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Genetics

Analysis of malignancy in human cells: Malignant and transformed phenotypes are under separate genetic control

(human cell hybrids/fibroblasts/HeLa cells/tumorigenicity/neoplastic progression)

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ABSTRACT **Human cell hybrids derived from malignant HeLa and normal fibroblast parental cells expressed many of the transformed properties of the HeLa parent but their tumor-producing capability was suppressed. Hybrids derived from HeLa/HeLa fusions retained both their transformed and malignant phenotypes. Thus, an apparent separation of the control of the transformed versus malignant phenotype is indicated. Furthermore, several transformed properties—including lack of density-dependent inhibition of growth, lectin agglutination, lowered requirement for serum growth factors, and anchorage independence—are expressed coordinately in the nontumorigenic hybrids. This finding suggests that none of these properties by themselves, or in concert, endows a cell with tumorigenic potential.**

The transition of a normal cell to a neoplastic one is reflected by a complex array of phenotype changes that are amenable to study. Many of these changes—which include lack of contact inhibition of division or topoinhibition (1, 2), reduced requirement for serum growth factors (3, 4), agglutination of cells by lectins (5, 6), anchorage independence (7, 8), altered cyclic nucleotide levels (9, 10), increased protease activity (11), and surface membrane changes such as altered ganglioside profiles (12) and fluctuations in the expression of glycoproteins [e.g., large external transformation sensitive glycoprotein (LETS)] (13, 14)—have been the subjects of intense investigation. Apparent correlations between one or more of these properties and the neoplastic (malignant) state of transformed* cells have been suggested (3–18), but in most cases the dependence of the malignant state on any one of these properties has been questioned (19–22). In many of these investigations, cells of differing origins, including different animal species, have been used for comparative purposes.

We report here our findings with intraspecific human hybrids, in which malignancy (defined here as the capacity of cells to produce a progressively growing tumor in a suitable host) is suppressed, whereas many of the *in vitro* properties that have been associated with malignant transformation continue to be expressed. Our results indicate that these two functional states—that is, the malignant and transformed phenotypes—are under separate genetic control in this cell system.

MATERIALS AND METHODS

Parental and Hybrid Cells. The parental and hybrid cell lines are presented in Table 1. Details of hybridization procedure and selection of hybrids have been presented elsewhere (23). All cell populations were regularly tested for the presence of mycoplasma contaminants by cultural methods, uridine/uracil ratio (24), and 4′,6-diamidino-2-phenylindole assay (25). The only cell line that was contaminated with mycoplasmas was ESH6.

Growth Curves. Topoinhibition was measured by plating approximately 1×10^5 cells into each 60-mm petri dish containing 5 ml of Eagle's minimal essential medium supplemented with 2 mM glutamine, 5% fetal calf serum, penicillin (100 units/ml), and streptomycin (50 μg/ml), hereafter designated growth medium. The cells received fresh growth medium on days 3 and 5 of the assay. At 48-hr intervals, cells were removed from dishes with 0.1% trypsin/7 mM EDTA and counted in a model ZBI Coulter counter.

The requirement for serum growth factors was assayed by plating cells as described above, but in this case only 0.25% fetal calf serum was present in the medium. The cells were not re-fed with fresh medium during the 7-day period of this assay.

Lectin Agglutination. Cells were removed from their substrate by treatment with 7 mM EDTA or 10 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid and suspended in sterile phosphate-buffered saline. Cell clumps were removed by gravity sedimentation and the resulting single cell suspensions were adjusted to a concentration of 1×10^6 cells per ml. One-half milliliter of each cell suspension was placed onto the surface of a siliconized 35-mm petri dish and mixed with 0.5 ml of phosphate-buffered saline or conconavalin A (Con A) at final concentrations of 20 and 100 μg/ml. The degree of agglutination was scored at 10-min intervals for a period of 30 min at room temperature.

Growth in Soft Agar. The method used was essentially that described by Macpherson and Montagnier (26). Briefly, an agar base layer containing growth nutrients was pipetted into 60-mm petri dishes. The base layer was allowed to solidify and then suspensions of cells ranging from 5×10^4 to 1×10^6 per dish were layered over the base layer in 0.3% agar medium. The cultures were incubated in a humidified CO_2 incubator at 37° for periods up to 21 days, with periodic re-feeding.

Malignancy Assays. Congenitally athymic *nu/nu* (*nude*) mice were used as the *in vivo* assay system for determining the malignant state of the various cell populations. We have demonstrated the efficiency of this system in earlier reports (23, 27). Cells were harvested by trypsinization and suspended in serum-free minimal essential medium; 0.2-ml samples of these cell suspensions, containing $1–50 \times 10^6$ cells, were inoculated subcutaneously into the ventral midline. Certain selected cell lines were inoculated via several routes (subcutaneous, intramuscular, intraperitoneal, intrathoracic, and intracerebral). Finally, selected cell lines were grown on glass beads (4-mm

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Abbreviations: LETS, large external transformation sensitive glycoprotein; Con A, concanavalin A.

* Our definition of a transformed cell is one that has become an immortal (established) line and exhibits any or all of the *in vitro* characteristics described above.

Table 1. Parental and hybrid human cell lines

Cell line designation	Genetic constitution
D98/AH-2	Parental HeLa
HBU	Parental HeLa*
WI-38	Parental fibroblast
ESH2	HeLa × fibroblast (H/F)
ESH3	H/F
ESH5	H/F
ESH6	D98/AH-2 × HBU*
ESH20	H/F

The HeLa parent of the HeLa × fibroblast fusions was D98/AH-2 in all cases.
* Mycoplasma contaminated.

diameter) until confluency was reached and the beads were then implanted under the skins of *nude* mice. All animals were examined for the presence of tumors and checked for physical signs of distress at regular intervals. At autopsy, tissues were taken for histopathologic study.

RESULTS

Topoinhibition. The only cell population to exhibit density-dependent inhibition of division was the fibroblast parent WI-38 (Fig. 1). All hybrid cell populations (including those not shown in the figure) continued to proliferate throughout the 8-day growth period. When the incubation continued beyond this period, the multilayered cells sloughed off the surface of the dishes. Both D98/AH-2 and the hybrids had high glycolytic activity and at the same time were very sensitive to an acidic environment. Under the conditions of the growth assay, in which the cells were only re-fed twice on days 3 and 5, increased acidity of the medium prevented meaningful measurements from being made beyond 8 days.

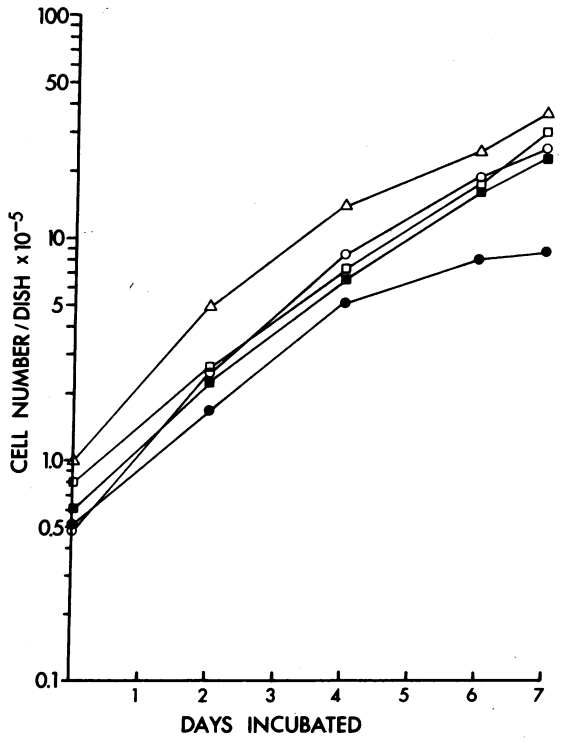

FIG. 1. Growth of parental and hybrid cell lines in medium containing 5% calf serum. Each point is a mean of triplicate samples. ●, WI-38; △, D98/AH-2; □, ESH3; ○, ESH5; ■, ESH6.

Table 2. Agglutination response to Con A

Cell type	Con A 20 μg/ml	Con A 100 μg/ml	Control
D98/AH-2	++	+++	0
WI-38	+	+	+
ESH2	+++	+++	0
ESH3	+++	+++	0
ESH5	+++	+++	0
ESH6	++	++++	+

The degree of agglutination was scored after 30-min incubation at room temperature. Agglutination was graded from 0 to ++++: 0, no aggregation; +, few small aggregates but mostly free cells; ++, 3–20 cell aggregates plus free cells; +++, most cells aggregated in clumps of varying sizes; ++++, large cell aggregates plus a few small aggregates and free cells. Control, phosphate-buffered saline.

Requirement for Serum Growth Factors. The normal fibroblast parent WI-38 failed to proliferate in minimal essential medium containing 0.25% calf serum (Fig. 2). All the other cell lines, including the HeLa/HeLa hybrid ESH6 and the various HeLa/fibroblast hybrids, underwent several population doublings in the low-serum medium. One exception was ESH3 which appeared to be intermediate in its ability to proliferate in this serum concentration.

Lectin Agglutination. All cell lines, with the exception of WI-38, were agglutinated in the presence of Con A. Interestingly, the HeLa/fibroblast hybrids agglutinated to a greater extent in low concentrations of Con A compared to D98/AH-2 or ESH6 (Table 2). Some autoagglutination was seen with the WI-38 and ESH6 preparations, which we were unable to prevent; however, this did not interfere with the assay.

Growth in Soft Agar. The results (Table 3) indicate that all of the cell lines, with the exception of WI-38, formed colonies in soft agar. The HeLa/fibroblast hybrid lines produced larger colonies at the lower cell densities. This may be due, in part, to their larger cell volume and capacity to condition medium more efficiently. The reason that ESH6 was not as efficient as the other hybrids may be related to the fact that it is contaminated with mycoplasmas. We found that it was possible to score the dishes for colonies after 8 days of incubation. At periods

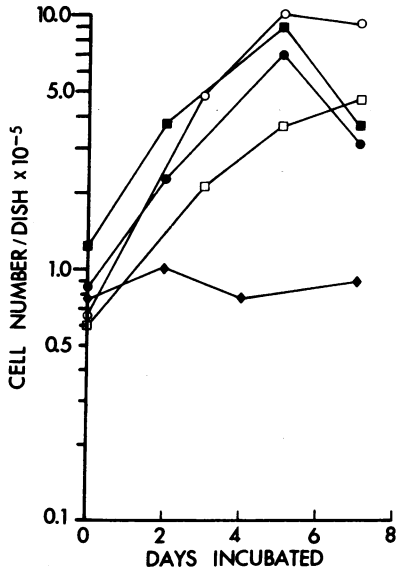

FIG. 2. Growth of parental and hybrid cell lines in medium containing 0.25% calf serum. Each point is a mean of triplicate counts. ♦, WI-38; ■, D98/AH-2; ●, ESH2; □, ESH3; ○, ESH5.

Table 3. Growth of parental and hybrid cells in soft agar

Cell type	Formation of clones			
	1×10^6	5×10^5	1×10^5	5×10^4
D98/AH-2	3+	3+	2+	1+
WI-38	0	0	0	0
ESH2	4+	4+	3+	2+
ESH3	ND	ND	3+	2+
ESH5	4+	4+	3+	2+
ESH6	4+	4+	2+	1+

Cells were seeded at four different inocula per dish (5×10^4 to 1×10^6). Dishes were scored for the presence of colonies after an 8-day incubation period. Cell clumps containing more than 10 cells were scored as positive. Absolute counts of the number of colonies per dish were not attempted. Our score range from 0 to 4+ represents an arbitrary estimate of the number of colonies per dish at each cell concentration. ND, not done.

thereafter, the colonies progessively increased in size but no further increase in the relative numbers of colonies occurred. The largest colonies were observed in the dishes seeded with 5×10^4 cells, ranging up to 1.5 mm in diameter.

Colonies were not detected in dishes seeded with less than 1×10^4 D98/AH-2 or hybrid cells. The possibility that at this low concentration the cells require conditioned medium in order to propagate has not been investigated.

A summary of the *in vitro* properties of the parental and hybrid cell lines is given in Table 4 and includes characteristics published elsewhere (23).

Malignancy Assays. The only cell lines that produced progressively growing tumors in *nude* mice were D98/AH-2 and the HeLa/HeLa hybrid ESH6, irrespective of the route of inoculation (Table 5). Selected tumors were reestablished *in vitro* and their human origin was confirmed by chromosomal and enzyme analyses (data not shown). Animals that received HeLa/fibroblast hybrid cells have remained free of tumors for periods of up to 1 year. When animals were sacrificed, histopathologic study of tissues taken at autopsy showed no evidence of neoplasia in all cases.

DISCUSSION

Transformed cells in culture exhibit a number of properties that distinguish them from their normal counterparts. Several of these properties have been described in this report. Most of them, at one time or another, have been correlated with the capacity of the cell line in question to produce tumors in a suitable animal host (3–18). Our results are clearly at variance with this suggestion. We show here that, when a malignant human cell was hybridized with a normal human fibroblast, the malignant phenotype of the resulting hybrid was suppressed. However, several properties associated with transformation continued to be expressed. One interpretation is that the transformed phenotype is expressed as a dominant or codominant trait whereas malignancy behaves as a recessive trait, a view shared by other investigators (28, 29). It should be noted here that hybrids between normal human diploid cells continue to behave as normal cells and undergo senescence (30).

The continued expression of several properties associated with the transformed phenotype in our hybrid cells is compatible with the concept of pleiotypic control advanced by Tomkins and his colleagues (31, 32). However, in other experimental systems it has been found that one or more of the transformed traits described above may escape from the effects of this postulated coordinate control (20, 21).

Assuming that our results, which were derived from hybrid combinations, accurately reflect the genetic status of individual cells, one may ask why spontaneous transformation of human cells in culture is an extremely rare phenomenon. One explanation could be that the mutational event leading to transformation may occur at a relatively low frequency in human cells and is, therefore, not detected under conventional culture conditions.

Our premise of separate genetic control for the expression of malignancy versus transformation obviously relies on an adequate *in vivo* assay for malignancy. We have expended considerable effort in developing assays that will reproducibly detect malignant animal cell populations (23, 27). In addition to the *nude* mouse we have used neonatally thymectomized, antithymocyte serum-treated mice and thymectomized, whole-body irradiated, bone marrow-reconstituted mice and have found similar results (27). We used several routes of inoculation in these experiments because we (33) and others (34) have shown that certain cell lines, especially lymphoblastoid lines, will produce tumors only in specific anatomical sites. Finally, although Boone *et al.* (35, 36) showed that certain cell lines require solid substrates in order to fulfill their tumorigenic potential, this does not appear to be the case with our hybrid cell lines. In studies to be reported elsewhere, we found that the inability of our hybrids to form tumors is not due to lack of angiogenesis factors or cytotoxic humoral immunity. Furthermore, both the HeLa parental cell population and the HeLa/HeLa hybrid will produce tumors in 100% of animals when as few as 1×10^4 cells are inoculated. As many as 5×10^7 HeLa/fibroblast hybrid cells have failed to produce tumors in experiments involving several hundred mice (unpublished data). Thus, unlike the experimental model systems of other investigators who have to contend with varying degrees of tumorigenicity of their suppressed hybrids (28, 29), there is total suppression of malignancy in our intraspecific human hybrids, and this suppression is stable. This stability is probably related to the relatively small degree of chromosomal segregation from the hybrid cells. In a detailed study of one hybrid line we noted a mean loss of only one or two chromosomes after 120 population doublings after an initial chromosomal loss during the first few divisions after fusion (B. Weissman and E. Stanbridge, unpublished data).

Table 4. Summary of the *in vitro* properties of parental and hybrid cells

Property	Parental cells		HeLa/fibroblast hybrids			HeLa/HeLa hybrid, ESH6
	Fibroblast	HeLa	ESH2	ESH3	ESH5	
Morphology	Fibroblastic	Epithelial	Variable	Variable	Variable	Epithelial
Population doublings	Finite	Infinite	Infinite	Infinite	Infinite	Infinite
Topoinhibition	+	−	−	−	−	−
Requirement for serum factors	High	Low	Low	Low	Low	Low
Growth in soft agar	−	+	++	++	++	+
Lectin agglutination	±	++	++	++	++	+

Table 5. Tumor nodule formation by human hybrid cells

Hybrid population	Route of inoculation					
	Subcutaneous	Intraperitoneal	Intramuscular	Intrathoracic	Intracerebral 5×10^5 cells	Subcutaneous (glass beads)*
ESH6 (HeLa/HeLa), 1×10^6 cells	+ (4/4)	+ (4/4)	+ (4/4)	+ (4/4)	+ (4/4)	+ (3/3)
ESH5 (H/F), 1×10^7 cells	− (0/6)	− (0/6)	− (0/6)	− (0/6)	− (0/4)	− (0/3)
ESH20 (H/F), 1×10^7 cells	− (0/4)	− (0/4)	− (0/4)	− (0/4)	− (0/4)	ND
ESH3 (H/F), 1×10^7 cells	− (0/10)	− (0/6)	− (0/6)	ND	ND	ND
ESH2 (H/F), 1×10^7 cells	− (0/10)	− (0/6)	− (0/6)	ND	ND	ND

Results as positive (+) or negative (−) and, in parentheses, number of animals with tumors/number of animals inoculated. ND, not done.
* Approximately 5×10^4 cells per bead.

Our data are compatible with the concept of neoplastic progression (37) and the two-step theory of cancer (38). Also, the recessive nature of the malignant phenotype reported here fits Coming's hypothesis of carcinogenesis (39) but, unlike his model, we find a distinct separation in the genetic control of the transformed versus malignant phenotype.

Owing to their stable nature, the use of human intraspecific cell hybrids provides a powerful tool in the determination of those traits that are *specifically* correlated with malignancy.

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