A contact-insensitive subpopulation in Syrian hamster cell cultures with a greater susceptibility to chemically induced neoplastic transformation

(contact inhibition/neoplastic transformation/differentiation/somatic mutation)

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ABSTRACT We previously have identified a subpopulation of contact-insensitive (CS-) cells which lacks density-dependent inhibition of cell division in primary and low-passage cultures of Syrian hamster embryonic (SHE) fibroblastic cells. Further, we have shown that the proportion of these CS- cells declines as a result of the stable phenotypic conversion of the CS- cells to contact-sensitive (CS+) cells. To determine whether these transient CS- cells are more sensitive to carcinogenic/mutagenic perturbation, the susceptibility to neoplastic transformation and somatic mutation induced by N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) was examined in clonally isolated cell cultures containing various proportions of CS- cells (0.02–4%). The frequencies of morphological transformation, focus formation, and neoplastic transformation showed a positive correlation to the proportion of CS- cells in the treated cultures. In contrast, the frequency of MNNG-induced somatic mutation at the Na+,K+-ATPase locus was similar among cultures varying in their proportion of CS- cells. Thus, there is a transient subpopulation of CS- cells in primary SHE cell cultures that is more susceptible to neoplastic transformation although equally susceptible to induced point mutation. This dissociation between somatic point mutation and neoplastic transformation indicates a fundamental difference in the nature of these two phenomena. A possible relationship between the propensity of CS- cells (versus CS+ cells) to carcinogen-induced neoplastic transformation and the state of differentiation of the CS- cells is discussed.

A number of studies have indicated that the carcinogenic process involves crucial changes in the differentiation process (1–5). However, the role of cellular differentiation in the process of neoplastic transformation has not been defined. Specifically, it is of interest to determine how a cell's state of differentiation affects its susceptibility to carcinogenic perturbation. Among mesenchymal cell systems, few cell types have been developed to investigate the relationship between these processes in a single cellular system. Although some established mouse cell lines have been used for this purpose (6, 7), cell lines have two serious drawbacks. The cells are aneuploid and exhibit certain preneoplastic characteristics (8) and, because these cells are established in culture, developmental changes induced in these cells cannot be compared directly with naturally occurring in vivo developmental processes.

Low-passage Syrian hamster embryonic (SHE) cells are normal, diploid mesenchymal cells, which have been used for concomitant studies on neoplastic transformation and somatic mutation (9, 10). Recently, we identified a contact-insensitive (CS-) cellular subpopulation, which lacks density-dependent inhibition of cell division, in primary and low-passage cultures of SHE fibroblastic cells (11–13). CS- cells can be identified by their ability to proliferate and form sizeable colonies on lethally irradiated confluent monolayers (cell mats) of contact-sensitive (CS+) cells. These CS- cells in low-passage SHE cell cultures are transient and are lost through phenotypic conversion to CS+ cells during passage in vitro (11–13). Further, we have observed that CS- cells can differentiate terminally into mature adipocytes, whereas CS+ cells do not exhibit terminally differentiated phenotypes (13). Based on these observations, we propose that the conversion of CS- cells to CS+ cells may represent an early stage of differentiation prior to the appearance of a specific phenotype or function that defines the terminally differentiated state.

In this study we investigated the different susceptibility of CS- cells versus CS+ cells to carcinogenic perturbation by comparing the propensity toward neoplastic transformation and somatic mutation among cell cultures that vary in the proportion of CS- cells. The data show that cultures enriched for CS+ cells are more susceptible to neoplastic transformation than cultures depleted of CS- cells, although both cultures were originated from the same pool of cells and have the same propensity toward somatic point mutation. This observation is discussed with reference to our hypothesis that CS- cells are less differentiated than CS+ cells and that less differentiated cells may have a greater propensity toward induced neoplastic transformation.

MATERIALS AND METHODS

Cells, Culture Medium, and Chemical Treatment. SHE cell cultures were established from 13-day gestation fetuses collected aseptically by caesarian section from strain LSH inbred golden Syrian hamsters (Lakeview Hamster Colony, Newfield, NJ) as previously described (9). Cells were cultured in modified Dulbecco's Eagle's medium (22) (Biobals, Northbrook, IL) supplemented with 10% heat-inactivated fetal bovine serum (Biobals) and 0.1 mg of garamycin (Schering) per ml. Cells were grown at 37°C in humidified 5% CO2/95% air. All cultures were tested and found to be free of mycoplasma contamination. Preparation of N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) stock solutions and treatment of cells with MNNG were as described (12).

Preparation of Clonally Isolated CS- Enriched and CS- Depleted Cultures. It was not possible to isolate pure CS- clones by standard cell cloning techniques because, as the original CS- parental cell proliferated, CS+ progeny were.

Abbreviations: SHE, Syrian hamster embryo; CS-, contact-insensitive; CS+, contact-sensitive; AD-, anchorage-independent; MNNG, N-methyl-N'-nitro-N-nitrosoguanidine.
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produced. Therefore, to generate cultures enriched for CS- cells, passage 1 SHE cells from frozen stock were inoculated onto 10 cell mats, prepared as previously described (12), and incubated for 10 days with a medium change every 2 days. Cell mat colonies (100–150) were locally trypsinized for 5 min with 1 mm² sterile filter paper soaked with 0.02% EDTA/0.05% trypsin, resuspended in fresh medium, and pooled to obtain sufficient cells (4 × 10⁶) for transformation and mutation experiments. To obtain cultures that varied in the proportion of CS⁻ cells, pooled clonal CS⁻ cells were further cultivated either on a cell mat or on a plastic surface for 8 days, resulting in a 50-fold difference (0.2–1.1%) in the proportion of CS⁻ cells without a large difference in in vitro age. These cells were either used immediately or frozen until use. At the beginning of each experiment, the cloning efficiency on cell mat was determined as described (12) and expressed as the percentage of CS⁻ cells for each culture.

**Cytotoxicity, Morphological Transformation, and Focus Assays.** Cytotoxicity and morphological transformation were determined simultaneously as described (9, 10). Survival was expressed as the fraction of the number of colonies in plates exposed to MNNG over the number of colonies in plates treated with solvent. The morphological transformation frequency was calculated on the basis of 1 × 10⁵ surviving colonies. Focus formation was assayed by a modification of the method of Casto et al. (14). Cells were inoculated at a density of 6 × 10⁴ per 100-mm dish in control cultures. In MNNG-treated cultures, the number of colonies plated was adjusted by the anticipated survival, so that all cultures had nearly the same density after recovery from treatment. Twenty-four hours after seeding, the cells were treated with various concentrations of MNNG for 2 hr at 37°C. Then the cultures were washed twice with phosphate-buffered saline, replenished with growth medium, and incubated for 4 wk, with medium changes every 3 days. The cultures were fixed with methanol, stained with Giemsa, and scored with a stereomicroscope for malignantly transformed foci consisting of basophilic, randomly growing cells with atypical nuclei. Data were expressed as the frequency of transformed foci per 1 × 10⁵ surviving cells.

**Progressive Acquisition of the Permanent CS⁻ Phenotype, Anchorage-Independence (AD⁻), and Tumorigenicity.** Cells were inoculated into 75 cm² flasks at a density of 1 × 10⁶ per flask. After 24 hr, cells were treated with MNNG for 2 hr at a concentration previously determined to result in ~30% survival. Following exposure time, cultures were washed twice with phosphate-buffered saline and replenished with fresh medium. Control flasks were inoculated with 3 × 10⁵ cells and treated with 0.1% dimethyl sulfoxide for 2 hr. Approximately 3 days after treatment, the cultures reached confluence, and each flask was subsequently subcultured into three flasks with an inoculum of 5 × 10⁶ cells per flask. Every two or three passages, each culture was examined for the CS⁻ phenotype by cloning on cell mats and for the AD⁻ phenotype by cloning in soft agar as described (12). After cultures acquired the AD⁻ phenotype, the cells were assayed for tumorigenicity as described (12).

**Mutation Assay.** The frequency of mutation at the Na⁺,K⁺-ATPase locus after a 3-day expression time was quantitated by a respreading assay as described (9). Mutation frequencies were corrected for cytotoxicity and cloning efficiency and were expressed on the basis of 10⁵ clonogenic cells.

**RESULTS**

**Derivation of Cultures and Cell Survival.** To investigate the effect of the frequency of CS⁻ cells in a target population, we compared cytotoxicity, morphological transformation, focus formation, and the progressive acquisition of neoplasia-related phenotypes in three types of cultures: (i) cultures highly enriched for CS⁻ cells derived from pooled clones isolated directly from cell mat (~4% CS⁻ cells); (ii) cultures internally enriched for CS⁻ cells obtained by culturing pooled CS⁻ cells for 8 days on cell mats (0.2–1.1% CS⁻ cells); and (iii) cultures depleted of CS⁻ cells obtained by culturing pooled CS⁻ cells for 8 days on plastic surfaces (0.02–0.2% CS⁻ cells).

A dose–response curve for cell survival was determined for these cultures. Cells derived from the CS⁻-depleted cultures were more sensitive to MNNG than those from CS⁻-enriched cultures (Fig. 1A). This difference in cytotoxicity implied that the target size of the CS⁻ cells could be larger than that of the CS⁻ cells or that repair may be more efficient in the CS⁻ cells. Repair capacity has been reported to decline with both in vitro senescence (15) and cellular differentiation (16).

![Fig. 1. Dose–response curves for cultures that vary in the proportion of CS⁻ cells for MNNG-induced cytotoxicity (A) and morphological transformation based on survival fraction (B) and MNNG concentration (C). CS⁻-enriched cultures: 4.0% (○), 0.6% (△), 0.21% (○), and 0.20% (○). CS⁻-depleted cultures: 0.03% (△), 0.02% (○), and 0.02% (○).](attachment:figure1.png)
Morphological Transformation. Because the degree of cytotoxicity was different among these cultures at the same MNNG concentration, we compared the transformation frequency at biologically equivalent doses of MNNG by plotting transformation frequencies against survival fraction (Fig. 1B) as well as against different concentrations of MNNG (Fig. 1C). The frequency of morphological transformation exceeded the frequency of CS− cells, suggesting that CS− cells were not the only cells susceptible to carcinogen-induced morphological transformation. However, the frequencies of morphological transformation in the CS−-enriched cultures were 7- to 16-fold higher than those in the CS−-depleted cultures. Further, there was a positive correlation between the transformation frequency and the percentage of CS− cells in the initial cell culture treated with MNNG, with the highest frequencies of morphological transformation exhibited by cultures with the highest initial number of CS− cells. The frequency of spontaneous morphological transformation appeared to be slightly higher in CS−-enriched cultures (1.8% versus 0.4%). This may have been due to the fact that CS− cells per se have a tendency to exhibit a criss-cross pattern of colony growth.

Induction of morphological transformation was dose-dependent. However, at high concentrations of MNNG, the frequency of transformation leveled off, partly because a longer recovery time was required for the cells treated with higher concentrations of MNNG. Because of the high frequency of spontaneous morphological transformation in our CS− cell system, the possibility of selection of preexisting morphological transformants could not be totally excluded. However, transformation frequencies in CS−-enriched cultures were higher than theoretical selection frequency (17). For example, with the assumption that MNNG was only cytotoxic to the nontransforming cells, at 50% survival the transformation frequency should have been twice that of the control (100% survival)—i.e., 3.6% versus 1.8%, respectively. However, the experimental value was 16%, significantly above the theoretically calculated value of 3.6%. Therefore, morphological transformation in the CS−-enriched cell culture cannot be explained by selection phenomenon alone and is most likely due to induction by carcinogens, as reported for mixed cultures of SHE cells (10, 18, 19). Further, lower proliferative capacity as an explanation of the lower incidence of transformation in CS− cells is unlikely because the frequencies of morphological transformation were calculated on a surviving colony basis and, therefore, should not have been affected by proliferative capacity.

Focus Formation Assay. Focus formation 4 wk after carcinogen treatment appeared to be a more reliable neoplasia-related phenotype than morphological alteration occurring within 1 wk after carcinogen treatment. The frequency of focus formation increased linearly with increased dose of MNNG in all cultures (Fig. 2). Moreover, the frequency of transformed foci correlated well with the initial frequency of CS− cells in the culture at the time of treatment. Transformed foci consisted of variously sized, randomly growing, basophilic cells, which appeared histologically more malignant than the morphological transformants.

One spontaneously transformed focus was found in one of two control dishes from highly CS−-enriched cultures; no spontaneous foci were observed in eight control dishes of intermediate CS−-enriched cultures. These low incidences of spontaneous and induced focus formation (Fig. 2) were in contrast to the high incidences of spontaneous and induced morphological transformation (Fig. 1 B and C). This observation that the frequency of the morphological transformation was higher than that of the focus formation suggests that morphological transformation could be a reversible event not necessarily linked definitively with focus formation and neoplastic progression.

**Fig. 2.** Dose–response curves for MNNG-induced transformed focus formation in cultures that vary in the proportion of CS− cells. Symbols are as in Fig. 1.

Neoplastic Progression To AD−. To demonstrate the effect of the frequency of CS− cells on neoplastic transformation, seven cultures, all derived from a single pool of cell mat clones but containing different proportions of transiently CS− cells, were treated with equally cytotoxic doses of MNNG (=30% survival), passed in vitro, and examined for crisis, senescence, and the temporal acquisition of a heritable or permanent CS− phenotype, AD−, and tumorigenicity. Each of the seven initial cultures was divided into three flasks after confluence, and these triplicate cultures were carried out until the AD− phenotype was observed. A low frequency of the dimethyl sulfoxide-treated control cultures in each group spontaneously escaped senescence after a long crisis period.

**Table 1.** Frequency of escape from senescence and acquisition of the permanent CS− and AD− phenotypes in cultures initially containing various proportions of transient CS− cells

<table>
<thead>
<tr>
<th>Type of culture and treatment</th>
<th>CS− enriched (0.2−4.0%)*</th>
<th>CS− depleted (&lt;0.2%)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Data</td>
<td>MeSO (0.1%)</td>
<td>MNNNG (10 μM)</td>
</tr>
<tr>
<td>Cytotoxicity, % survival</td>
<td>100</td>
<td>25−30</td>
</tr>
<tr>
<td>Cultures analyzed, no.</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>Cultures established</td>
<td>1†</td>
<td>12</td>
</tr>
<tr>
<td>(escaped senescence), no.</td>
<td>0</td>
<td>12</td>
</tr>
<tr>
<td>Cultures neoplasticly</td>
<td>0</td>
<td>100†</td>
</tr>
<tr>
<td>transformed (AD−), %</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

MeSO, dimethyl sulfoxide.

*Percentage cloning efficiency on cell mat.
† Cultures spontaneously escaped from senescence after a long crisis period (1−2 months) but did not acquire the AD− phenotype.
‡ All cultures have acquired permanent CS− and AD− growth properties; all cultures tested for tumorigenicity are positive (see text).
of 1–2 months, but these cultures did not transform neoplastically (i.e., did not acquire AD− phenotype) after further passages (Table 1). In contrast, among the treated cultures, all of the CS−-enriched cultures (0.2–4.0% CS−) transformed, while only 56% of the treated CS−-depleted cultures (<0.2% CS−) transformed (Table 1). Further, all of these transformed cultures eventually acquired both the permanent CS− and the AD− phenotypes. All AD− cell cultures that have been tested for tumorigenicity yielded tumors with 25–100% incidence within 2–5 months after injection of newborn hamsters with 2 × 10⁶ cells. We conclude from these observations that CS−-enriched cultures treated with MNNG have a higher frequency of escape from senescence, acquisition of in vitro neoplasia-related growth properties, and tumorigenicity than do MNNG-treated CS−-depleted cultures.

The frequency of CS− cells in a culture at the time of treatment with MNNG also affected the chronological acquisition time of the CS− phenotype (Fig. 3, closed arrows). Although the permanent CS− phenotype was acquired in all nonsenescent cultures 15–17 population doublings after treatment, as in the case with mixed SHE cell cultures (12), the acquisition of this growth property was delayed in chronological time in CS−-depleted cultures (50 days versus 20–30 days after treatment) (Fig. 3). This phenomenon was presumably associated with the fact that CS−-enriched cultures were less likely to exhibit a crisis period in contrast to CS−-depleted cultures, which frequently experienced long crisis periods (Fig. 3).

The acquisition time of the AD− phenotype varied among the individual cultures, ranging between 30 and 80 posttreatment population doublings (Fig. 3, open arrows) as previously observed with mixed SHE cell cultures (19). Although no strong correlation has yet been detected between the proportion of CS− cells in the initial culture and the chronological time of acquisition of the AD− phenotype, CS−-depleted cultures did tend either to acquire the AD− phenotype at a later chronological time or to not acquire it at all.

![Figure 3](image-url)  
**Figure 3.** Cumulative population doublings of three MNNG-treated cultures, carried in triplicate, which varied in the proportion of transient CS− cells (1.1% (○), 0.2% (△), and 0.03% (□)). Closed arrows indicate first detection of permanent CS− cells; open arrows indicate first detection of AD− cells; and the asterisk indicates senescence of culture.

### Table 2. Frequency of MNNG-induced mutation at the Na+,K+-ATPase locus in cultures that vary in the proportion of CS− cells

<table>
<thead>
<tr>
<th>Type of culture treated with 10 µM MNNG</th>
<th>Data</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>CS−-enriched (0.9)*</td>
<td>30.5</td>
<td>26.7</td>
</tr>
<tr>
<td>CS−-depleted (0.006)*</td>
<td>659</td>
<td>666</td>
</tr>
</tbody>
</table>

Experiments were done after allowing 3 days for expression. The untreated cultures had <1 mutant per 10⁶ clonogenic cells. The dose–response (mutants per clonogenic cells) between 1, 5, and 10 µM MNNG was linear (9).

*Percentage of CS− cells.

### DISCUSSION

We have demonstrated that primary and low-passage cultures of SHE cells are heterogeneous, and we have identified a transient CS− cellular subpopulation in these mixed cell cultures. Further, we have shown that the frequencies of morphological transformation, focus formation, and neoplastic transformation decrease with a decrease in the proportion of CS− cells in treated cultures. Other laboratories have reported similar heterogeneity in SHE cell cultures. Huberman and Sachs demonstrated in cloning experiments that different SHE cell clones showed different cytotoxicity and transformation by benzo[a]pyrene (18). Pienta et al. reported that morphological transformation could be induced with chemical carcinogens only with certain pools of SHE cells (20). Borek and Sachs reported that, in mixed cultures of SHE cells, the frequency of x-ray-induced transformation decreased with an increase in the passage level of the culture at the time of treatment and that no transformed colonies were obtained after irradiation of cells from cultures that had been passaged three times (21). Together, these data and our data suggest that a subpopulation of cells in SHE cell cultures is more susceptible to carcinogen-induced neoplastic transformation than is the population as a whole.

The existence of a subpopulation of cells with differential susceptibility to neoplastic transformation leads to a consideration of the significance or function of these transient cells. In separate studies, we further characterized the CS− subpopulation specifically in terms of its relationship to in vitro cellular senescence and cellular differentiation. We have shown that phenotypic conversion of CS− cells to CS− cells accompanied reduced proliferative capacity and increased cell volume, which are markers for in vitro senescence (13). In addition, cultures with higher initial numbers of CS− cells exhibited longer in vitro proliferative life spans than did cultures with smaller initial numbers of CS− cells, and continuous exposure to tumor promoters both retarded the decline in the proportion of CS− cells and increased by ≈2-fold the in vitro proliferative life span of 13-day gestation SHE cell cultures (13). Thus, the loss of CS− cells paralleled
in vitro senescence. Further, CS⁺ cells differentiated terminally into mature adipocytes, whereas CS⁻ cells were not observed to exhibit terminally differentiated phenotypes (13). These data suggest that the CS⁻ to CS⁺ conversion is associated with differentiation. We propose that it represents an early stage in differentiation possibly associated with altered control of proliferation prior to the acquisition of a morphologically or biochemically recognizable differentiated phenotype.

We conclude from these experiments that the CS⁻ cells are less-differentiated cells and that these less-differentiated cells are more susceptible to chemically induced neoplastic transformation than are more-differentiated CS⁺ cells. These results suggest that genes, or the control of the expression of genes, associated with neoplastic transformation could be altered with cellular differentiation, thereby changing their susceptibility to carcinogens. Neoplastic transformation of mixed SHE cell cultures has been described as a multistep, progressive process (19), and the induction of both morphological transformation and a heritable AD⁻ phenotype has been shown to be distinct from the induction of somatic mutation observed at the Na⁺,K⁺-ATPase and HGPRT (hypoxanthine/guanine phosphoribosyltransferase) loci (10). Neoplastic transformation may be initiated by a single gene mutational change, but it appears to be more complex than a single-gene mutational process. In the present comparative study, the frequency of MNNG-induced forward point mutation to ouabain resistance is similar between CS⁻ cells and CS⁺ cells, while the susceptibility to MNNG-induced neoplastic transformation is different between the two cell types. This dissociation between mutation and neoplastic transformation indicates a fundamental difference in the nature of these two processes and emphasizes the importance of epigenetic mechanisms, possibly coupled with differentiation, in neoplastic progression.

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