Cellular differentiation and neoplasia: Characterization of subpopulations of cells that have neoplasia-related growth properties in Syrian hamster embryo cell cultures

(anchorage independence/contact inhibition/neoplastic transformation)

SHUJI NAKANO AND PAUL O. P. TS'O

Division of Biophysics, School of Hygiene and Public Health, The Johns Hopkins University, 615 North Wolfe Street, Baltimore, Maryland 21205

ABSTRACT   Cellular subpopulations having two of the growth properties of neoplastically transformed cells—lack of postconfluence inhibition of cell division (CI^-) and anchorage independence of growth (AD^-)—were found in cell cultures established from 10- to 13-day-old Syrian hamster embryos. The subpopulations having these properties decrease with increasing gestation period of the embryo as well as with continuing passage in vitro. The decrease in these subpopulations was also observed when they were cultured on a lethally irradiated confluent monolayer of contact-inhibited cells (cell mat), a selection condition for CI^- cells. Therefore, negative selection cannot be the explanation for the loss of CI^- cells in the population, leaving two other possibilities: either the loss of proliferative capacity of the CI^- cells or the acquisition of sensitivity to postconfluence inhibition of cell division (CI^+) of this subpopulation on in vitro culture or in vivo growth. The CI^- subpopulations were isolated clonally from cell mats and were cultured continuously on both cell mats or plastic dishes. The results indicate that these cells did not lose proliferative capacity but acquired the contact-inhibited phenotype. This result, together with the fact that embryonic development in vivo also decreases CI^-/AD^- subpopulations, suggests that the disappearance of these subpopulations is due to cellular differentiation of the CI^-/AD^- cells to become CI^+/AD^+ cells.

Neoplastic transformation of normal diploid fibroblasts has been investigated by using Syrian hamster embryo (SHE) cells as a model system (1–5), particularly concerning the basic relationship between neoplastic transformation and somatic mutation (2, 6). However, other studies also have suggested that the carcinogenic process involves crucial changes in the differentiation process (7, 8). Therefore, it will be useful if the basic mechanisms of neoplastic transformation, somatic mutation, and differentiation can all be investigated in a single cellular system.

Although numerous carcinogenesis experiments have been done with hamster embryonic cells, no system has been developed for the study of the correlation between carcinogenesis and differentiation. It has been reported that hamster embryo tissue derived from fetuses of 6- to 8-day gestation gives rise to teratomas when transplanted into syngeneic animals (9). However, these in vivo studies have difficult problems and technical restrictions in experimental design. Therefore, an in vitro system closely correlated to the in vivo developmental stage is needed to study the role of differentiation in carcinogenesis. This prompted us to search for subpopulations in normal SHE cell culture that may exhibit properties of neoplastically transformed cells, particularly the SHE cell culture from the early gestation period and at early passage after putting these cells into culture. These properties include the lack of postconfluence inhibition of cell division (CI^-) and anchorage independence of growth (AD^-), both of which are closely correlated with tumorigenicity (10–12).

To detect and select CI^- cells, a quantitative assay has been developed by measuring the number of colony-forming cells on lethally irradiated confluent monolayers of contact-inhibited cells (cell mat) as described by Aaronson and Todaro for mouse cells (12). By using a soft agar assay for AD^- cells (13, 14), we have quantified the number of cells with those properties in SHE cell culture as a function of in vivo gestation period and of in vitro passaging. These CI^-/AD^- subpopulations have been found in the early passages of these cultures. Moreover, these subpopulations decrease rapidly with increasing gestation period of embryos from which the cultures were derived, as well as with continuing passage in vitro. The mechanisms of the decrease of these subpopulations are discussed with reference to cellular differentiation.

MATERIALS AND METHODS

Cells and Growth Media. SHE cell cultures were established from 10- to 13-day-gestation fetuses collected aseptically by caesarian section from inbred Syrian hamsters (Lakeview Hamster Colony, Newfield, NJ). Pools of primary cultures from litter mates were used immediately after they had been checked for viability or stored in liquid N_2 until use. Various tumorigenic cell lines—BP6, BP6T, BP12, and BP12B—were previously established in this laboratory by treatment of 13-day-old SHE cells with benzo[a]pyrene (10). SHE 21F CL 2/1 cells are a clonal subtetraploid cell line isolated from 11-day-old SHE culture. This cell line is strictly sensitive to postconfluence inhibition of cell division and produced a uniform monolayer. The culture medium used was IBR-modified Dulbecco's modified Eagle's reinforced medium (Bio-Labs, Northbrook, IL) supplemented with 10% fetal bovine serum (Reheis Chemical, Kankakee, IL) without antibiotics. Cells were grown at 37°C in humid 5% CO_2/95% air.

Cell Mat Assay. To detect and select cells insensitive to postconfluence inhibition of cell division (CI^- cells), lethally irradiated confluent monolayers, or cell mats, of SHE 21F CL 2/1 cells were used. Cell mat plates were prepared by inoculating $1-5 \times 10^5$ SHE 21F Cl 2/1 cells into plastic dishes (100 mm Falcon) and incubating until confluence. Then the medium was changed to fresh medium and incubation was continued for an

Abbreviations: SHE, Syrian hamster embryo; CI^- cells, cells insensitive to postconfluence inhibition of cell division; CI^+ cells, cells sensitive to postconfluence inhibition of cell division; AD^- cells, cells that are anchorage independent in growth; AD^+ cells, cells that are anchorage dependent in growth; PDL, population doubling level.

additional 2 days. Confluent cell mats were lethally irradiated with γ-rays (4000 rads; 1 rad = 1.0×10^{-2} gray). The cells to be tested were superinoculated onto these irradiated cell mat plates and incubated for 7–14 days with medium change every 3 days. Cultures were methanol-fixed, Giemsa-stained, and scored for colonies under a stereoscopic microscope. Alternatively, colonies were clonally isolated for clonal experiments or the entire dishes were trypsinized for replating experiments. The cloning efficiency of CI⁻ colonies was expressed as the percentage of plated cells that formed colonies containing >50 cells on cell mats.

Soft Agar Assay. The efficiency of colony formation in semisolid medium was measured as described by MacPherson and Montagnier (13) and modified by Kakunaga and Kamahora (14). Briefly, cells suspended in 4.0 ml of 0.3% Difco agar supplemented with complete medium and 0.1% bacto-peptone were plated in 60-mm dishes over a layer of 0.6% agar containing complete medium. Plates were incubated at 37°C in humid 5% CO_2/95% air for at least 4 weeks with periodic refeeding. Colony formation efficiency in semisolid agar was expressed as the percentage of plated cells that formed colonies containing at least 50 cells.

Clonal Isolation. To characterize clonally isolated CI⁻ cells, CI⁻ colonies grown on cell mat plates were marked and washed twice with phosphate-buffered saline. Then, sterile filter papers soaked with 0.02% EDTA/0.05% trypsin were put on each colony with incubation for 5 min at 37°C. For pure clonal experiments, each filter paper was transferred to a separate 30-mm plate containing fresh medium and subcultured. Alternatively, for pooled clonal experiments, fresh medium was added to the cell mat plates to suspend the cells from trypsinized colonies. CI⁻ cells were collected from each plate, pooled, and used for the experiment.

RESULTS

Effect of Lethally Irradiated Confluent Monolayers of CI⁺ Cells (Cell Mat) on Growth of Normal and Transformed Cells.
As shown in Table 1, all the independently derived benzo[a]pyrene-transformed cells exhibited high cloning efficiency on plastic surfaces (60–85%) but had various degrees of cloning

efficiency on cell mats (7–50%) and in soft agar (4–85%), with a good correlation between the latter two properties. On the contrary, the SHE 21F CL 2/1 cells, which showed a relatively high cloning efficiency on plastic surfaces (46%), failed to form colonies on cell mats and in soft agar, even when plated at up to 5×10^5/plate. A reconstruction experiment using a mixed culture of both CI⁻ tumorigenic cells (BP6T) and normal CI⁺ SHE cells showed that the cell mat assay could discriminate a single CI⁻ cell among 1×10^5 CI⁺ cells. Therefore, the cell mat assay is effective for detecting a small number of CI⁻ cells among a large population of CI⁺ cells (unpublished results).

In contrast to tumorigenic cells, normal SHE cells had a relatively low cloning efficiency on plastic surfaces (1–2%) and showed no colony formation in soft agar when plated with an inoculum as much as 1×10^5/60-mm dish (Table 1). However, when early passage SHE cells were inoculated onto cell mats, they formed very small fibroblastic colonies (Fig. 1) at very low frequency (up to 0.38%). Moreover, the cloning efficiency on cell mats decreased with further passaging but there was only a slight reduction of cloning efficiency on plastic surfaces. From passage 3 to passage 8, the decrease of cloning efficiency on cell mats is much more rapid than the loss of cloning efficiency on plastic surfaces (efficiencies at passage 8 are $\frac{1}{400}$ and $\frac{1}{4}$, respectively, of those at passage 3) during the same time (Table 1). These observations suggest that SHE cell cultures in early passages contain a subpopulation of CI⁻ cells within the majority of CI⁺ cells and that this subpopulation disappeared on further passaging on plastic surfaces. The disappearance of the CI⁻ subpopulation could be due to the following possibilities: (i) selection pressure in favor of CI⁺ cells over CI⁻ cells, (ii) loss of proliferative potential of CI⁻ cells, or (iii) phenotypic conversion from CI⁻ to CI⁺ cells.

This observation was further investigated with another SHE cell preparation (SHE 30F) from 13-day-gestation fetuses. This culture at early passage was characterized with respect to population doubling level (PDL), CI⁻ subpopulation, and AD⁻ subpopulation during in vitro passaging. As shown in Table 2, CI⁻ cells were demonstrated at a frequency of 0.16% at passage 2

Table 1. Comparison of cloning ability of various normal and transformed SHE cells on plastic surfaces and cell mats and in semisolid agar

Cells	Cloning efficiency,%			Diameter of colony on cell mat, mm
	On plastic surfaces	On cell mats*	In soft agar	
BP12†	67.0	6.7	3.9	≈2
BP12B†	57.0	18.0	19.6	≈4
BP6†	82.5	33.5	42.0	≈4
BP6T†	84.5	49.6	85.0	≈6
SHE 21F CL 2/1‡	45.5	<0.0002	<0.0002	—
SHE 3P§	1.25	0.38	<0.001	≈1
SHE 4P§	1.38	0.13	<0.001	≈0.5
SHE 6P§	0.51	0.05	<0.001	≈0.5
SHE 7P§	0.26	<0.01	<0.001	—
SHE 8P§	0.30	<0.001	<0.001	—

* Incubation times on cell mats were 14 days for normal SHE cells and 7 days for transformed BP cells.
† All transformed SHE cells induced by benzo[a]pyrene treatment are described in ref. 10.
‡ Spontaneously derived established clonal cell line of SHE cells that shows contact inhibition of cell growth.
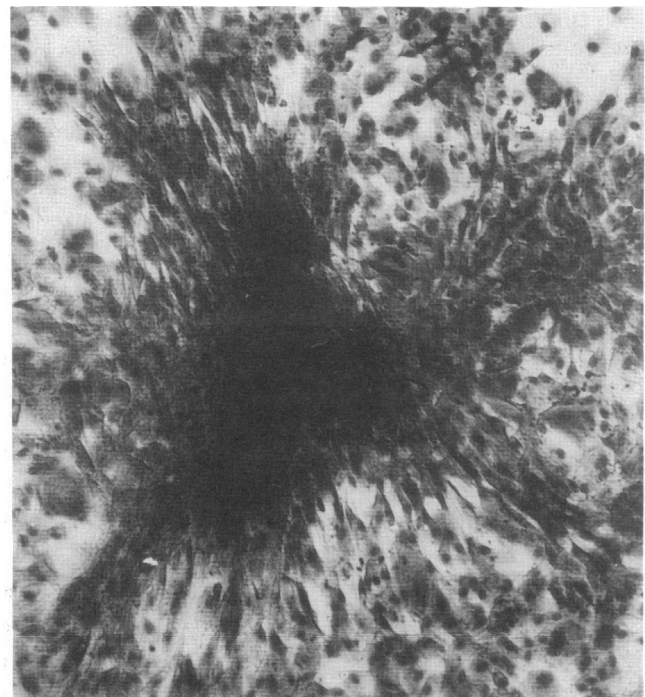§ Approximately passage 3–8 (PDL undetermined) SHE 17F cells derived from 13-day-gestation fetuses.

FIG. 1.   Typical SHE cell colony growing on cell mat. (×40.)

Table 2. Concomitant quantitation of CI⁻ cells (cell mat assay) and AD⁻ cells (agar assay) during serial passage of SHE 30F cells derived from 13-day-old embryos

		Cloning efficiency, %		
Passage	PDL, no.	On plastic surfaces*	On cell mats*	In semi-solid agar†
2	1.1	2.50	0.16	0.008
3	4.7	0.64	0.04	0.002
5	8.5	1.04	<0.001	<0.0002
6	11.0	0.94	<0.001	<0.0002

Population doublings were calculated from number of initial cells per culture and number of cells obtained at time of subculture.
* Means of duplicate dishes.
† Each agar plate was inoculated with 5×10^5 cells. Data are means of triplicate dishes.

and AD⁻ cells were detected at an extremely low cloning efficiency (0.008%). Although the cloning efficiency on plastic surfaces did not change significantly, both the CI⁻ and AD⁻ subpopulations greatly decreased with increasing PDL and could not be detected (at levels of 0.001% and 0.0002%, respectively) after 8.5 population doublings.

Detection of CI⁻ Cells and AD⁻ Cells in SHE Cell Culture Derived from 10-Day-Gestation Fetuses: Influence of Developmental Stages. As shown in Table 3, the 10-day embryo cell cultures (SHE 50) at 0–0.3 PDL had a very high cloning efficiency on cell mats, 3.7% and 3.0%, respectively, while the cloning efficiency on plastic surfaces was 0.2–0.3%. These CI⁻ subpopulations decreased quickly with increasing population doubling in spite of increased cloning efficiencies on plastic surfaces. This suggests that the cloning ability on cell mats is not necessarily correlated with the cloning potential on plastic surfaces. The size of colonies on cell mats also decreased with higher population doubling, but the decrease on plastic surfaces was not significant, suggesting that the proliferative capacity on cell mats is reduced faster than that on plastic surfaces. Colonies on cell mats consisted of three fibroblastic types: piled-up colony, elongated colony, and crisscross colony.

AD⁻ cells were also detected when 5×10^5 cells were inoculated into a soft agar plate. The cloning efficiency in soft agar decreased from 0.014% at 1.0 PDL to <0.0002% at 6.1 PDL. Colonies in soft agar were much smaller than those formed by the tumorigenic cell lines; >4 weeks was needed to obtain macroscopically visible colonies (Fig. 2).

At 1.0 PDL, the cloning efficiencies on cell mats (0.88%) and in soft agar (0.014%) for the culture derived from 10-day-ges-
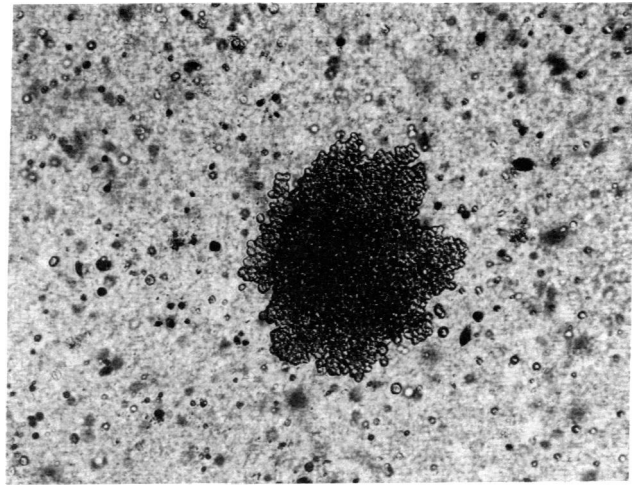

FIG. 2. Colony in 0.3% semisolid agar at 40th day of culture. (×30.)

tation fetuses were much higher than those of 13-day-gestation fetuses at 1.1 PDL (0.16% on cell mats and 0.008% in soft agar). At 1.6 PDL, however, the cloning efficiencies on cell mats and in soft agar became comparable with those of the 13-day-gestation fetuses at 1.1 PDL (Tables 2 and 3). This observation suggests that cell cultures derived from the earlier embryo (10-day-old versus 13-day-old) appeared to have a larger subpopulation of CI⁻ and AD⁻ cells, and in vitro passaging and in vivo growth have similar effects in decreasing these subpopulations.

Properties of Cultures Maintained on Cell Mats: Is Negative Selection a Cause of the Loss of CI⁻ Cells? As shown in Table 4, after a week of growth on cell mats, the recovered culture showed an increased percentage of CI⁻ cells (7.8%; ≈10-fold higher than that of the corresponding culture of SHE 50 passage 2 cells shown in Table 3), with no significant change of cloning efficiency on plastic surfaces. However, the percentage of CI⁻ cells capable of forming colonies on cell mats gradually decreased to 4.8% after 2 weeks of incubation and to 1.5% after 3 weeks of incubation, despite the fact that the total number of CI⁻ cells growing on the original cell mat plate increased with incubation time.

This gradual and relative loss of CI⁻ cells capable of forming colonies on cell mats cannot be explained by negative selection

Table 3. Concomitant quantitation of CI⁻ cells (cell mat assay) and AD⁻ cells (agar assay) during serial passage of SHE 50 cells derived from 10-day-old embryos

		Cloning efficiency,%			Diameter of colony on cell mat, mm
Passage	PDL*	On plastic surfaces†	On cell mats†	In soft agar‡	
Primary	0	0.23	3.66	—	1.0–2.0
1	0.3	0.25	3.00	—	1.0–2.0
2	1.0	1.25	0.88	0.014	0.5–1.0
3	1.6	1.17	0.16	0.006	0.5–1.0
4	3.3	0.91	0.02	0.003	<0.5
5	6.1	0.20	<0.0002	<0.0002	—

* Population doubling was calculated from number of initial cells per culture and number of cells obtained at time of subculture.
† Means of duplicate dishes.
‡ Each soft agar plate was inoculated with 5×10^5 cells. Data are means of triplicate dishes.

Table 4. Quantitation of CI⁻ cells (cell mat assay) and AD⁻ cells (agar assay) after various incubation times on cell mats of SHE 50 cells established from 10-day-old embryos

			Cloning efficiency, %		
Incubation time, weeks	Total cells on cell mat, no.	PDL*	On plastic surfaces†	On cell mats†	In soft agar‡
0	1×10^6	0.3	0.25	3.0	—
1	5.5×10^6	2.8	1.48	7.8	0.027
2	9.7×10^6	3.6	1.24	4.8	0.020
3	19.5×10^6	4.6	1.33	1.5	0.008

Replicate cell mat plates were inoculated with 1×10^6 SHE 50 passage 1 cells derived from 10-day-old embryos and incubated for 1, 2, or 3 weeks, changing the medium every 3 days. After each incubation period, the cultures were trypsinized, counted for cell number (ignoring larger cells from the cell mat), and assayed for CI⁻ and AD⁻ cells.
* Population doublings were calculated from initial cell number and number of cells obtained at time of experiment.
† Means of duplicate dishes.
‡ Each agar plate was inoculated with 5×10^5 cells. Data are means of triplicate dishes.

because the cell mat is a favorable condition for CI⁻ cells. The data·simply imply that the population of CI⁻ cells that retain colony-forming ability on cell mats was gradually diluted by the appearance of CI⁻ cells incapable of forming colonies. Therefore, two hypotheses can be considered: (*i*) CI⁻ cells lost their proliferative capacity and (*ii*) CI⁻ cells lost CI⁻ growth property or acquired CI⁺ phenotype.

Properties of Clonally Isolated CI⁻ Cells. To characterize them further, CI⁻ cells were isolated clonally from the cell mats. Among 25 colonies isolated individually, only 6 cultures reached more than 5×10^5 cells while the remaining colonies senesced at next subculture. However, none of these cultures contained cells capable of forming colonies on cell mats 3 or 4 weeks after isolation. Therefore, to obtain sufficient numbers of CI⁻ cells for experimentation, 50 CI⁻ clones were collected and pooled; these cells were used directly. These clonally isolated CI⁻ cells were cultured serially either on cell mats or on plastic dishes, and the increases in cell number and the cloning efficiencies were examined. It should be noted that when clonally isolated CI⁻ cells were immediately inoculated onto cell mats, only 10% were able to form colonies. This may be due to a change in growth potential occurring during the isolation and assay for CI⁻ cells.

According to the growth curves shown in Fig. 3 *Inset*, the growth rate of CI⁻ cells was faster on plastic dishes than on cell mats, implying higher proliferative capacity of CI⁻ cells on plastic dishes. As shown in Fig. 3, the percentage of these CI⁻ cells capable of forming colonies on cell mats gradually decreased with continued growth—i.e., increased population doublings. The decrease of this subpopulation was much faster on plastic dishes than on cell mats. On the other hand, the proliferative capacity, as shown by the cloning efficiency on plastic surfaces, of these isolated CI⁻ cells was not significantly different between cultures grown on cell mats and cultures grown on plastic dishes. These discrepancies between proliferative capacity on plastic surfaces and colony-forming ability on cell mats suggest that the loss of colony-forming CI⁻ cells is not due to the loss
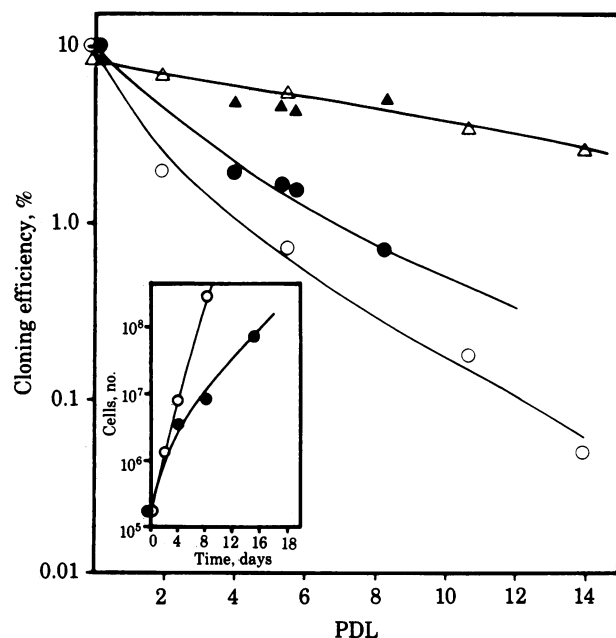
Table 5. Split culture experiment

Culture	CI⁻ clones, no.	Culture	CI⁻ clones, no.
1	0	11	0
2	0	12	3
3	1	13	3
4	2	14	0
5	4	15	15
6	1	16	4
7	0	17	5
8	1	18	2
9	33	19	—
10	1	20	—

Clonally isolated CI⁻ cells sampled from the bulk culture were inoculated into 20 separate 60-mm dishes (2×10^3 per dish). After 8 days of culture on plastic surfaces, all the cells in each dish were inoculated onto a cell mat and incubated for 14 days. Resultant colonies were fixed, stained, and counted. Mean = 4.17; variance = 60.4; variance of the mean = 14.5.

of proliferative potential—i.e., senescence of CI⁻ cells.

Adaptation of the cultures grown on cell mats as a possible explanation for their higher cloning efficiency can be rejected because of the retarded growth of CI⁻ cells on cell mats (Fig. 3 *Inset*), as cellular adaptation is usually associated with increase in proliferative potential. Therefore, the observed difference of cloning efficiency curves between cultures grown on two different surfaces (Fig. 3) indicates that proliferation of CI⁻ cells induces a loss of CI⁻ growth property or an acquisition of CI⁺ phenotype (failure to divide on cell mats). Such a phenotypic change in the cell culture results in the dilution of CI⁻ cells by CI⁺ cells emerging during cultivation. This dilution process is faster in cultures on plastic dishes, in which there is no restriction on proliferation of CI⁺ cells.

Split Culture Experiment. To probe the nature of the process by which CI⁻ cells become CI⁺ cells in terms of cellular differentiation, a "split culture" similar to Luria–Delbrück fluctuation analysis (15) was performed. Approximately 2×10^3 cells from the pooled culture of cloned CI⁻ cells were inoculated separately into 20 dishes (60 mm) and incubated for 8 days. A significant variation in the number of CI⁺ colonies was observed in individual split cultures when all the cells in each dish were inoculated onto cell mats. This experiment indicates that the cloned CI⁻ cell culture is heterogeneous in generating CI⁺ progenies, suggesting that each CI⁻ cell is in a different stage of cellular differentiation from CI⁻ to CI⁺ phenotype. With a high variance/mean ratio, this experiment can also be interpreted as suggesting that the conversion from CI⁻ to CI⁺ is a random event, reminiscent of a mutational event (15).

As shown in Table 5, short-term cultivation on cell mats for selecting CI⁻ cells also increased the AD⁻ subpopulation in these cultures as compared with similar cultures at corresponding passages and PDL. This phenomenon suggests that within the CI⁻ subpopulation, there is a subpopulation that possesses both CI⁻ and AD⁻ growth phenotypes. Therefore, it is conceivable that the origins of these subpopulations are similar if not identical. So far, attempts to obtain AD⁻ cell cultures from AD⁻ clones in soft agar have been unsuccessful.

FIG. 3. Cloning efficiency of CI⁻ cell culture grown continuously on cell mats (● and ▲) and in plastic dishes (○ and △) as assayed on cell mats (○ and ●) and on plastic surfaces (△ and ▲). (*Inset*) Cumulative number of CI⁻ cells in culture grown in plastic dishes (○) and on cell mats (●).

DISCUSSION

In SHE cell cultures, subpopulations of cells that have CI⁻ or AD⁻ (or both) growth properties have been found. The number of cells that have these properties decreased with increasing age of the embryos from which cultures were derived and with continuing passage *in vitro*. Although the cultures established from 10-day-old embryos contain larger subpopulations of these cells

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as compared with those from 13-day-old embryos, their disappearance from culture is faster in the 10-day embryo cells (Tables 2 and 3). This may be due to inappropriate culturing condition for the cells established from 10-day-old embryos; *in vitro* life span of these 10-day-old embryo cultures is shorter than that of 13-day-old embryos when cultured in the same growth media. We have also demonstrated that the loss of CI^- cells from culture is caused neither by negative selection nor by loss of proliferative capacity of CI^- cells but by phenotypic conversion from CI^- to CI^+ cells on growth. We interpret this phenotypic conversion as an indication of cellular differentiation. This hypothesis is supported by the split culture experiment, which shows heterogeneity of cloned CI^- cells in generating CI^+ progenies, suggesting various stages of commitment in the differentiation process from CI^- to CI^+ phenotype. Moreover, we have found that the culture that has a larger subpopulation of CI^- cells has a longer lifespan *in vitro* than the culture that has a smaller subpopulation of CI^- cells (data not shown). All these observations are compatible with the hypothesis that the finite life span of cultured cells may be due to the decrease or loss of subpopulation of immortal cells (16) and that *in vitro* cellular aging is caused by terminal differentiation (17). Therefore, the CI^- cells reported here are considered to correspond to less differentiated cells capable of undergoing more cell divisions than CI^+ cells before reaching terminal differentiation.

The AD^- subpopulations also disappeared on growth and cell division on both cell mats and plastic surfaces. It was also shown that the enrichment procedure of CI^- cells increased the subpopulation of AD^- cells and that this subpopulation decreased in parallel with the CI^- subpopulation. Moreover, cloning efficiency of AD^- cells in soft agar appears to increase with larger inoculum (data not shown), indicating a crossfeeding effect. This is in contrast to the neoplastically transformed cells, which cannot form colonies efficiently in high density culture, probably due to the nutrient depletion (18). As bone marrow progenitor cells require a feeder layer, colony-stimulating factor, or conditioned medium for colony formation in semisolid agar (19), the nutritional requirement of AD^- cells is similar to that of the hematopoietic progenitor cells rather than to that of the neoplastic cells. All of the above results suggest that the disappearance of the AD^- subpopulation is also caused by cellular differentiation.

There have been a number of studies on the induced differentiation of transformed cells (20–23) but none on the transformation propensity of differentiated vs. undifferentiated cells. The unusual nature of our system is that subpopulations of normal diploid embryonic cells are found to exhibit CI^- and AD^- growth properties highly correlated with malignancy. Moreover, on *in vitro* replication, these subpopulations differentiate to acquire CI^+ and AD^+ growth phenotypes, properties of normal diploid embryonic fibroblasts. Thus, this system should enable us to study the interrelationship between regulation of normal differentiation and that of malignant gene expression in normal diploid cells in culture.

It has been reported that in SHE cells, the propensity toward x-ray-induced neoplastic transformation decreases with increasing passage, suggesting the presence of a subpopulation more susceptible to neoplastic transformation in early passages (24). These cells could correspond to the CI^-/AD^- cells reported here, as the CI^-/AD^- cells already exhibit phenotypes associated with neoplasia. To understand the basic interrelationship among differentiation, somatic mutation, and neoplastic transformation, it is necessary to clarify the differences in the processes of neoplastic transformation and somatic mutation between undifferentiated and differentiated cells. This early SHE cell system appears to be unique for such a study, as all three processes can be investigated concomitantly after a single carcinogenic perturbation.

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