart 1). There were 2 waves of cells in the S phase, and the 1st wave was more pronounced than the 2nd. The 1st wave occurred on the 2nd day of cultivation.
the hormonal stimulation of DNA synthesis both in vivo and in organ culture (2, 22).

The results obtained regarding the relationship between DNA synthesis and time of DMBA treatment on NLAL incidence in the mammary gland cultivated in presence of IPAF are shown in Chart 2. Glands cultivated in IPAF medium were treated with DMBA for a period of 24 hr at different times during the initial 6-day cultivation. The incubation was then continued in carcinogen-free fresh medium containing the same hormones for a total period of 9 days. The results show that nearly 80% of the glands treated with DMBA for the 24-hr period between the 3rd and 4th day (72 to 96 hr) of cultivation contained NLAL. The incidence of NLAL in glands treated with DMBA during the 5th day (96 to 120 hr) of cultivation was reduced, but the frequency remained relatively high. The 24-hr DMBA treatment of the glands during the initial 3 days of culture produced only a moderate NLAL incidence. The 24-hr period of carcinogen treatment of glands during the 3rd and 4th day, which produced the highest NLAL incidence, also correlates with the period when a relatively high frequency of epithelial cells are expected to be in G1 to S phase (immediately before entering the S phase) of the cell cycle during the 2nd wave of DNA synthesis.

Hormone-Carcinogen Interaction. The results delineating the influence of different combinations of hormones on frequency of NLAL are shown in Table 1. In medium with IPF, DMBA did not induce any NLAL, regardless of the time of treatment. In IPA-containing medium, a low NLAL incidence was observed in glands that were treated with DMBA on the 4th or 5th day of culture. In glands cultivated with medium containing IPAF, however, the incidence of NLAL induced by DMBA was greatly enhanced. The frequency of NLAL after each period of DMBA treatment increased progressively, reaching the highest level (nearly 80%) when the glands were treated during the interval between the 3rd and 4th day (72 to 96 hr) of culture.

The results suggest that cortisol in the presence of aldosterone along with insulin and prolactin enhances DMBA-induced NLAL of mammary cells, but only cortisol in combination with insulin and prolactin did not induce any NLAL. Aldosterone alone with insulin and prolactin induced a low frequency of NLAL at the same time period.

When mammary glands were treated with MCA (10 μg/ml) for 24 hr during the 4th day of cultivation in medium with IPAF, 8 out of 20 glands (40%) contained NLAL, with an average of 2.2 lesions per gland.

Cytotoxic Effect of Carcinogenic Hydrocarbons on Mammary Cells. Most chemical carcinogens are known to exert some cytotoxic effect, and the density of cell population at the time of treatment is believed to influence the cloning efficiency of the “transformed” cells (10). Accordingly, the cytotoxic effect of DMBA on hormone-stimulated mammary cells was studied. The pattern of IPAF-induced alveolar growth of the gland after DMBA (2 μg/ml) treatment at different times is shown in Figs. 8 to 13. Glands treated with DMBA during the 1st or 2nd 24 hr in culture showed poor alveolar growth during subsequent cultivation in carcinogen-free medium, but the control gland treated with DMSO developed normal alveolar structure (Figs. 11 to 13). A similar pattern of cytotoxic effect of DMBA was also observed in glands cultivated in IPA-containing medium (data not shown).

DISCUSSION

It has been demonstrated that the biological cycle of lobuloalveolar growth, lactation (including formation of casein), and alveolar regression of the mouse mammary gland can be mimicked in culture medium within 3 weeks by sequential cultivation of the whole gland with appropriate steroid and polypeptide hormone combinations (23, 24). The present study shows that combination of the hormones, insulin + prolactin + aldosterone + cortisol contained in the medium, allowed full lobuloalveolar growth and some alveolar secretory activity within 9 days in the culture medium. The observation that medium with IPAF promoted growth of lobuloalveolar structure in a manner similar to that with IPA strongly suggests that, in the presence of insulin and prolactin, growth-promoting action of aldosterone is compatible with the lactogenic action of cortisol and vice versa. Subsequent cultivation for 12 to 15 days in IA-containing medium led to the degeneration of the alveolar structures, leaving a ductal parenchyma.

Treatment of the gland with DMBA or MCA during alveolar growth induced NLAL, which were sustained in the gland as nonregressed lobuloalveolar lesions in a hormone-deficient medium. These results are consistent with an earlier preliminary report (4) from our laboratory about induction of nodule-like lesions by 1 μg DMBA or 5 μg MCA per ml of medium in whole mammary gland of C3Hf mice in organ culture. However, the present study reveals that factors such as increased dosage (2 μg DMBA and 10 μg MCA per ml of medium) of carcinogens, time of treatment during the culture period, and combination of polypeptide and corticosteroid hormones play a role in significantly enhancing NLAL incidence. Treatment of the gland with DMBA between the 3rd and 4th day of cultivation in a medium with IPAF raises the NLAL incidence as high as 80%, with an average of 8.4 lesions per gland, compared to 26% (4.1 lesions/gland) observed during the earlier studies (4). A similar treatment with MCA also increased NLAL incidence, but it was not as high as that with DMBA. This relatively lower frequency (40%) of NLAL after MCA treatment of the mammary gland in the culture medium seems to be consistent with similar lower frequency of MCA-induced HAN in vivo (15). The HAN induced by MuMTV, DMBA, or hormones in mouse mammary gland in vivo (7, 14), and their outgrowths after serial fatpad transplantation, mostly maintain a hyperactive lobuloalveolar structure and sometimes lactate in a nonpregnant or virgin host. This is believed to be indicative of an altered response of the HAN cells to host hormonal control. Their tumorigenic potential, however, may vary from none to very high (7, 14). Like the HAN in vivo, the lobuloalveolar structures of the NLAL induced in organ culture are also sustained, and some contain milk-like secretory material in a hormone-deficient medium. This characteristic transformed condition of DMBA- or MCA-induced NLAL cells with respect to hormonal regulation in...
organ culture is similar to the conditions of the HAN in vivo (14, 15, 19). Therefore, it is likely that some of the NLAL induced by the carcinogenic hydrocarbons in organ culture may also possess the characteristics of the HAN, including tumorigenic potential.

The results show that, in IPAF medium, DNA synthesis in the mammary epithelium occurs in 2 waves; the 1st reaches a peak at 48 hr and, the 2nd, at 96 hr. It has been reported that after 9-day priming with estradiol and progesterone, a large proportion of mammary epithelial cells are in the S phase (DNA synthesis) in vivo (2), and it is known that mammary cells that already entered the S compartment in vivo complete the DNA replicative process during the initial hours of cultivation in the culture medium (17). Therefore, the 1st wave of DNA synthesis observed in the culture medium should represent mostly those cells that were in the S compartment in vivo while the 2nd wave consists of the DNA replicating cells which were initiated by the hormones in the culture medium. The high level of DNA synthesis on the 4th day of culture of whole mammary gland with hormone combination IPA is consistent with similar results previously observed (16). The absence of the 2nd wave of DNA synthesis and the reduced lobuloalveolar development in IPF medium indicate that this hormone combination is not optimal for lobuloalveolar growth of the mammary gland.

There was either no or little incidence of NLAL in glands treated with DMBA during the initial 2 days of cultivation in medium with IPAF. The same treatment period corresponds to the 1st wave of DNA synthesis in the cells, most of which already entered the S compartment in vivo. Recently, it has been shown that cells in the S compartment are more susceptible to the cytotoxic effects of chemical carcinogen at concentrations conducive to high transformation frequency in culture and cytotoxicity of MNNG has been found to be inversely related to transformation frequency (5). Consistent with these findings, present results also demonstrate that highest cytotoxicity of 2 μg DMBA per ml corresponds to the period of more pronounced 1st wave of DNA synthesis when 38% of the epithelial cells are in the S compartment of the cell cycle. Furthermore, evidence indicates that chemical carcinogen-induced transformation frequency is related to survival of the target cells following treatment (5). A poor survival of the mammary cells is also indicated by the virtual absence of lobuloalveolar development of the glands treated during the initial 2 days in culture. Thus, the lack of NLAL incidence after the initial 2 DMBA treatments may be due to reduced survival of the mammary cells. On the other hand, DMBA-induced NLAL incidence reaches the highest level in glands treated during the interval between the 3rd and 4th day of culture. This period corresponds to one when a substantial number of cells are expected to be in transit from G1 to S phase before entering DNA synthesis. Accordingly, we suggest that high incidence of NLAL in glands treated between the 3rd and 4th day may be due to exposure of the more susceptible mammary epithelial cells in the G1 to S-phase boundary, and this interpretation is in agreement with the phenomenon of increased sensitivity of G1 to S-phase cells for chemical carcinogen-induced transformation in culture (5). It may be noted that NLAL incidence after treatment between the 4th and 5th day of culture is reduced, even though lobuloalveolar development after carcinogen exposure is substantial in these glands. Another factor that needs consideration relates to the cell density at the time of treatment. The amount of alveolar epithelium during the initial 2 days of culture is low; consequently, the survival probability of alveolar cells after the cytotoxic effects of DMBA is expected to be reduced, whereas, prior cultivation for 3 days in carcinogen-free growth-promoting medium permitted some lobuloalveolar development, and loss of some cells due to cytotoxicity may not retard subsequent lobuloalveolar development in an acute manner.

Interestingly, factors such as cell cycle dependency and survival of cells after cytotoxicity found conducive to MNNG-induced increased transformation frequency of mouse fibroblasts in culture(s) also seem to be associated with enhanced incidence of NLAL in DMBA-treated mammary gland in culture. The growth-stimulating effects of aldosterone in the presence of insulin and prolactin (16) may provide a promoting environment by inducing epithelial cells into the more susceptible G1 to S compartment of the cell cycle at the time of carcinogen treatment. As indicated by the results, cortisol in the presence of IPA enhances DMBA-induced NLAL of mammary cells. However, only cortisol with insulin and prolactin is not favorable for NLAL-inducing action of DMBA. At this time we are unable to explain the mechanisms involved in enhancement of DMBA-induced NLAL in the presence of cortisol. However, there have been reports that glucocorticoid may enhance the metabolism of DMBA (21) by increasing the activity of the enzyme arylhydrocarbon hydroxylase (9, 10), and the presence of glucocorticoids was found to augment DMBA-induced mouse skin tumorigenesis (6).

Finally, we would like to emphasize that, although NLAL produced in organ culture morphologically resemble the HAN in vivo, the biological properties of NLAL remain to be determined. At this time it is premature to conclude that mammary NLAL induced in organ culture is necessarily equivalent to HAN in vivo. Nevertheless, it is of interest to note that NLAL are present only in glands treated with the carcinogenic hydrocarbons. Furthermore, cell cycle dependency and survival of the treated cells associated with enhanced NLAL incidence appear to be consistent with similar conditions that produce MNNG-induced increased transformation frequency of mouse fibroblasts in culture (5).

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REFERENCES


Carcinogen-induced Mammary Lesions in Organ Culture
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Fig. 1. Whole mount of an uncultivated gland after 9-day priming of the mouse with estradiol + progesterone. Note the ductal architecture with end buds and no alveolar structures. x 10.

Fig. 2. Glands cultivated in medium containing IPA for 9 days. Note the extensive lobuloalveolar structures. x 10.

Fig. 3. Glands cultivated in medium containing IPAF for 9 days. Note the extensive lobuloalveolar structures. x 10.

Fig. 4. Glands cultivated in medium containing IPF for 9 days. Lobuloalveolar development is not extensive. x 10.

Fig. 5. Glands initially cultivated in medium containing IPAF, for 9 days, followed by cultivation in medium containing IA for 15 days. Note the regression of the lobuloalveolar structure leaving a ductal architecture. Histology of these glands failed to show any viable lobuloalveolar structure. x 10.

Fig. 6. Presence of NLAL in a gland cultivated in medium containing IPAF and treated with DMBA (2 μg/ml), followed by cultivation in medium containing IA for 15 days (see "Materials and Methods"); a, lower magnification, x 10; b, portion of the same gland at higher magnification, x 20. Note the presence of alveolar structures. Morphology of control glands treated with DMSO was as in Fig. 5.

Fig. 7. Histology of one of the NLAL shown in Fig. 6. Note the lobuloalveolar characteristic and the retained "milk-like" secretory material in the lumen. x 50.

Fig. 8. A DMSO-treated control gland cultivated in medium containing IPFA for 9 days. Note the full lobuloalveolar growth. x 10.

Fig. 9. A gland treated with DMBA (2 μg/ml) for a 24-hr period during the 1st day of 9-day culture. Note the virtual absence of lobuloalveolar structure. x 10.

Fig. 10. A gland treated with DMBA (2 μg/ml) for a 24-hr period during the 2nd day of 9-day culture. Morphology of this gland is essentially similar to that in Fig. 9. x 10.

Fig. 11. A gland treated with DMBA (2 μg/ml) for a 24-hr period during the 3rd day of 9-day culture. Note the much improved lobuloalveolar growth. x 10.

Fig. 12. A gland treated with DMBA (2 μg/ml) for a 24-hr period during the 3rd day of 9-day culture. Morphology of this gland is essentially same as that shown in Fig. 11. x 10.

Fig. 13. A gland treated with DMBA (2 μg/ml) for a 24-hr period during the 3rd day of 9-day culture. Morphology of this gland is essentially same as that shown in Fig. 11. x 10.
Cell Cycle-related Hormone Carcinogen Interaction during Chemical Carcinogen Induction of Nodule-like Mammary Lesions in Organ Culture

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