Transplantation of Mouse Mammary Epithelial Cells Grown in Primary Collagen Gel Cultures

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ABSTRACT

A technique for the transplantation of mouse mammary epithelial cells, grown in collagen gels, has been developed that demonstrates that the phenotype of the cells prior to culture was not altered by the culture conditions. Mammary epithelial cells from virgin and midpregnant C57BL/Crl mice; virgin, midpregnant, and multiparous nonpregnant BALB/cfC3H/Crl mice; a BALB/c hyperplastic alveolar nodule line, and mammary tumors from BALB/cfC3H/Crl mice were embedded inside collagen gels and grown for 10 to 14 days in the presence of 25% swine serum plus choler toxin (0.01 μg/ml). The epithelial cells increased in number during the culture period. At the end of the culture period, the cells were removed from the collagen gels and transplanted to the gland-free mammary fat pads of 3-week-old syngeneic female mice. Culture in collagen gels increased the number of cells necessary to obtain a high percentage of mammary outgrowths as compared to cells not grown inside collagen gels. In general, mammary cells grown inside collagen gels gave rise to outgrowths, similar in phenotype to those from noncultured cells, and were representative of the tissue of origin. Mammary epithelial cells from C57BL/Crl virgin donors grown in collagen gels for 10 to 14 days retained their ability to respond to the endogenous hormones of pregnancy and lactation of the host and formed lobuloalveolar structures full of secretion similar to the host's own mammary gland. The data indicate that the growth of mammary epithelial cells in collagen gels and subsequent transplantation into the gland-free fat pads of syngeneic hosts provides a simple system, wherein cells can be grown in vitro and their phenotypes determined in vivo.

INTRODUCTION

A cell culture system has been developed recently that permits the prolonged and continuous growth of both normal and tumor mouse mammary epithelial cells in primary culture (23–27). This enhanced growth is achieved by embedding the cells in a collagen gel matrix.

The mammary cells embedded in collagen gels gave rise to 3-dimensional colonies with projecting arms. These colonies were composed of epithelial cells (25–27), and the arms of the colonies frequently had lumina reminiscent of ductal structures in vivo. Horse serum, growth factors, and agents known to increase cyclic adenosine 3':5'-monophosphate levels have all been demonstrated to be growth promoting for primary cultures of mouse mammary cells embedded in collagen gels (23).

This collagen gel culture system thus provides a unique opportunity to analyze the role of hormones and other agents on the regulation of growth, differentiation, and transformation of mammary epithelial cells. However, the establishment of epithelial cell cultures often results in the selection of cell populations and the subsequent loss or reduction of functional activity (12). In addition, spontaneous transformation of normal cells is also known to occur in vitro (20). Thus, it is of the utmost importance to determine the nature of the mammary epithelial cells undergoing growth inside the collagen gel matrix.

Since it has been established that the transplantation of mammary tissues to the gland-free mammary fat pads of syngeneic hosts produces outgrowths characteristic of the tissue of origin (7, 9, 10, 13), we used this technique to assess the nature of the cultured mammary epithelial cells. Transplantation studies were initiated with normal, preneoplastic, or neoplastic mammary epithelial cells grown in a collagen gel matrix to answer the following questions: (a) Did the cells retain their epithelial nature and original phenotype (normal, hyperplastic alveolar nodule, or tumor)? (b) Did the cells retain their capability to respond to mammogenic and lactogenic hormones in vivo? (c) Did collagen gel cultures, containing a mixture of normal and transformed mammary cells, produce mixed outgrowths when transplanted?

In this report, the term "colonies" refers to the structures formed by epithelial cells inside the collagen gels, and "outgrowth" refers to the structures formed in the mammary fat pads after transplantation of mammary epithelial cells.

MATERIALS AND METHODS

Animals. All mice were obtained from the inbred mouse colony maintained at the Cancer Research Laboratory, University of California, Berkeley, Calif. They were housed in plastic cages on wood shavings in temperature-controlled rooms with a fixed light cycle. Food pellets (Wayne Lab-Blox F-G; Allied Mills, Inc., Chicago, Ill.) and water were available ad libitum.

The normal mammary tissues were obtained from the C57BL/Crl (virgin and 5- to 10-day pregnant) and the BALB/cfC3H/Crl (virgin, midpregnant, and nonpregnant multiparous) strains of mice. The C57BL strain is a M-MTV unexpressed strain with a very low mammary tumor incidence and no detectable mammary dysplasias demonstrable by the cell-dissociation technique (10, 13). The BALB/cfC3H strain, on the other hand, shows a high degree of murine mammary tumor virus expression with a high spontaneous mammary tumor incidence in parous mice and is positive for nodule-transformed cells by the cell-dissociation technique (9). A hyperplastic alveolar nodule outgrowth line D2 recovered from BALB/cfCrl mice was used. This outgrowth line was established in 1969 and has been maintained by serial transplantation (8). Mammary tumors were obtained from BALB/cfC3H/Crl retired breeders.

Cell Dissociation. Mammary tissues were dissociated according to the procedure described previously (24, 26) in 0.1% collagenase type III (Worthington Biochemical Corp., Freehold, N. J.), which was fol-
...stomral and other contaminating cell types by centrifugation in a density gradient of Percoll (Pharmacia Fine Chemicals Inc., Piscataway, N. J.). Two ml of the cell suspension containing 3 to 5 x 10^6 cells were placed on top of 28 ml of a preformed Percoll gradient (10.8 ml of Percoll, 1.2 ml 10X Waymouth medium, 16 ml Puck’s Saline A (Grand Island Biological Co., Grand Island, N. Y.), and 0.5 ml of 0.05% DNase (Sigma Chemical Co., St. Louis, Mo., 625 units/mg). The gradient was preformed by centrifugation at 10,000 x g for 1 h, and the cells were layered on top and centrifuged at 800 x g for 15 min at 20°. Epithelial cells which band preferentially at a higher density (1.07 to 1.08 g/ml) than do stromal cells were collected by aspirating the gradient to just above the epithelial band and then removing the band with a pipet. The band was suspended in 50 ml of Medium 199 and pelleted by centrifugation at 180 x g for 5 min, and an aliquot was counted in 0.02% crystal violet.

Culture Procedure. Rat tail collagen solutions and gels were prepared as described previously (24). For transplantation experiments, 1 to 1.5 x 10^6 cells in 5 ml of cold gelation mixture were overlaid on 4 ml of gelled collagen in 100-mm tissue culture plates ( Falcon Plastics, Oxnard, Calif.) and allowed to gel. Cells (5 to 10 x 10^5) in 2.5 ml of cold gelation mixture were overlaid on 0.3 ml of gelled collagen in each well of multiwell plates (Falcon) for determination of increase in cell number. Cultures were fed every 2 days with a medium that consisted of Dulbecco’s modified Eagles’ medium (Grand Island Biological Co.), Ham’s F-12 (Grand Island Biological Co.) (1:1) ( Dulbecco’s modified Eagles’ medium:F-12), 25% swine serum (Grand Island Biological Co.), 0.01 μg of cholera toxin per ml (Sigma), 0.05 μg of gentamicin sulfate per ml (Sigma), and 2.5 μg of amphotericin B per ml (Sigma).

Determination of Cell Number. Individual gels, in triplicate, were taken at the start (0 time) and at the end of the culture period for cell number determination by a fluorometric assay for DNA (15).

Transplantation into Mice. At the end of a 10- to 14-day culture period, the entire collagen gels, which contained colonies, were removed from the tissue culture plates and placed in approximately 10 ml of 0.1% collagenase ( Worthington type III) in Medium 199 (Grand Island Biological Co.) per 100 mm gel. The collagen gels were dissolved by gentle shaking at 37° for approximately 30 min. Colonies of epithelial cells remain nearly intact after this treatment. To prepare cell suspensions suitable for transplantation, the colonies were washed and centrifuged twice at 180 x g. The cell pellets were resuspended in 10 ml of 10% swine serum in Dulbecco’s modified Eagle’s medium/F-12 per collagen gel and were placed in 100-mm tissue culture plates (Falcon). Two days after plating, the colonies had attached and spread, forming a pavement-like monolayer. The media was removed by aspiration, and 10 ml of a solution of Puck’s Saline A, trypsin, and Versene were added. The plates were incubated at 37° for 10 to 15 min. The cells detached and were washed twice with Medium 199 and collected by centrifugation. DNase (0.04%) was added to reduce clumping. The cells were resuspended in 2 ml of Medium 199, and aliquots that were suspended in 0.01% crystal violet were counted in a hemocytometer.

The volumes of the cell suspensions were adjusted to give the necessary concentrations of cells to be injected in 0.01 ml. Controls were enzymatically dissociated noncultured mammary cells injected at the appropriate concentrations. Plating the colonies in plastic culture plates was necessary to obtain a suspension of cells that could be counted and was in small enough clumps to permit injection. We have found that when epithelial colonies were removed from the collagen, they were recovered as large intact colonies. We attempted to dissociate the colonies into smaller clumps with protease and found that such a procedure resulted in the loss of many cells and a low frequency of outgrowths following transplantation.

The cells were injected in a volume of 0.01 ml into the gland-free mammary fat pads of 3-week-old syngeneic female mice (9). The host mice were maintained as virgins and were terminated 8 to 10 weeks after injection. The day before termination, each mouse received 0.75 ml of 0.5% trypan blue in 0.85% NaCl solution i.p. to permit the tentative identification and classification of mammary gland outgrowths. To determine whether the outgrowths derived from cells grown in collagen gels would respond in an appropriate manner to the endogenous hormones of pregnancy or lactation, host mice were selected that had visible ductal outgrowths. These mice were mated and allowed to go through a pregnancy and lactation. During pregnancy or lactation, host mice were terminated. The injected inguinal fat pads and a part of one of the thoracic glands were removed, spread in Tissue-Tek (Miles Laboratories, Inc., Elkhart, Ind.), capsules, fixed in Telynesiczky’s fixative, defatted in acetone, stained in iron-hematoxylin, and examined in methyl salicylate by means of a dissecting microscope.

Samples of selected outgrowths from the fixed and stained whole mounts were cut out, embedded in paraffin, sectioned at 7 μm, and stained with hematoxylin and eosin for histological examination.

Classification of Outgrowths. The outgrowths that resulted from the injection of dissociated cells were classified according to the scheme used previously (13).

RESULTS

Growth of Cells Embedded in Collagen Gels. Mammary epithelial cells from normal virgin or pregnant (BALB/cfC3H/Crlg and C57BL) mice, BALB/c D2 nodule outgrowth line, and BALB/cfC3H mammary tumors grew as 3-dimensional colonies inside the collagen gels (Figs. 1 and 2). The colonies from normal or transformed mammary tissues were not distinguishable morphologically (Figs. 1 and 2). Cells embedded in collagen gels increased in number over the 10- to 14-day culture period in the presence of 25% swine serum + cholera toxin (0.01 μg/ml), as determined by DNA assay (Chart 1).

Transplantation of Mouse Mammary Epithelial Cells Grown in Collagen Gels. The effect of culture on the number of outgrowths produced by a given number of injected cells was determined. Noncultured cells (5 x 10^4, 10^5, and 5 x 10^6), cells grown for 10 to 14 days in collagen gels, and cells maintained as monolayers for 2 days were injected into gland-free fat pads of syngeneic mice, and the number of outgrowths was enumerated (Table 1). To achieve a high percentage of mammary outgrowths, a greater number of cells grown in collagen gels is required than either noncultured or monolayer cultured cells.

Morphology of Mammary Outgrowths. Table 2 shows the types of outgrowth morphologies recovered when mammary cells from various sources were cultured inside collagen gels and subsequently transplanted. C57BL mammary cells produced ductal outgrowths (Fig. 3) that were morphologically indistinguishable from the virgin host’s own glands (Fig. 3A) or from ductal outgrowths derived from noncultured dissociated cells. Cells from BALB/cfC3H midpregnant mice produced ductal, lobuloalveolar, or mixed outgrowths. Cells from BALB/cfC3H parous nonpregnant mice produced ductal, lobuloalveolar and ductal, and mixtures of lobuloalveolar and tumor outgrowths (Fig. 4). Lobuloalveolar outgrowths have been shown to be nodule-transformed populations (9). The BALB/c D2 nodule outgrowth line has a lobuloalveolar morphology and also produced lobuloalveolar outgrowths when transplanted after culture (Fig. 5). When cells from BALB/cfC3H mammary adenocarcinomas were cultured and transplanted, the tumors...
found at the injection sites were also mammary adenocarci-
nomas (Fig. 6). In general, mammary cells grown inside colla-
gen gels for 10 to 14 days gave rise to outgrowths similar to
those from noncultured cells or those cultured on monolayer
for 2 days and are representative of the tissue of origin.

Effects of Pregnancy and Lactation on Outgrowths De-
rived from Mammary Cells Grown in Collagen Gels. Although
hormones have been demonstrated to be growth promoting for
mammary epithelial cells in vivo, mammogenic hormones have
not been demonstrated to be growth promoting in the collagen
gel system (23, 26, 27). To determine whether the cells grown
in collagen gels retained the ability to respond to the hormones
of pregnancy and lactation in vivo, C57BL mammary cells
grown in collagen gel were injected into the gland-free mam-
mary fat pads of syngeneic female mice. Eight to 10 weeks
after transplantation, the host mice were examined for ductal
outgrowths by injection with trypan blue and observation of the
mammary fat pads was made by means of a dissecting micro-
scope. Mice with ductal outgrowths (Fig. 3) were mated and
allowed to go through a pregnancy and/or lactation. During
pregnancy or lactation, host mice were terminated. Mammary
outgrowths derived from cultured cells responded to the host’s
endogenous hormones of pregnancy and lactation in a manner
similar to the host’s own mammary glands. During pregnancy,
the ductal outgrowths became more branched and gave rise to
lobuloalveolar structures (Figs. 7 and 7A). During lactation, the
lobuloalveolar structures became full of milk-like secretion
(Figs. 8 and 8A).

DISCUSSION

These data demonstrate that the collagen gel provides a
suitable matrix for the growth of normal, dysplastic, and neo-
plastic mouse mammary epithelial cells. Mammary epithelial
cells can be recovered from the collagen gels and transplanted
into the gland-free mammary fat pads of syngeneic mice. The
cells gave rise to outgrowths, which were phenotypically similar
to outgrowths from noncultured cells, and ductal outgrowths
from normal cells retained the capability to respond, like in situ
mammary tissues, to the endogenous hormones of the host.

DeOme et al. (7) demonstrated that the transplantation of
pieces of normal mammary tissues into the gland-free fat pads
of syngeneic mice produced outgrowths that were character-
istic of the type of tissue transplanted. In addition, the out-
growths were responsive to endogenous hormones of the host
mouse, and their development paralleled that of the host’s own
glands. Normal mammary tissues maintained in organ cultures,
in which growth is limited, produced normal outgrowths either
when transplanted as pieces (2) or when dissociated into a cell
suspension (21).

### Table 1

<table>
<thead>
<tr>
<th>Time in culture (days)</th>
<th>No. of cells transplanted</th>
<th>No. of outgrowths/No. of transplants</th>
<th>% of take</th>
</tr>
</thead>
<tbody>
<tr>
<td>Noncultured</td>
<td>$5 \times 10^4$</td>
<td>13/18</td>
<td>72</td>
</tr>
<tr>
<td></td>
<td>$5 \times 10^6$</td>
<td>8/8</td>
<td>100</td>
</tr>
<tr>
<td>2 (monolayer)</td>
<td>$5 \times 10^4$</td>
<td>10/16</td>
<td>63</td>
</tr>
<tr>
<td></td>
<td>$5 \times 10^6$</td>
<td>9/13</td>
<td>69</td>
</tr>
<tr>
<td>10-14 (collagen gel) + 2 (monolayer)</td>
<td>$5 \times 10^4$</td>
<td>0/12</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>$5 \times 10^6$</td>
<td>2/12</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>$5 \times 10^8$</td>
<td>9/14</td>
<td>64</td>
</tr>
</tbody>
</table>

* Obtained from BALB/cfC3H midpregnant mice.

### Table 2

**Morphology of mammary outgrowths**

<table>
<thead>
<tr>
<th>Origin of tissue</th>
<th>Morphology of outgrowths (noncultured)</th>
<th>Morphology of outgrowths (cultured)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL (virgin)</td>
<td>Ductal</td>
<td>Ductal</td>
</tr>
<tr>
<td>C57BL (pregnant)</td>
<td>Ductal + lobuloalveolar</td>
<td>Ductal + lobuloalveolar</td>
</tr>
<tr>
<td>BALB/cfC3H (midpregnant)</td>
<td>Ductal + lobuloalveolar</td>
<td>Ductal + lobuloalveolar</td>
</tr>
<tr>
<td>BALB/cfC3H (parous)</td>
<td>Lobuloalveolar + tumor</td>
<td>Lobuloalveolar + tumor</td>
</tr>
<tr>
<td>BALB/c (D2 nodule line)</td>
<td>Lobuloalveolar + tumor</td>
<td>Lobuloalveolar + tumor</td>
</tr>
<tr>
<td>BALB/cfC3H (mammary tumor)</td>
<td>Mammary adenocarcinoma</td>
<td>Mammary adenocarcinoma</td>
</tr>
</tbody>
</table>

* Derived from cells enzymatically dissociated and not cultured.

** Chart 1. Growth of mammary epithelial cells from virgin, midpregnant, or mammary tumor-bearing mice inside collagen gels. Stippled bars (TO), number of cells placed in each well. Cross-hatched bars, number of cells at the end of 10 days (10 d) or 14 days (14 d). The growth of epithelial cells is variable between experiments and usually a 5- to 20-fold increase in cell number is observed. Bars S.D. **
Mouse mammary cells have been cultured and transplanted by other researchers. Anderson et al. (1) established an in vitro cell line from the transplantable outgrowth line D2. This cell line had epithelial characteristics in vitro but had no tumorigenic potential when transplanted into the gland-free mammary fat pads, although the morphology of the outgrowths was not described. Butel et al. (3) established epithelial cell lines from mouse mammary tumors which induced epithelial tumors when injected s.c. into mice. In this study, we have described an in vitro-in vivo system in which normal mammary cells can be propagated in primary culture and following transplantation produce outgrowths morphologically similar to their tissues of origin and retain their ability to respond, like the host's own mammary glands, to endogenous hormones.

Culture inside collagen gels decreases the transplantability of the mammary cells, since it is necessary to transplant a greater number of cells to achieve the same percentage of takes as noncultured cells or cells maintained as monolayers for 2 days. One possible explanation for our observations is that because of enhanced growth in vitro, the cells lose some ability to proliferate after transplantation. Serial transplantation of mouse mammary epithelium in vivo has been reported to cause a decline in growth rate, and the decline was related to the number of cell divisions undergone (5, 6).

We have not determined whether the mammary cells have a finite proliferative potential, as has been demonstrated for some cell lines (14), or lose their ability to grow because of cells becoming terminally differentiated, since the cells are grown in an undefined medium.

An alternate explanation is that the cells in culture acquire an enhanced immunogenicity because of the heterologous sera included in the media, as has been demonstrated in other culture systems (16, 18). In our experiments, dissociated cells were placed in monolayer for 2 days in the presence of swine serum and compared with cells grown inside collagen gels and then placed on monolayer. Thus, both groups were exposed to swine serum, but the transplantability of collagen gel-cultured cells remained less than the cells grown in monolayer.

Enzymatic dissociation of mammary tissues from mammary tumor virus-infected mice (9, 10), chemical carcinogen-treated mice (13), or preneoplastic mammary nodule lines (17) increases the recovery of outgrowths containing either ductal dysplasias, hyperplastic alveolar nodules, or mammary tumors and are characteristic of the original tissue. When mammary cells, either normal or mixtures of normal and transformed cells, were cultured inside collagen gels and then transplanted, they gave rise to outgrowths with the same phenotype as those derived from cells not cultured inside collagen gels. For example, when cells from parous BALB/cfC3H mice were cultured, they produced mixed outgrowths containing normal and transformed populations identical to noncultured cells. This suggests that the phenotypes of the cells that were placed inside the collagen gels were maintained, and little or no selection or spontaneous transformation occurred during the culture period. Daniel and DeOme (4) cultured mouse mammary tissue in monolayer from 6 days to 6 weeks and transplanted the cells into the gland-free fat pads of syngeneic mice. Some of the mice used were C57BL/Crl mice from the same colony as was used in our study. A variety of abnormal outgrowths developed from the cultured cells, including lobuloalveolar structures and ductal abnormalities in virgin hosts. Cells from C57BL/Crl mice grown in collagen gels have not produced similar abnormalities in vivo. However, cell proliferation is greater in collagen gels than in monolayers (25, 27).

Tumors developed from the D2 nodule outgrowth line by the end of the 8-week transplant period in the experiments reported here. The D2 outgrowth line was established here at the Cancer Research Laboratory by DeOme and Medina (8) in 1969 and has been maintained by serial transplantation. In our studies, the D2 nodule outgrowth line produced tumors at a higher incidence than did that reported by Medina et al. (17). We do not know whether selection or divergence has occurred in our D2 outgrowth line as compared with that maintained by Medina.

The collagen gel system provides a system in which growth of normal mammary epithelial cells can be sustained. Transplantation of these cells to the gland-free mammary fat pad provides evidence that, after collagen gel culture, the mammary cells retain their original phenotype (normal, hyperplastic alveolar nodule, or tumor) and are capable of responding to mammogenic and lactogenic hormones in vivo and that when mammary tissues containing a mixture of normal and transformed cells are cultured they produce a mixture of normal and transformed outgrowths when transplanted. Recently, Flynn (11) has demonstrated that mouse mammary epithelial cells can be grown inside collagen gel and with the appropriate hormonal stimulation can be shown to undergo differentiation in terms of casein production. The ability to grow mammary epithelial cells in vitro and to analyze the effects of carcinogens or substances that may enhance transformation among these cell populations provides a useful model for studying the process of transformation of mammary epithelial cells.

ACKNOWLEDGMENTS

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REFERENCES

Hemeoxylin, x 10.

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normal mammary gland from an early pregnant mouse. The host mouse, however, was a virgin female BALB/c animal. Hematoxylin. x 16.5.

17. Medina, D., Shepherd, F., and Gropp, T. Enhancement of the tumorigenicity


Fig. 1. Three-dimensional colony inside the collagen gel matrix composed of mammary epithelial cells from BALB/cfC3H midpregnant mice after 8 days in culture. × 48.

Fig. 2. Three-dimensional colonies inside the collagen gel matrix composed of mammary tumor cells from BALB/cfC3H mice after 8 days in culture. × 48.

Fig. 3. Whole-mount preparation of a mammary fat pad with normal ductal outgrowth 8 weeks after injection of C57BL virgin mouse cells cultured in collagen gels. Note the structural similarity to the virgin host’s own gland (3A). Hematoxylin, × 10.

Fig. 3A. Whole-mount preparation of a piece of the virgin host’s thoracic mammary gland. Hematoxylin, × 10.

Fig. 4. Mixed lobuloalveolar and tumor (arrow) outgrowth 8 weeks after injection of BALB/cfC3H nonpregnant parous cells. A portion of the outgrowth resembles normal mammary gland from an early pregnant mouse. The host mouse, however, was a virgin female BALB/c animal. Hematoxylin, × 16.5.

Fig. 5. Lobuloalveolar outgrowth 8 weeks after injection of BALB/cD2 nodule outgrowth line cells cultured in collagen gels. Hematoxylin, × 10.

Fig. 6. Histological section of an adenocarcinoma produced by collagen gel-cultured BALB/cfC3H mammary tumor cells. H & E, × 100.

Fig. 7. Whole-mount preparation of an outgrowth from C57BL mammary cells in a pregnant host. Note the similarity to the host’s own gland (7A). Hematoxylin, × 10.

Fig. 7A. Whole-mount preparation of a piece of the pregnant host’s thoracic mammary gland. Hematoxylin, × 10.

Fig. 8. Whole-mount preparation of an outgrowth from C57BL mammary cells in a lactating host (LN, lymph node). Note the similarity to the host’s own gland (8A). Hematoxylin, × 10.

Fig. 8A. Whole-mount preparation of a piece of the lactating host’s thoracic mammary gland. Hematoxylin, × 10.
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