Growth of Nontumorigenic Cells in Millipore Diffusion Chambers Implanted in Mice and Implications for in Vivo Growth Regulation

Lola M. Reid, Charles D. Stiles, Milton H. Saier, Jr., and Michael J. Rindler

ABSTRACT

We have been studying the growth regulation of nontumorigenic versus tumorigenic cells in vitro and in vivo. In these studies, we have found that a variety of mammalian cell cultures which are nontumorigenic in athymic nude mice or in syngeneic hosts grow exponentially if implanted in vivo within sealed Millipore diffusion chambers. The growth rates of the cells in vivo within the diffusion chambers were comparable to those observed in vitro. Moreover, the cells grown in vivo within the chambers exhibited density-dependent growth inhibition which was similar, at least superficially, to the in vitro phenomenon.

The ability of the nontumorigenic cells to grow when implanted in vivo within diffusion chambers was correlated with and was thought due, in part, to the formation of serous clots within the implanted chambers. Studies of the clots by light, scanning electron, and electron microscopy showed cells throughout the clot and adherent to the clot fibers. We tested the growth of each of the cell lines for dependence on serum growth factors by observing the ability of the cells to grow in medium supplemented with serum versus defibrinogenated, platelet-poor plasma (PPP). BALB/c-3T3 cells and other nontumorigenic, fibroblast-like cell lines failed to proliferate in vitro when defibrinogenated, PPP was substituted for serum, demonstrating a requirement by the cells for growth factors unique to serum. Virally transformed, tumorigenic fibroblasts grew in media supplemented with either PPP or serum. Thus, for these fibroblast-like cell lines, the ability of the cells to grow in PPP is correlated with tumorigenicity in athymic nude mice.

Since the interstitial fluid surrounding cells in vivo resembles PPP more than serum and since many of the nontumorigenic fibroblast-like cell lines demonstrated a requirement for serum growth factor, the growth of these cell lines within the implanted diffusion chambers suggests that serum growth factors might be required for growth of such cells in vivo and that serum growth factors can accumulate within the diffusion chambers. Analysis of the serous clot material provided evidence to corroborate this hypothesis. The fluid from implanted chambers can stimulate growth of BALB/c-3T3 cells. The growth factor activity of the serous chamber fluid is more comparable to that of mouse serum than to that of mouse PPP.

Our studies suggest that two of the possible factors provided by implanted Millipore chambers in vivo and required for growth of nontumorigenic fibroblast-like cells are: (a) a substrate for cell anchorage provided in this case by the fibrous clots and by the nitrocellulose surface of the Millipore filters; and (b) growth factors which accumulate within the microenvironment of the diffusion chamber implants.

INTRODUCTION

The congenitally athymic nude mouse (23, 33, 35) is deficient in thymus-dependent immune function and has been shown to accept skin xenografts from animals as phylogenetically remote as chicken and lizard (19). Primary cultures of mammalian cells and many established animal cell lines can be transplanted into nude mice with a minimal likelihood of immunological rejection (12, 26-28, 34, 41). Studies on the growth of these cultured cells in nude mice may, therefore, generate information relevant to the growth regulation of normal tissue.

Many studies have tried to find a particular in vitro property which is causally related to malignant transformation. In vitro phenotypes associated with neoplastic growth of cells include loss of density-dependent inhibition, growth in low serum concentrations, and anchorage-independent growth (12, 14, 18, 39, 41, 43, 46). Although there is no known single correlate to tumorigenicity, studies by several investigators show that the tumorigenicity of cell lines in nude mice is significantly related to anchorage-independent growth; cells which are tumorigenic in nude mice will almost always proliferate under anchorage-independent conditions in vitro.

In preliminary studies attempting to find in vivo conditions in which nontumorigenic cell lines would grow, we discovered that a variety of tumorigenic and nontumorigenic cells proliferate in vivo if enclosed within Millipore diffusion chambers by the technique of Algire and Legallais (2) implanted s.c. or i.p. in mice. A loose gelatinous clot was found to form within the chamber, and the cells were observed to attach to the clot fibers and to proliferate throughout the clot. The presence of the fibrin clot initiated interest in the role of clot formation in the stimulation of growth of the cells. A preliminary report of this work has been presented (29).

MATERIALS AND METHODS

Cell Cultures. The cell lines and cell strains used in this study are listed with their origins and appropriate literature references in Table 1. Cell culture media, balanced salt solutions, and animal sera were obtained from Grand Island Biological Company, Grand Island, N. Y., unless otherwise indicated. All cell cultures were routinely grown in Ham's F-10 or DME6.

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2 Supported by NIH Postdoctoral Fellowship Award 5-F22-AM00274. Present address: Department of Molecular Pharmacology, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, N. Y. 10461. To whom requests for reprints should be addressed.
3 Supported by NIH Postdoctoral Fellowship Award DE-03366. Present address: Sidney Farber Cancer Institute, 44 Binney Street, Boston, Mass. 02115.
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supplemented with 12.5% horse serum and 2.5% fetal bovine serum. All cell lines were screened for Mycoplasma by the uridine/uracil ratios test (36) prior to use and were negative for infection by this test.

**Mice.** BALB/c mice were obtained from Strong Laboratories, La Jolla, Calif. A colony of athymic "nude" mice was established with breeding stock obtained from the Bomholtgard Ltd., Denmark, and maintained as described previously (28, 41).

**Millipore Diffusion Chambers.** Diffusion chambers were assembled from 13-mm Lucite rings sealed with Millipore filters (HAWP01300) with a pore diameter of 0.45 μm. Rings, filters, and glue were obtained from the Millipore Company.

The filters were boiled in distilled water for 1 to 2 min and then glued onto the rings. Sealed chambers were sterilized by exposure to 5,000 rads of 60Co irradiation. The cells were prepared as single-cell suspensions in serum-free medium and were seeded into the chambers through a needle hole in the Lucite ring. The hole was then sealed with glue.

**Measurement of Growth Rates in Vivo.** Identical aliquots of 104 to 106 cells were seeded into the chambers as described, and the chambers were implanted i.p. or s.c. into the mice (identical results were obtained with either i.p. or s.c. implantation). Empty chambers were implanted into some mice for the purpose of background subtraction. At periodic intervals, mice were killed, and the chambers were recovered. Mouse mesenchymal cells adhering to the outside of the chambers were removed first by wiping the chamber with a Kimwipe and then by incubating the sealed chambers for 1 hr in 0.1% trypsin solution. The chambers were then thoroughly rinsed with Dulbecco’s modified PBS, placed in fresh trypsin, and opened. This pretreatment of the sealed chambers with trypsin always solubilized a portion of the clot within. The cells released from the clot were counted immediately by the trypan blue exclusion test to assay both viability and cell number. Despite the long trypsin treatment of the sealed chamber, more than 90% of the cells released from the clot excluded trypan blue dye. The clot material remaining within the chamber was then digested with 0.1% trypsin for up to 12 hr. Since the clots required so many hours to be totally digested and since cells left in trypsin for more than 1 hr would lyse, the clots were left in trypsin for 1-hr intervals and then rinsed with Dulbecco’s modified PBS; the cells released were counted by both the Coulter electronic cell counter and the trypan blue exclusion test for viability and cell number. After each hourly treatment, if there was any clot remaining, the trypsin treatment, rinsing, and counting procedures were repeated. Thus, the cells within the clot were gradually released over a 5- to 12-hr period. The total cell number in the chamber was then the sum of the counts from all the separate determinations.

Empty Millipore chambers which were inoculated with serum-free medium only, were implanted in the mice and subsequently processed just as were the chambers containing cells. The empty chambers always yielded a low but significant cell count due to mouse mesenchymal cells not removed by the initial trypsin digestion; these background counts were subtracted from the total for the cell-filled chambers.

All of the cell lines grown in the chambers in vivo were cultured following their dissociation from the clot, and their identity with respect to the cells injected confirmed by karyotype and morphological studies. To ensure that growth of the cells in the chambers did not result from malignant transfor-
and either mouse serum, fetal bovine serum, mouse plasma, or the fluid collected from the Millipore chambers implanted in the mice. All cultures were incubated with \(^{3}{}^{3}H\)thymidine (5 µCi/ml) for 48 hr and then processed for autoradiography as described by Scher et al. (35).

RESULTS

The Growth of Cell Cultures within Millipore Chambers in Vivo. Secondary cell cultures of mouse and of human embryo cells do not form tumors in nude mice. Also, many animal cell lines established from nonneoplastic tissue do not form tumors in adult nude mice including the MDCK (dog kidney), BRLC (rat liver), 31A (rat ovary), and BALB/c-3T3 cell lines (39). Table 1 provides a listing of cell cultures tested in this study for their ability to grow in vivo when enclosed within Millipore chambers. The growth response in the chambers occurs irrespective of the ability to form tumors when injected into athymic nude mice or into natural hosts.

Two features of the growth curves in Chart 1 are worthy of comment. (a) A phenomenon superficially analogous to density-dependent growth inhibition in vitro occurs within Millipore chambers in vivo. The cell population ceases to increase at a point long before the chambers are filled to capacity with

Table 1

<table>
<thead>
<tr>
<th>Designation</th>
<th>Origin</th>
<th>Morphology</th>
<th>Tumorigenicity in nude mice</th>
<th>Comments and literature references</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary cultures</td>
<td>Human pituitary adenomas</td>
<td>Epithelioid</td>
<td>No</td>
<td>Single-cell suspensions from surgical specimens inoculated into chambers. Cells proliferated for 1 to 2 passages in mice, but then growth ceased and did not reinitiate with transplantation or in vitro culturing. The carcinomas proliferated indefinitely (27, 28).</td>
</tr>
<tr>
<td>Human prostate, benign prostatic hyperplasia</td>
<td>Epithelioid</td>
<td>No</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human prostatic carcinomas</td>
<td>Epithelioid</td>
<td>Yes</td>
<td></td>
<td></td>
</tr>
<tr>
<td>BALB/c</td>
<td>Mouse embryo</td>
<td>Fibroblast-like</td>
<td>No</td>
<td>Embryonic cell strain derived from disaggregated mouse embryos.</td>
</tr>
<tr>
<td>HFL-Johnson</td>
<td>Human fetal lung</td>
<td>Fibroblast-like</td>
<td>No</td>
<td>Cells from fetus afflicted with inborn metabolic disorder, cystinosis. Cells donated by J. Schneider, University of California, San Diego.</td>
</tr>
<tr>
<td>5NA10</td>
<td>Human skin</td>
<td>Fibroblast-like</td>
<td>No</td>
<td>Cells from normal, adult skin (shoulder). Cells donated by J. Larner, University of Virginia, Charlottesville, Va.</td>
</tr>
<tr>
<td>Cell lines</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>31A</td>
<td>Rat ovary</td>
<td>Epithelioid</td>
<td>No</td>
<td>Cloned cell line from hyperplastic rat ovary grown in spleen (9).</td>
</tr>
<tr>
<td>BRLC</td>
<td>Baby rat liver</td>
<td>Epithelioid</td>
<td>No</td>
<td>Cloned cell line from normal rat liver (11).</td>
</tr>
<tr>
<td>MDCK</td>
<td>Dog kidney</td>
<td>Epithelioid</td>
<td>No</td>
<td>Cloned cell line from normal dog kidney, American Type Culture Collection CCL 34. The cells form nodules (but not tumors) in baby nude mice and do not form nodules or tumors in the adults.</td>
</tr>
<tr>
<td>BALB/c-3T3</td>
<td>Mouse embryo</td>
<td>Fibroblast-like</td>
<td>No</td>
<td>Cloned line from disaggregated 14-17-day-old mouse embryos (1).</td>
</tr>
<tr>
<td>VA2-aza-G'</td>
<td>Human embryonic lung</td>
<td>Fibroblast-like</td>
<td>No</td>
<td>SV40-transformed W18 cells selected for resistance to 8-azaguanine (45). The cells do not form tumors in nude mice within 4 mos. after injection (42).</td>
</tr>
<tr>
<td>SVT2</td>
<td>Mouse embryo</td>
<td>Fibroblast-like</td>
<td>Yes</td>
<td>SV40-tranformed mouse embryonic cells. Developed by S. A. Aaronson, NIH, Bethesda, Md.</td>
</tr>
<tr>
<td>Y-1</td>
<td>Mouse adrenal tumor</td>
<td>Epithelioid</td>
<td>Yes</td>
<td>Steroid-secreting cell line from an adrenal cortical carcinoma of a male LAFI mouse (48).</td>
</tr>
<tr>
<td>3B4</td>
<td>Mouse adrenal tumor</td>
<td>Epithelioid</td>
<td>Yes</td>
<td>Cyclic 3',5'-AMP-resistant cell line derived from Y-1 (20).</td>
</tr>
</tbody>
</table>

\( ^{a} \) Tumorigenicity studies done by Reid et al. (27) and by Reid and Shin (28).

\( ^{b} \) Tumorigenicity studies by Stiles et al. (41).

\( ^{c} \) Tumorigenicity of these cell cultures assayed for this study. BALB/c nude mice (5 mice/group) were given injections of \( 10^{6} \) cells s.c. and examined regularly for tumors for 4 months.

\( ^{d} \) Tumorigenicity studies by Masui et al. (20).
material. The relative saturation densities of these cell lines cultured in Millipore diffusion chamber implants are the same as those observed for growth in tissue culture in a previous study (41). (b) As shown in Chart 1, growth within the chambers is exponential. The population-doubling times (hr) in all cases are similar to those measured when the cells were cultured in vitro in DME with 10% fetal bovine serum.

Morphological Studies on Diffusion Chamber Clots. Diffusion chambers removed from mice contain a loose gelatinous clot. This clot is formed within 10 hr after implantation. The amount of clot material found within the chambers is independent of the cell type sealed within the chambers. Chambers implanted without cells accumulate the same amounts of clot material. Tissue culture cells sealed within the chambers are embedded within the clot (see Fig. 1) and are found proliferating throughout it. Studies of the clot by scanning and transmission electron microscopy show the association of the cells on clot fibers and the presence of cells throughout the clot.

The Requirement for Serum-specific Growth Factors. In previous studies, s.c. inoculation of BALB/c embryo fibroblasts and BALB/c-3T3 cells did not form tumors in athymic nude mice with the BALB/c genetic background. Chart 2 depicts the requirement for serum-specific growth factors expressed by nontumorigenic fibroblasts and “fibroblast-like” cells such as BALB/c-3T3. The cell line SVT2, an SV40-transformed derivative of BALB/c embryo fibroblasts, is highly tumorigenic in athymic nude mice and grows equally well in either plasma- or serum-supplemented media (Chart 2). SVT2 is representative of the tumorigenic, fibroblast-like cell lines studied in its lack of requirement for serum-specific growth factors. The requirement for serum-specific growth factors is not a universal characteristic of nontumorigenic cells. The “epithelioid-like” cell line, MDCK, grows equally well in plasma- or serum-supplemented media (Chart 2) although it is not tumorigenic in adult nude mice (41).

DISCUSSION

We have found that a wide range of primary and secondary cell cultures as well as a number of cell lines, whether tumorigenic or not in immunosuppressed hosts, will proliferate exponentially in vivo within sealed Millipore diffusion chambers. The growth of the cells within the chambers is associated with and is thought to be due to the formation of a fibrous clot (assumed to be fibrin) which provides anchorage and growth factors, both thought to be essential for the growth of normal cells but not for tumorigenic cells. The evidence for this is discussed below.

A number of nontumorigenic and nontransformed, fibroblast-like cells such as BALB/c-3T3 cells were shown to require

Growth Factor Activity of PRS, PPP, and DF from Mice. Chart 3 reveals how PRS, PPP, and DF from mice compared as to their ability to initiate replicative DNA synthesis in quiescent cultures of BALB/c-3T3 cells. For reference, the mitogenic activity of these agents was compared to that of fetal bovine serum.

The addition of fresh fetal bovine serum to levels of 10% stimulated nearly 80% of the BALB/c-3T3 cells to undergo an additional round of replicative DNA synthesis as shown by autoradiography (Chart 3). When fresh bovine serum was added to final concentrations of 30% or greater, 100% of the 3T3 cells underwent a fresh round of division; to maintain viable cell cultures in 30 and 100% fetal bovine serum, it was necessary first to dialyze the serum against DME. Mouse PRS proved to be a more potent source of growth factor activity than did fetal bovine serum for BALB/c-3T3 cells (Chart 3). By comparison to mouse PRS, mouse PPP was only weakly mitogenic. Relative to mouse PPP, the diffusion chamber fluid that accumulated within diffusion chamber implants was significantly enriched in growth factor activity and was roughly comparable to fetal bovine serum as a growth stimulator.
growth factors found in PRS but not in PPP for growth in vitro. Our data corroborate those of others (4, 15, 40) in the observation that tumorigenic fibroblasts require reduced amounts of the PRS-specific growth factor(s) and grow equally well in media supplemented with either PRS or PPP. The observation that nontransformed, nontumorigenic cells require PRS-specific growth factor(s) for growth in vitro seems true for rodent and human "fibroblast-like" cells but not for "epithelioid-like" cells. For example, MDCK which is nontumorigenic in adult male mice is an epithelial cell line that grows equally well in plasma or serum. Further studies exploring this phenomenon have been done by Scher et al. (35) and Pledger et al. (25).

The fibroblast growth activity is normally released during the formation of blood clots (3, 4, 16, 17, 32). The interstitial fluid surrounding cells in vivo probably resembles PPP more than PRS with respect to its content. Nontumorigenic "fibroblast-like" cells do not grow in PPP-supplemented culture medium in vitro. Nevertheless, these same nontumorigenic fibroblast-like cells grow very well within Millipore diffusion chamber implants in vitro. The paradoxical growth of nontumorigenic cells in vivo within Millipore diffusion chambers (29) suggests that cells within the diffusion chambers are (a) exposed to higher local concentrations of growth factors such as those found in PRS and/or (b) better able to respond to constitutive levels of growth factor activity within the interstitial milieu by virtue of their anchorage to an insoluble substrate. Support for the presence of growth factor activity within the diffusion chamber implants comes from experiments in which cells unable to grow in PPP were stimulated to proliferate in vitro by the addition of diffusion chamber fluid to the culture medium.

The findings presented here show that growth of nontumorigenic cells within Millipore chambers could be promoted both by the anchorage afforded by the gelatinous clots within the diffusion chambers and by the localized concentration of growth factors in the diffusion chamber fluid. Since tumorigenic cells tend to be anchorage independent for growth in vitro and since our studies show that certain tumorigenic cells in addition to being anchorage-independent have lost the requirement for certain growth factors (in the case of fibroblast-like cells, serum mitogens), it is possible that malignancy of a cell may be correlated with multiple factors or that some factors may substitute for others in tumorigenic mechanisms.

A wide range of insoluble materials including Bakelite (44), metal foils (22), plastic discs, and sheets of Teflon (7, 8, 10, 21) have been shown to induce sarcomas when implanted into animals. Boone et al., 5, 6, have demonstrated that non-tumorigenic cell lines such as BALB/c-3T3 produce malignant hemangiomas when implanted attached to glass beads. In man, asbestosis dust appears to be a potent carcinogen or cocarcinogen for lung neoplasms (37). The induction of tumors by this heterogeneous group of insoluble agents is puzzling and not obviously reconcilable with only the mutagenic mode of action (3). A hypothesis worthy of further evaluation is that the induction of sarcomas by inert materials and the growth of nontumorigenic cells in vivo within diffusion chambers may be manifestations of the same phenomenon, a localized release of serum-like "wound hormones" in response to chronic irritation. Local concentrations of growth factors and the anchorage to the plastics could act as promoters in the carcinogenic process.

The observation that all cell cultures tested will grow within diffusion chambers implanted in vivo may have practical applications in addition to the implications for in vivo growth regulation. Diffusion chamber implants could be used to establish continuous cell lines from primary explants of normal, benign, or malignant tissues or to transplant functioning cells such as pancreatic islet cells into patients to provide the patient with a source of a hormone or factor missing in the patient due to disease. Diffusion chamber implants could also be used in experimental cancer therapy studies. By implanting biopsy material from a patient's malignant tumor into chambers, implanting the chambers into mice, treating the mice with various therapeutic regimens, and evaluating their effects on the cells within the chambers, one could ascertain within weeks an appropriate chemotherapeutic or hormonal treatment for that patient.

REFERENCES


Growth Regulation of Cultured Animal Cells
Fig. 1. BALB/c-3T3 cells contained within the fibrous clots which formed in diffusion chambers implanted in mice. A, phase microscopy of BALB/c-3T3 cells within a clot removed from a diffusion chamber. Arrow, edge of the clot. It is still attached to one of the filters from the diffusion chamber. X 350. B, scanning electron microscopy of 3T3 cells within a clot from a diffusion chamber. Note the fibrous nature of the clot and the 3T3 cells (arrows) attached to the surface. X 1000.
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