A Qualitative and Quantitative Assay for Cells Lacking Postconfluence Inhibition of Cell Division: Characterization of This Phenotype in Carcinogen-treated Syrian Hamster Embryo Cells in Culture

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ABSTRACT

We have developed a qualitative and quantitative assay system for detecting cells lacking postconfluence inhibition of cell division (contact insensitivity, CS*) in golden Syrian hamster embryo cells in culture by measuring the number of cells able to form colonies on a lethally irradiated, confluent monolayer of a contact-sensitive established cell line. A subpopulation in normal low-passage cultures of golden Syrian hamster embryo cells temporarily exhibits this CS* phenotype at very low frequency (\(\sim 4 \times 10^{-2}\)) but quickly loses the property within a few passages in vitro. This phenotype is invariably exhibited by various tumorigenic cell lines at very high frequency (7 to 50 \(\times 10^{-2}\)) and appears to correlate with the anchorage-independent growth phenotype. The temporal acquisition of the CS* phenotype by tertiary-passage golden Syrian hamster embryo cells following exposure to \(N\)-methyl-\(N\)'-nitro-\(N\)'-nitrosoguanidine was examined. Cells with a stably heritable CS* phenotype are detected after approximately 20 posttreatment population doublings. In contrast, anchorage-independent cells are not detected until 35 to 95 posttreatment population doublings. These CS* cells appear to be preneoplastic cells, since clonally isolated CS* cells did not exhibit anchorage-independent growth until after further passageing in vitro. The results suggest that acquisition of the CS* phenotype represents an early stage in neoplastic progression.

INTRODUCTION

Tumor development in vivo is a progressive, multistep process which occurs through qualitatively different stages (13, 14). Correspondingly, evidence indicates that both chemically and virally induced neoplastic transformation in vitro is a multistep, progressive process, which cannot be described adequately by a simple extrapolation of a single-gene mutational event (Refs. 6 and 12; Footnote 4). The phenomenon of progression in normal low-passage cultures of golden Syrian hamster embryo cells temporarily exhibits this CS* phenotype at very low frequency (\(\sim 4 \times 10^{-2}\)) but quickly loses the property within a few passages in vitro. This phenotype is invariably exhibited by various tumorigenic cell lines at very high frequency (7 to 50 \(\times 10^{-2}\)) and appears to correlate with the anchorage-independent growth phenotype. The temporal acquisition of the CS* phenotype by tertiary-passage golden Syrian hamster embryo cells following exposure to \(N\)-methyl-\(N\)'-nitro-\(N\)'-nitrosoguanidine was examined. Cells with a stably heritable CS* phenotype are detected after approximately 20 posttreatment population doublings. In contrast, anchorage-independent cells are not detected until 35 to 95 posttreatment population doublings. These CS* cells appear to be preneoplastic cells, since clonally isolated CS* cells did not exhibit anchorage-independent growth until after further passageing in vitro. The results suggest that acquisition of the CS* phenotype represents an early stage in neoplastic progression.

Several in vitro growth properties associated with neoplastic transformation, including morphological transformation (7), enhanced fibrinolytic activity (18, 27, 28), and anchorage independence (5, 15, 20, 22), have been well characterized, and their temporal acquisition during neoplastic progression has been studied (4, 6). For the system of GSHE cells, both morphological transformation and enhanced fibrinolytic activity are expressed within 2 weeks after carcinogen treatment (4), while cells capable of growth in soft agar, a property highly correlated with tumorigenicity (5, 20, 33), are not detected until 32 to 75 population doublings (6). Another important malignant growth property, the lack of contact-mediated growth control (contact insensitivity, CS*), which allows autonomy from the growth-regulatory effect of contact with normal cells, has been reported to correlate well with tumorigenicity in many tumorigenic systems (1, 23, 29, 37). Qualitatively, colony-forming ability on confluent monolayers of contact-inhibited cells has been used as an indicator of the lack of contact inhibition of cell division. In mouse cells, there is a strong correlation between tumorigenicity and the ability to form colonies on CS* confluent monolayer of BALB/3T3 cells (1), leading to the use of this assay as an in vitro parameter indicative of malignancy (30). In human cells, this method has been used for distinguishing normal cells from tumor-derived CS* variants that have not been characterized, and the temporal acquisition of this phenotype during neoplastic progression has not been defined.

In this paper, we report the application of this method to the development of a qualitative and quantitative assay system for the detection and selection of CS* cells in GSHE cell cultures. Using this method, the temporal acquisition of CS* phenotype in GSHE cells following carcinogenic perturbation was examined in relation to the acquisition of anchorage independence.

MATERIALS AND METHODS

Cells and Growth Medium. GSHE cell cultures (Preparation 17F) were established from 13-day gestation fetuses collected aseptically by cesarean section from inbred Syrian hamsters (Charles River Lakeview Hamster Colony, Newfield, N. J.). Pooled primary cultures from littermates were stored in liquid nitrogen. Secondary cultures were initiated from the frozen stocks, and all transformation experiments were performed with tertiary- or later-passage cultures. Various tumorigenic cell lines BP6, BP6T, BP12, and BP12B were established previously in this laboratory by exposure of GSHE cells to \(N\)-methyl-\(N\)'-nitro-\(N\)'-nitrosoguanidine (5). BHK-A cells were derived from soft-agar colony of BHK 21/c1 13, originally isolated by Stoker and MacPherson (35) and obtained from the American Type Culture Collection (Rockville, Md.). Detailed growth characteristics other than contact-mediated growth

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control were described previously (5). GSHE 21F CI 2/1 cells are a spontaneously established clonal, subtetraploid cell line isolated from a GSHE strain derived from 11-day gestation fetuses. This cell line is strictly sensitive to postconfluence inhibition of cell division and produces a uniform monolayer of “cobblestone-like” appearance. GSHE AP17 is a spontaneously established cell line derived from GSHE cells established from 13-day gestation fetuses. Cells were cultured in IBR modified Dulbecco’s Eagle’s reinforced medium (Biolabs, Northbrook, Ill.) supplemented with 10% fetal bovine serum without antibiotics. Cells were grown at 37°C in a humid atmosphere of 5% CO2 and 95% air. All cultures were free of Mycoplasma contamination.

Chemicals and Treatment. MNNG was obtained from Sigma Chemical Co. (St. Louis, Mo.). A 1000X stock solution of MNNG dissolved in dimethyl sulfoxide was prepared, and aliquots were frozen until use. Exponentially growing cultures were treated with 5 μM MNNG for 2 hr, resulting in approximately 30% survival. Control cultures were treated with 0.1% dimethyl sulfoxide for 2 hr, and this treatment was found to be nontoxic.

Determination of Growth Curves. Replicate plates (60 mm) inoculated with 2 to 5 x 10⁴ cells were incubated for 6 hr for attachment. Then, the medium was changed to either normal or conditioned medium, and the plates were incubated with medium changes on Days 2 and 4. Cell number was determined each day by Coulter Counter after removing the cells from replicate plates with 0.05% trypsin and 0.02% EDTA in Ča⁺⁻ and Mg²⁺-free phosphate-buffered saline [0.14 mM NaCl-3 mM KCl-1.8 mM Na₂HPO₄-1 mM KH₂PO₄ (pH 7.4)].

Cell Mat Assay. To detect and select CS⁻ cells, lethally irradiated, confluent monolayers (cell mats) of GSHE 21F CI 2/1 cells were used. Cell mat plates were prepared by inoculating 100-mm plastic dishes (Falcon Labware, Oxnard, Calif.) with 1 to 5 x 10⁶ GSHE 21F CI 2/1 cells and incubating the plates for 3 to 6 days. The medium was changed at confluence, and the plates were incubated for an additional 2 days to ensure complete confluence. Confluent cell mats were lethally irradiated by γ-irradiation (4000 rads). Cells grown and irradiated in this manner rarely detached from the substrate for at least 2 weeks following irradiation and have never recovered in subsequent subculturing. The cells to be tested were superinoculated in fresh medium onto these lethally irradiated cell mats and incubated with medium changes every 3 days. After 7 to 14 days, CS⁻ colonies were isolated for further analysis or measured quantitatively with a stereoscopic microscope after the plates were methanol fixed and Giemsa stained. The cloning efficiency of CS⁻ cells is expressed as the percentage of plated cells that formed colonies containing more than 50 cells on the cell mat.

Soft-Agar Assay and Tumorigenicity. The efficiency of colony formation in semisolid agar was measured by the procedure described by MacPherson and Montagnier (22), as modified by Kakunaga and Kamahora (20). Briefly, cells suspended in 4.0 ml of 0.3% Difco agar (Difco Laboratory, Detroit, Mich.) supplemented with complete medium and 0.1% Bactopeptone (Difco Laboratory) (21) were plated in 60-mm dishes over a layer of complete medium solidified with 0.6% agar. The cloning efficiency in semisolid agar was expressed as the percentage of plated cells which formed colonies containing at least 50 cells. This linearity also not shown). These data indicate that the cloning ability on cell mats generally correlated quantitatively with their cloning efficiency in soft agar (Table 1). One exception to this correlation is represented by AP17 cells, a spontaneously derived, established cell line, which showed a very low cloning efficiency in soft agar (0.3%) in spite of high cloning efficiency on cell mat (49%) comparable to that of BP6T cells. Therefore, the Kendall coefficient of rank correlation (5) between cloning efficiencies on cell mats and in soft agar is 0.76. The coefficient of rank correlation between cloning efficiencies on plastic dishes and cell mats was slightly less, 0.69.

Characteristics of Colony Growth on Cell Mat. The number of colonies on cell mats increases in a linear manner with increased inoculation of test cells as shown by superinoculating GSHE passage 4 cells in the range of 1 x 10² to 1 x 10⁶ per cell mat; a similar relationship was observed for BP6T cells inoculated in the range of 1 x 10² to 4 x 10⁵ cells/dish (data not shown). These data indicate that the cloning ability on cell mat is independent of inoculation density. This linearity also suggests that these colonies are of single-cell origin.

Reconstruction Experiment. To determine the resolution of
detection of CS- transformed cells among normal cells, known ratios of BP6T cells and late-passage normal diploid GSHE cells were cocultivated on the cell mat. The presence of GSHE cells in the cell mat cultures did not inhibit the efficiency of colony formation of BP6T cells, even if 1 x 10^6 GSHE cells were inoculated together with 10 BP6T cells (Table 2). Thus, the resolution of detection of CS- cells among CS+ cells is ~1 x 10^{-5}. Since highly tumorigenic BP6T cells were used in these reconstruction experiments, the sensitivity of detection could be lower in early transformed cells, occurring in a population of normal cells following carcigenogen treatment.

**Effect of Conditioned Medium on the Growth of Normal and Transformed GSHE Cells.** To examine the growth-inhibitory effect of conditioned medium on the growth of normal and transformed GSHE cells, growth curves were obtained by culturing those cells in either normal or conditioned medium. Conditioned medium was prepared by overlaying confluent flasks (75 sq cm) of GSHE 21F Cl 2/1 cells with 10 ml of fresh medium for 2 days. As shown in Chart 1, conditioned medium significantly inhibited the growth of normal GSHE cells but not the growth of BP6T cells as compared with normal medium.

These data suggest that conditioned medium either contains inhibitors most effective against normal GSHE cells or lacks certain nutritional factors for the growth of normal GSHE cells.

**Temporal Acquisition of CS- Cells and AGA+ Cells**

<table>
<thead>
<tr>
<th>Seeding cell density</th>
<th>Av. no. of transformed colonies</th>
</tr>
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<tbody>
<tr>
<td>GSHE</td>
<td>BP6T</td>
</tr>
<tr>
<td>10^5</td>
<td>0</td>
</tr>
<tr>
<td>10^6</td>
<td>0</td>
</tr>
<tr>
<td>10^7</td>
<td>0</td>
</tr>
<tr>
<td>10^8</td>
<td>0</td>
</tr>
<tr>
<td>10^9</td>
<td>2.4 ± 0.5^c</td>
</tr>
<tr>
<td>10^10</td>
<td>2.5 ± 0.5^c</td>
</tr>
<tr>
<td>10^11</td>
<td>3 ± 1.2^c</td>
</tr>
</tbody>
</table>

- GSHE cells at passage 6 were used.
- A number of very small dense areas were seen due to the very high inoculation density, but these were easily discriminated from transformed foci.
- Mean ± S.D. (2 separate determinations).

**Normal GSHE Cells Exposed to MNNG.** Table 3 shows the temporal relationship between the appearance of CS- colonies (CS- phenotype) and the appearance of colonies capable of growth in soft agar (AGA+ phenotype) in mass-treated cultures following exposure of passage 3 GSHE cells to 5 μM MNNG. Similar to control cultures, the CS- cell population in the MNNG-treated cultures decreased with continued passage. Solvent-treated control cells senesced within 9 posttreatment passages. In contrast, the MNNG-treated cultures survived crisis, and CS- cells reappeared at PTP 7 and increased in number with further passaging (Figs. 1B and 2B). The acquisition of this phenotype thus required approximately 20 population doublings. In comparison, AGA+ cells were not observed until PTP 12 (35 population doublings). The acquisition time of these growth alterations in 2 major experiments ranges between 21 and 22 population doublings for CS- phenotype and between 35 and 95 population doublings for AGA+ growth.
Tumors within 2 months following injection of newborn hamsters with $2 \times 10^6$ observed for 8 months.

Each clone, 3 to 5 newborn hamsters were given injections of $2 \times 10^6$ cells and observed for 8 months. The growth-inhibitory effect of this monolayer was evident in the conditional medium, resulting in many local dense areas. Moreover, these clones and truly transformed foci. In developing the cell mat assay as that described in this report using a lethally irradiated cell mat, provides consistent and reproducible results and allows high resolution ($1 \times 10^{-5}$) and quantitative detection of CS- cells induced by carcinogenic perturbation. Moreover, characterization of these carcinogen-induced CS- cells is possible, since CS- colonies can be isolated from the lethally irradiated cell mats.

The mechanism of the growth-regulatory effect of the clonal cell line has not been determined. This growth inhibition could be mediated by cell-to-cell interaction (10, 11, 32), which becomes effective only at confluence (36) and thus may not be due to simple cell-to-cell contacts (23). This hypothesis is supported by the observation that conditioned medium from density-inhibited cultures has been shown to contain substances which inhibit DNA synthesis (16, 17, 25). We have found a similar growth-inhibitory effect of conditioned medium from clonal cultures of GSHE 21F CI 2/1 cells. Therefore, both factors released from the cell line and direct cell-to-cell interaction may be involved in the growth inhibition by the clonal cell line.

The growth properties of CS- cells found in early passages of GSHE cell cultures have been fully characterized (24). Briefly, the frequency of these CS- cells reduces with passage in vitro by phenotypic conversion from CS- to CS+ cells presumably through cellular differentiation. Following treatment of tertiary-passage GSHE cells with MNNG, the CS- cells initially decrease to below the level of detection (<$10^{-5}$) in a manner similar to the controls. However, in MNNG-treated cultures, cells which have CS- phenotype reappear at 21 to 22 PTPD, followed by AGA+ cells which appear between 35 and 95 PTPD. Clonal cultures of these permanent CS- cells, isolated within a few population doublings of their appearance, are morphologically transformed (Fig. 2C) but exhibit neither the AGA+ phenotype nor tumorigenicity until after later passage in culture. These clonal experiments suggest that the appearance of AGA+ cells is due to the progression of variant clones, such as the CS- clones, and not due to selection of preexisting transformed cells capable of growth in soft agar.

**DISCUSSION**

Cells which escape from postconfluence inhibition of cell division are able to proliferate among density-inhibited cells (3). Based on this phenomenon, focus assays have been developed as short-term detection systems for transformed cells (9, 19, 31). Although this type of assay has been used in hamster cell systems (8), it appears to be less suitable for our GSHE cell system, because low-passage embryonic fibroblasts are heterogeneous in their contact sensitivity (24) and tend to grow to higher saturation density, therefore making it difficult to distinguish between naturally occurring high-density colonies and truly transformed foci. In developing the cell mat assay system, normal diploid GSHE cell strains were initially used for generating cell mats. These cells grew to higher saturation density, resulting in many local dense areas. Moreover, these cells easily detached from the substrate after irradiation. Furthermore, the growth-inhibitory effect of this monolayer was insufficient to suppress even the growth of highly contact-inhibited GSHE 21F CI 2/1 cells (data not shown). This failure of growth inhibition could be ascribed to the incomplete contact inhibition of GSHE cell monolayers. A similar growth suppression failure was reported when mouse embryonic cells were used for a monolayer (34). In contrast, an assay system, such as that described in this report using a lethally irradiated confluent monolayer of established CS+ cells, circumvents these problems, provides consistent and reproducible results, and allows high resolution ($1 \times 10^{-5}$) and quantitative detection of CS- cells induced by carcinogenic perturbation. Moreover, characterization of these carcinogen-induced CS- cells is possible, since CS- colonies can be isolated from the lethally irradiated cell mats.

**REFERENCES**


Fig. 1. Cell mat dish inoculated with $1 \times 10^5$ BP6T cells (A) and $1 \times 10^3$ GSHE cells 12 PTPD after MNNG treatment (B). The difference in the background in these cell mat plates is due to a difference in the staining time rather than a difference in the density of the cell monolayer. Stained with Giemsa.

Fig. 2. A, a colony of BP6T cells growing on cell mat. Giemsa stain, x 30. B, MNNG-induced CS\textsuperscript{-} colony growing on cell mat. Giemsa stain, x 30. C, clonally isolated CS\textsuperscript{-} cells from MNNG-treated cultures. Phase contrast, x 125.
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