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Colony-forming Ability in Calcium-poor Medium *in Vitro* and Tumorigenicity *in Vivo* Not Coupled in Clones of Transformed Rat Hepatic Epithelial Cells[1]

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ABSTRACT

The ability of eukaryotic cells in culture to proliferate in calcium-poor medium has been found to characterize populations of transformed cells, but the relationship between this phenotypic property and tumorigenicity at the cellular level is unclear. Thus, we have isolated 14 clonal subpopulations, based on their ability to colonize in calcium-poor medium, from a parental tumorigenic rat hepatic epithelial cell line which was transformed by multiple exposures to *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. These clonal subpopulations of cells were tested for their ability to grow in soft agar, to express γ-glutamyl transpeptidase activity, and to form tumors upon back-transplantation into isogeneic newborn rats. The results indicated that clonal subpopulations of cells selected by their ability to grow in calcium-poor medium were phenotypically heterogeneous for γ-glutamyl transpeptidase activity and anchorage-independent growth, and, more importantly, they were not more tumorigenic than the phenotypically heterogeneous parental cell line. This observation suggests that the capability of cultured hepatic epithelial cells to grow in calcium-poor medium is not tightly coupled to the tumorigenic phenotype.

INTRODUCTION

Nontransformed mammalian cells require a relatively high concentration (1.0 to 2.0 mM) of calcium in their extracellular environments in order to initiate DNA synthesis and proliferate (2, 3, 5–7, 17, 21–26). In sharp contrast, neoplastically transformed populations of cells, both mesenchymal and epithelial, do not have a stringent requirement for environmental calcium, being able to initiate DNA synthesis, proliferate, and form colonies virtually as well in medium that is poor in calcium (0.02 mM) as in medium containing calcium at a concentration of 1.0 to 2.0 mM (2–4, 6, 7, 15, 19, 21–26). Indeed, the relative abilities of populations of eukaryotic cells to grow in calcium-poor and calcium-rich media have been found in several studies to distinguish accurately between tumorigenic and nontumorigenic populations of cells (19, 21, 23). It has been suggested that the ability to grow and form colonies in calcium-poor medium can be used to selectively isolate tumorigenic cells from mixed populations (15, 21). Even though the reported studies provide strong evidence that the ability to grow in calcium-poor medium is a characteristic feature of populations of transformed cells, the data available do not allow the conclusion that tumorigenicity and the ability to grow in calcium-poor medium are closely coupled at the cellular (clonal) level. Pretumorigenic and tumorigenic populations of cells are phenotypically heterogeneous (8–10), and tumorigenicity and ability to grow in calcium-poor medium may represent independent phenotypes. We have attempted to clarify this problem by comparing the relative tumorigenicity of a phenotypically heterogeneous population of transformed hepatic epithelial cells and of subclones of this population selected by isolation of colonies that form in calcium-poor medium. The results of this study show that several clonal subpopulations selected by their ability to colonize in calcium-poor medium are each less tumorigenic than is the phenotypically more heterogeneous population from which they are derived. Furthermore, our evidence suggests that each of several different clonal subpopulations selected for their ability to colonize in calcium-poor medium varies greatly in its relative tumorigenicity as well as in other phenotypic features. We conclude that tumorigenicity and ability to form colonies in calcium-poor medium are independent phenotypic traits in populations of transformed hepatic epithelial cells and that these traits are not coupled closely in individual cells from which clones are derived.

MATERIALS AND METHODS

The diploid hepatic epithelial cells used, termed the WB-F344 line, were isolated in our laboratory from the liver of a young adult, male rat of the Fischer 344 strain.[4] To obtain the tumorigenic cell line (WB-5-11), the WB-F344 cells were treated during logarithmic growth phases for 11 consecutive times with MNNG[5] (5 μg/ml). The schedule of treatment and the characterization of cell lines obtained after each treatment with MNNG will be described elsewhere.

Growth rates and colony-forming efficiency of cell lines grown in calcium-poor medium with or without supplemented calcium salt were determined using Falcon 6-well plates and 100-mm tissue culture plates. Calcium-poor medium was prepared from calcium-free Imemzo medium with zinc and insulin (Associated Biomedic System), buffered with 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Sigma Chemical Co.)-2.6 mM sodium bicarbonate, and supplemented with gentamycin (0.05 mg/ml), and 20% fetal bovine serum (Grand Island Biological Co.) which had been dialyzed against Chelex ion exchange resin (22). This medium contained less than 0.025 mM Ca^{2+} as determined by atomic absorption spectroscopy. To reconstitute the normal calcium concentration of the medium (2.0 mM), 2.0 ml of 1 M $CaCl_2$ solution were added to each liter of the calcium-poor medium.

Subclones were isolated in calcium-poor medium by seeding 10^2 to 10^3 tumorigenic WB-5-11 cells in 100-mm dishes (Falcon) in complete (calcium-rich) medium. After 24 hr, this medium was replaced with calcium-poor medium. Under these conditions of culture, plates of acetone-treated, control cells contained neither colonies nor visible single cells after 4 weeks. Colonies were observed in plates of transformed

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[5] The abbreviations used are: MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine; GGT, γ-glutamyl transpeptidase.

cells (WB-5-11) maintained in calcium-poor medium for 4 weeks, at which time calcium-poor medium was replaced with calcium-rich (basal) medium. Fourteen colonies were isolated with cloning rings and replated on 35-mm dishes in complete medium, and the populations gradually expanded. The clonal subpopulations were evaluated for the expression of GGT and the ability to form colonies in soft agar (13).

GGT activity was evaluated in 3 ways. (*a*) Cells (10^5) were seeded in 100-mm dishes, and confluent cultures were stained histochemically for GGT (18). The relative abundance of GGT-positive cells was scored 0, 1+ (1 to 10%), 2+ (10 to 49%), and 3+ (>50%). (*b*) One hundred cells were seeded in 100-mm dishes in duplicate, and 2 weeks later, the plates were stained histochemically for GGT for 15 min. Colonies which contained more than 30% of GGT-positive cells were scored as positive colonies. The total number of colonies was ascertained after staining with hematoxylin. (*c*) The specific activity of GGT in the clonal subpopulations was measured by Diagnostic Kit 545 (Sigma Chemical Co., St. Louis, MO).

For analysis of tumorigenicity, 10^6 cells in 0.1 ml of complete tissue culture medium were inoculated into the abdominal s.c. tissue of 1-day-old Fischer 344 rats. Tumor-bearing rats were killed when neoplasms were about 1 to 2 cm in diameter. Rats in which tumors could not be palpated were killed at the end of 16 months.

RESULTS

After 11 repeated treatments with MNNG (5 μ/ml), the tumorigenic WB cell population (WB-5-11) produced tumors in 34% of 1-day-old Fischer 344 newborn rats when 10^6 cells were inoculated s.c. into each animal. Tumors were first detected by palpation at 10 months after inoculation of cells. They were poorly differentiated carcinomas which showed foci of gland formation (adenocarcinoma) and other areas that resembled hepatocellular carcinoma. Control cells, which were paired with treated cells and were cultured and transferred identically, received no treatment (WB-0-11) or acetone vehicle alone (WB-A-11), and they failed to produce any tumors when inoculated under identical circumstances into newborn animals or when inoculated at a concentration of 10^7. The population of WB cells treated 11 times with 5 μg MNNG/ml (WB-5-11) was phenotypically heterogeneous for a number of characteristics, including DNA content and chromosome number, glycogen synthesis, isozymes of lactate dehydrogenase, NADH-diaphorase, GGT, and morphology.[6]

Control hepatic epithelial cells of the WB line (WB-0-0, WB-A-11, or WB-0-11) will not grow and form colonies in medium containing 0.02 mM calcium, even after 35 continuous population doublings *in vitro*. We were unable to establish any propagable clonal subpopulations in calcium-poor medium from untransformed populations of WB cells. In contrast, the phenotypically heterogeneous transformed population, derived from these diploid hepatic epithelial cells by 11 consecutive treatments with 5 μg MNNG/ml culture medium (WB-5-11), grew reasonably well in medium containing 0.02 mM calcium (Table 1). Furthermore, clonal subpopulations of the transformed population could be readily established by selective colonization in calcium-poor medium. The data presented in Table 2 suggest that these clonal subpopulations were heterogeneous for cellular phenotypes expressing the ability to form colonies in soft agar and the histochemically demonstrable presence of GGT and the biochemical activity of this enzyme. Five of the clonal subpopulations and the parental transformed and control populations were tested for tumorigenicity by back-transplanting cells s.c. into 1-day-old isogeneic rats. The combined results from all of the clonal subpopulations selected from the phenotypically heterogeneous transformed population by the ability to form colonies in 0.02 mM calcium are significantly less tumorigenic than are the unselected cells of the parental population ($\chi^2 = 7.8$; $p = < 0.01$). Results of tumorigenicity analysis of individual clonal subpopulations selected in calcium-poor medium suggest that tumorigenicity segregates heterogeneously in different clonal populations selected by the single criterion on their ability to form colonies in 0.02 mM calcium (Table 3). The apparent tumorigenicity of clonal subpopulations varies from nil to near that of the phenotypically heterogeneous, transformed parental population. However, no clonal subpopulation examined was more tumorigenic than was the phenotypically heterogeneous, transformed parental population.

Table 1

Relative abilities of WB hepatic epithelial cells treated various times with 5 μg MNNG/ml to grow in media containing 0.02 or 2.0 mM calcium

Cell group	Culture doublings	Cumulative concentration of MNNG (μg)	Ratio in medium containing 0.02/2.0 mM Ca^{2+} (increase in no. of cells in 1 wk)	Colony-forming efficiency
WB-0-0	5	0	0.09	0
WB-5-3	15	15	0.08	0
WB-5-5	20	25	0.13	0
WB-5-9	32	45	0.25	ND[a]
WB-5-11	38	55	0.68	0.37 ± 0.25[b]
WB-0-11	38	0	0.20	0.003 ± 0.001

[a] ND, not done.
[b] Mean ± S.D.

Table 2

Correlation between expression of GGT and ability to form colonies in soft agar by subpopulations of transformed hepatic epithelial cells selected for their ability to colonize in calcium-poor medium

	Expression of GGT			
	Relative abundance of histochemically positive cells	Mean % of colonies containing positively stained cells	Enzymatic specific activity (milliunits/mg protein)	Growth in soft agar
Transformed parental population (WB-5-11)	1+	9	6.1 ± 2.3[a]	+
Control (WB-0-11)	0	0	2.4 ± 0.9	–
Clonal subpopulations				
1	1+	ND[b]	ND	–
2	2+	ND	ND	+
3	3+	84	35 ± 1.8	+
4	1+	39	3.5 ± 0.1	+
5	1+	3	3 ± 0	+
6	0	ND	ND	–
7	0	ND	ND	–
8	1+	46	1.4 ± 0.1	+
9	0	ND	ND	–
10	2+	ND	ND	+
12	3+	81	25.9 ± 4.5	ND
13	2+	27	3.6 ± 0.2	ND
14	2+	37	1.2 ± 0	ND

[a] Mean ± S.D.
[b] ND, not done.

[6] M. S. Tsao, J. W. Grisham, K. G. Nelson, and J. D. Smith, manuscript in preparation.

Table 3
Tumorigenicity of a population of cultured hepatic epithelial cells transformed by in vitro treatment with MNNG, compared with the tumorigenicity of clonal subpopulations derived in calcium-poor medium and of nontransformed control populations

Source of cells	Ratio of no. of animals with tumors/no. of animals given injections of cells
Control population of cultured hepatic epithelial cells	0/11
Transformed population of cultured hepatic epithelial cells	21/61
Clonal subpopulations derived from transformed population by growth in calcium-poor medium	
Results of all combined studies	6/40
Individual subclones	
Clone 3	2/7
Clone 4	2/9
Clone 5	1/9
Clone 6	0/6
Clone 8	1/9

DISCUSSION

We conclude that tumorigenicity *in vivo* and the ability to grow in calcium-poor medium *in vitro* are independent phenotypic traits that are not closely coupled in individual cells (clones) isolated from a phenotypically heterogeneous, transformed population of hepatic epithelial cells. Although the ability to grow in calcium-poor medium distinguishes the tumorigenic transformed population from hepatic epithelial cell populations not treated with MNNG, and from populations that were treated with MNNG but are not yet tumorigenic, this phenotypic trait is not precisely linked to tumorigenicity at the clonal or cellular level. If the ability of transformed cells to colonize in calcium-poor medium was a precise marker of tumorigenicity at the cellular level, these clonal subpopulations would be expected to be more tumorigenic than the parental population. Instead, clonal subpopulations selected for their ability to form colonies from single cells in medium containing 0.02 mM calcium were not more tumorigenic than the phenotypically heterogeneous transformed population of hepatic epithelial cells from which they were derived. Although the numbers of clones tested are small, individual clones from calcium-poor medium appear to vary in their tumorigenicity from nil to near that of the population of cells from which they were isolated. The individual subclones also demonstrate considerable diversity for other phenotypic properties, including the ability to form colonies in soft agar and the expression of GGT. The scoring of GGT activity of individual subclones by the histochemical method demonstrated that most of these isolated subclones contained both GGT-positive and -negative cells, and the former have variable specific activities of this enzyme. The result also suggests that expression of GGT activity is not correlated with the ability of cells to grow in low-calcium medium.

Studies previously reported have suggested a close association in transformed hepatic epithelial cell populations between tumorigenicity, the ability to form colonies in soft agar, and the histochemical expression of GGT with the ability to form colonies in calcium-poor medium (19, 21). However, the relative tumorigenicity of cells that have been selected for their ability to grow in calcium-poor medium has not been addressed in these previous studies. Nevertheless, evidence from the previous studies raises the possibility that the relationships between these transformed phenotypes may not be precise. Nontransformed hepatic epithelial cells, tumorigenic populations of hepatic epithelial cells transformed *in vitro*, and cell lines isolated from hepatocellular carcinomas produced *in vivo* all demonstrate considerable variations in their relative abilities to form colonies in calcium-poor medium (19). Although cells of 2 of 3 nontransformed hepatic epithelial lines failed to form colonies in calcium-poor medium, one of 4 tumorigenic hepatic epithelial cell lines was less efficient in forming colonies in calcium-poor medium (6.8% of high calcium) than was one nontumorigenic cell line (8.6% of high calcium), and the other 3 tumorigenic lines showed efficiencies of 28.7 to 69.8%. The efficiency of colony formation in calcium-poor medium also varied markedly in cell lines derived from hepatocellular carcinomas, varying over the range of 13.9 to 96.8% of colony formation in calcium-rich medium (19). Recent studies also suggest that the ability of cultured hepatic epithelial cells to grow in calcium-poor medium is not a precise characteristic of chemically transformed (anchorage-independent) hepatic epithelial cell populations (20). Furthermore, calcium dependence of cultured cells appears to be affected by several conditions not directly related to their ability to produce tumors. Untransformed cultured cells acquired an improved ability to grow in calcium-poor medium after they had been grown *in vitro* for many generations (5, 6), and exposure of normal cells to epidermal growth factor (1, 12, 14) or 12-*O*-tetradecanoylphorbol-13-acetate (11) is said to reversibly reduce the requirement for extracellular calcium.

The mechanism(s) by which cells gain the ability to grow in calcium-poor medium is unknown. A scheme has been outlined that would relate the acquisition of this property to loss of growth control and possibly, therefore, to neoplastic transformation and tumorigenicity (21, 25). Although our results suggest that ability to grow in calcium-poor medium and tumorigenicity are not closely linked at the clonal or cellular level, it remains possible that this apparent lack of relationship is due to asynchrony in the appearance of these transformed phenotyes. For example, ability to grow in the presence of low levels of calcium may precede the acquisition of tumorigenicity. If such a pattern of development of transformed phenotypes does occur, then the observation of low-calcium clones that are not tumorigenic is to be expected. Further observations are required to determine whether tumorigenicity regularly develops in clonal populations that are able to grow in low-calcium medium but are not tumorigenic. However, a cultured malignant cell line which is unable to proliferate in low-calcium medium has been reported (16), providing further support that the acquisition of these 2 phenotypes by cells is not necessarily linked temporally or materially.

It is important to evaluate the material linkage between paratumorigenic phenotypes and tumorigenicity at the cellular level. Linkage of tumorigenicity and a paratumorigenic phenotype at the clonal (cellular) level would imply that (*a*) the gene controlling such a phenotype is located adjacent to the "transformation" gene on the same chromosome, supposing that such a single-locus transformation gene exists; (*b*) the paratumorigenic phenotype is controlled by the same regulatory gene as the transformation locus; and/or (*c*) the phenotypic property is required for the survival of the transformed cell. The analysis of the cellular coupling of phenotypic traits in clonal sublines of phenotypically heterogeneous, transformed hepatic epithelial cells may provide new information on the genetic nature of neoplastic transformation. Such studies may also lead to the identification or exclusion of markers that correlate precisely with tumorigenicity at the cellular level.

ADDENDUM

During the review of this manuscript, we were made aware of a manuscript by Boynton, Kleine, and Whitfield entitled "Relation between colony formation in calcium-deficient medium, colony formation in soft agar, and tumor formation by T51B rat liver cells," which has since been published (Cancer Lett., *21:* 293–302, 1984). This paper reported a study similar to ours with the same conclusion as described in this paper. We wish to thank the anonymous reviewer for providing us with a preprint of this manuscript before it was published.

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