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Expression of Transformation Markers and Suppression of Tumorigenicity in Human Cell Hybrids*

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Abstract—*Somatic human cell hybrids produced by fusion of HeLa cells and diploid fibroblasts were analysed in a study designed to test the coordinate expression of transformation markers and tumorigenicity. The great majority of these hybrids displayed a finite lifespan in culture, but some of them inherited from the HeLa parent the capability to grow as permanent cell lines. Hybrids from both groups all had a plasminogen activator activity 20 to 100-fold higher and a cloning efficiency in semisolid medium 2 to 10-fold lower than the HeLa parent. Cell surface fibronectin was expressed at variable levels, albeit in a disorganized form. No correlation between the level of plasminogen activator or fibronectin content and cloning efficiency in agar was observed. Two hybrid lines, assayed for tumorigenicity in nude mice, did not produce tumors, even at inocula 20-fold greater than those at which the HeLa cells formed tumors.*

INTRODUCTION

CELL fusion between normal and malignant cells is a useful tool for studying the expression and regulation of transformation properties and tumorigenicity (for reviews see [1–4]). The use of intraspecific crosses versus interspecific ones seems to provide the most reliable conditions for analysing the dominance or recessiveness of the transformed and malignant phenotypes [5–9].

Among the cell traits that have been correlated with tumor-forming ability, increased secretion of plasminogen activator (PA) [10–13] and decreased fibronectin (FN) deposition [14–16] have been extensively but independently studied.

In the present study, the parameters mentioned above, together with anchorage independence and tumorigenicity, were analysed in human intraspecific cell hybrids obtained from the fusion of tumorigenic PA^+FN^- and normal PA^-FN^+ cells. The main purpose of this investigation was to establish the dominance relationship for each marker and possible correlations among them.

As a result, we have been able to demonstrate that increased PA secretion, reduction in the content and organization of cell-surface FN and anchorage-independent growth are expressed concomitantly in different hybrid clones, although they are dissociated from tumor-forming ability.

MATERIALS AND METHODS

Parental cell lines

These were: a thymidine kinase (TK)-deficient derivative of HeLa cells resistant to 20 μg/ml of 5-bromodeoxyuridine (BUdR); human hypoxanthine guanine phosphoribosyltransferase (HGPRT)-deficient fibroblasts (GM 1362, provided by the Human Genetic Mutant Repository, Camden, NJ). These cell lines will be referred to as HeLa cells and fibroblasts respectively.

The HeLa cells were maintained in Eagle's minimum essential medium (MEM; Gibco) supplemented with 10% calf serum (Gibco) and containing 20 μg/ml of BUdR (Sigma). Fibro-

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blasts were grown in MEM supplemented with 10% fetal calf serum (Gibco).

Production of somatic cell hybrids

Somatic cell hybrids were isolated after polyethlyene glycol (PEG; M.W. 1000; Merck) fusion, as described by Davidson and Gerald [17], and selected in MEM-HAT medium [18] plus 10% fetal calf serum. Hybrid yield was approximately $1–2 \times 10^{-4}$. The 15 primary hybrids used in this study were derived from 14 different plates.

The parental and hybrid lines were tested for mycoplasma contamination by Hoechst 33258 staining [19] and found to be negative.

Chromosome analysis

Chromosome preparations were made according to the standard procedures. Conventional Giemsa and Quinacrine (Sigma) staining were used for chromosome counts (at least 30 metaphases for each line) and karyotyping respectively.

Assay for PA

Supernates from semiconfluent cells cultures, washed with Hanks solution and incubated for an additional 18 hr in the absence of serum, were harvested and aliquots assayed both in the presence and absence of 0.3% Na-dodecylsulphate (SDS) [20]. The assays were performed with and without plasminogen (PG, Kabi, Sweden) at a concentration of 6 μg/ml. PA levels were quantified using human urokinase (UK, Sigma) as a standard and the activity was expressed as UK Sigma unit equivalents. In order to normalize the data, PA levels were corrected for the number of cells and expressed as activity/cell/hr.

Test for FN by indirect immunofluorescence

Antisera: monospecific rabbit antiserum against human plasma fibronectin (kindly provided by Prof. Vaheri) and FITC-conjugated goat antiserum against rabbit Ig (Dako) were used. Immunofluorescent staining for FN was carried out as described by Vaheri *et al.* [16].

Cloning efficiency in liquid and semisolid medium

To test anchorage independence of growth, $10^3–10^5$ cells suspended in 3 ml of complete medium containing 0.36% Difco Noble Agar were plated in duplicate 60-mm dishes over a layer of complete medium containing 0.56% agar, as described by MacPherson and Montagnier [21]. The cultures were re-fed weekly, and after 14–21 days of incubation the number of colonies containing more than 25 cells was scored.

The plating efficiency of cells in liquid medium was determined in parallel by seeding $10^3–10^4$ cells in duplicate 60-mm dishes and the number of colonies with more than 25 cells scored after 8 days.

Tumorigenicity test

Cells to be tested were harvested by trypsinization and resuspended in serum-free medium at a concentration of $1 \times 10^6–10^7$ in 0.2 ml; 0.2–0.4 ml of cell suspension were injected subcutaneously at a single site in athymic nude mice (Charles River) and tumor development followed for at least five months after injection.

RESULTS

After PEG-mediated fusion of TK$^-$ HeLa cells with HGPRT$^-$ fibroblasts, 86 colonies were isolated from three independent experiments without selecting any particular morphologic type. Out of these, 64 showed exhaustion of growth *in vitro* after 1 month, 15 after 2 months and 7 after 4 months.

Transition from efficient growth to senescence was often accompanied by selective overgrowth within the heterogeneous hybrid cell populations of the subset of cells with a fibroblastic morphology. Since the hybrid clones that went into crisis were subcultured both in the presence and absence of selective medium, exhaustion of their growth cannot be ascribed to the loss of HGPRT and/or TK loci. Only two hybrid clones (H14 and B5) exhibited indefinite lifespan *in vitro*. Due to the progressive decline in growth rate, cytogenetic investigations and assays of transformation parameters were only performed on a fraction of the isolated colonies.

Cytogenetic analysis

Chromosome analysis proved the hybrid nature of the isolated colonies, as can be seen in Table 1, where modes and ranges are reported for 14 representative isolates. The modal chromosome numbers and variation intervals observed were both slightly lower than those expected from 1:1 hybrids of fibroblasts and HeLa cells, indicating that chromosome segregation had probably occurred in the very first phase of hybrid cell development. All hybrid lines, except for one (H14) with the lowest modal chromosome number (80), displayed the Y chromosome contributed from the fibroblast parent. The study of karyotype evolution performed on hybrid lines H14 and

Table 1. In vitro *properties of parental and hybrid lines*

Cells	Secreted P.A. (SU/cell/hr, $\times 10^{-11}$)	Pericellular FN pattern*	Plating efficiency (%) in liquid medium	Plating efficiency (%) in agar	Chromosome constitution Mode	Range	Y chromosome
Parental cells:							
GM 1362	<0.06	+++++	1×10^{-4}	$<5\times10^{-5}$	46	–	+
HeLa BUdR^R	2.7	–	22.4	17.0	60	56–61	–
Hybrid clones:							
H1	27	+++	n.f.†	n.f.†	97	93–100	+
H2	20	++	n.f.†	n.f.†	95	91– 98	+
H3	16	++	n.f.†	n.f.†	96	91– 97	+
H9	120	++	n.f.†	n.f.†	102	93–103	+
H23	30	++++	n.f.†	n.f.†	98	96–101	+
H25	46	++	n.f.†	n.f.†	99	93–101	+
E2	25	++	n.f.†	n.f.†	95	90–100	+
D2	n.d.‡	+++	n.f.†	n.f.†	n.d.	n.d.	n.d.
I1	n.d.	++	n.f.†	n.f.†	93	85–100	n.d.
H4	45	++	n.f.†	n.f.†	97	90–100	+
H21	63	+++	n.f.†	n.f.†	94	90–102	+
H14	42	+	7.9	1.9	80	73– 83	–
H35	144	++++	7.7	1.7	97	93–101	+
B5	113	++	13.4	9.0	91	84 –100	+
H45	230	+++	10.8	1.9	92	91–100	+

*Scored on a scale of – (HeLa) to +++++ (fibroblasts).
†Not feasible, since the clone died before reaching an adequate number of cells.
‡Not determined.

B5 that were continuously propagated for 6 and 9 months, respectively, showed a substantial chromosome loss, leading to a marked reduction in modal chromosome numbers (Fig. 1).

PA activity

A set of 13 hybrid clones and their corresponding PA^- (fibroblasts) and PA^+ (HeLa) parental cell lines were assayed for secreted PA activity. PA was dominantly expressed and found to be de-repressed, as enzyme levels in the hybrids were 20 to 100-fold higher than that of the PA^+ parent (Table 1, 1st column). Preferential selection of fusion products derived from HeLa cells with higher PA activity can be ruled out on the basis of the results of subcloning HeLa cells, which revealed an homogeneous cell population with respect to PA activity (data not shown). No enhancement of fibrinolytic activity was observed by co-cultivating fibroblasts and HeLa cells in a 1:1 ratio for periods of up to one week. After 5 days of co-cultivation, the PA secreted by the cell mixture was 1.6×10^{-5} SU/ml, a level intermediate between that of the PA^+ parent (2.7×10^{-5} SU/ml) and that of the PA^- parent ($< 1.5 \times 10^{-5}$ SU/ml) grown independently. All the hybrids expressed only plasminogen-dependent caseinolytic activity, as did the PA^+ parent. In order to evaluate the presence of PA or plasmin inhibitors in the conditioned media, the assays were also performed in the presence of SDS 0.3% [20]. A slight increase in PA activity ($\simeq 15\%$) was observed in all the SDS-treated samples.

In order to check stability in the production and regulation of PA, a time-course study of enzyme secretion was carried out on 4 hybrids. As shown in Fig. 2, the PA levels were almost unaffected by the number of passages.

FN pattern

The same hybrids were concomitantly tested for FN. Distribution of surface-exposed FN was

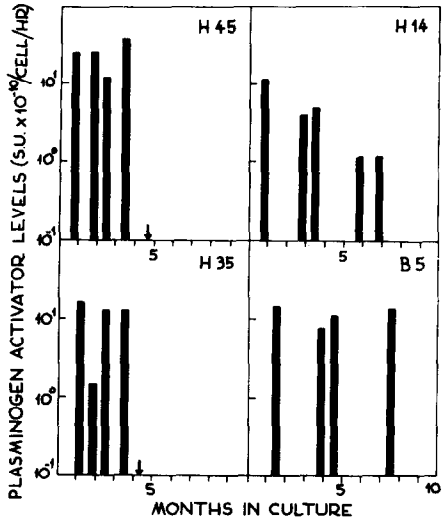

Fig. 2. Time-course analysis of plasminogen activator (PA) levels in four hybrid clones. The arrows indicate exhaustion of growth in vitro, *where applicable. The secreted PA activity is given as urokinase equivalents expressed as* SU/cell/hr.

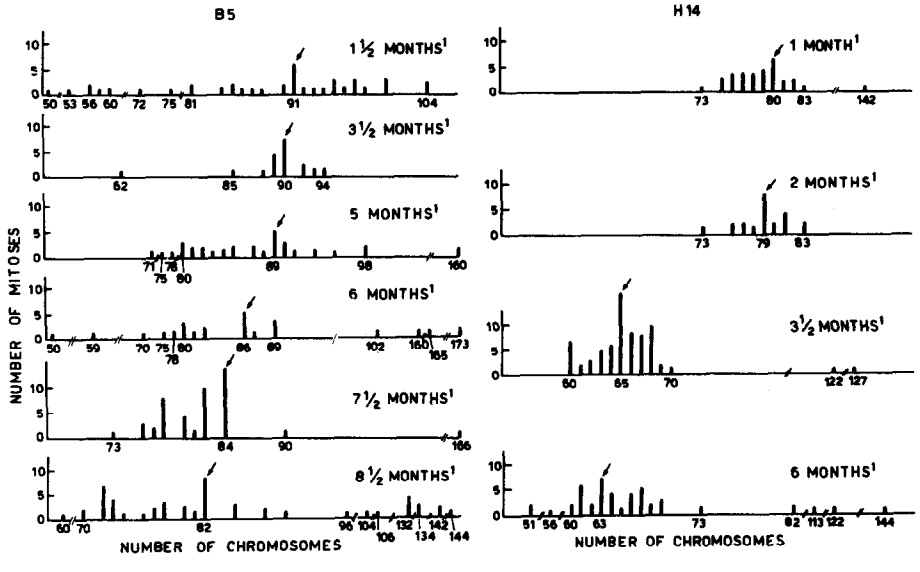

Fig. 1. Karyotype evolution of the B5 and H14 hybrid lines. The arrow indicates the modal chromosome number. (1) *Time in culture after isolation.*

evaluated by two separate criteria: a quantitative one, based on the percentage of FN-positive cells, and a qualitative one, based on determination of the organizational complexity of the fibrillar network, as compared to the parental fibroblast cells. All the hybrids analyzed exhibited surface-associated FN, albeit to varying degrees, since many intermediate patterns were observed between hybrids with a fibroblast-like pericellular matrix and others with an almost undetectable FN matrix (Table 1, 2nd column). None of the hybrids displayed the negative FN pattern typical of HeLa cells (scored as −). On the other hand, even in the most positive hybrids, the deposition of the FN matrix was never as organized as that of fibroblast cells (scored as +++++).

Growth in soft agar

Four hybrids tested for anchorage independence were able to form colonies in agar. Their plating efficiencies were 2 to 10-fold lower than that of the transformed parent (HeLa) (Table 1, 4th column). The plating efficiencies of the same hybrids in liquid medium were also lower than that of the established parent (Table 1, 3rd column). Unlike HeLa cells, hybrid lines gave rise to heterogeneous colony sizes, suggesting the presence of cells with different levels of anchorage independence.

Tumorigenicity assay

The two permanent hybrid cell lines, B5 and H14, together with the two parental lines, were tested for tumorigenicity in nude mice. Whereas the HeLa cells formed clearly detectable tumors at the site of injection 15 days following the inoculation of 1×10^6 cells per animal, no tumors were detected after 5 months of observation in any of the 4 animals injected with each of the two hybrid lines. The hybrids did not give rise to tumors even at inocula 20-fold greater than those used for HeLa cells. A histopathological study of tissues taken at autopsy confirmed the absence of neoplasia. The assay was performed after 6–8 months propagation of the hybrid lines, when chromosome segregation had led to a significant reduction in their modal chromosome numbers (Fig. 1).

DISCUSSION

A general feature of the hybrid clones derived from crossing HeLa cells with normal fibroblasts appears to be their inability to grow indefinitely in culture. If we consider permanent proliferation as an important marker of cell transformation, we might conclude that in somatic cell hybrids between a tumorigenic cell line and normal cells, the transformed phenotype is suppressed or behaves like a recessive trait, whereas finite lifespan characteristics are dominantly expressed. This is consistent with the results of Bunn and Tarrant [22], Muggleton-Harris and De Simone [23] and Pereira-Smith and Smith [24], who have observed that the majority of the hybrids obtained after fusion of normal cells with HeLa or SV40-transformed fibroblasts exhibit a finite life-span *in vitro*. However, in agreement with Stanbridge and Wilkinson [7] and Klinger [9], two of the hybrids here examined can be considered transformed since they were capable of multiplying indefinitely *in vitro* and giving rise to colonies in semisolid medium.

On the other hand, anchorage-independent growth was shown by hybrid lines whose proliferative potential became exhausted at later culture stages.

The most relevant feature common to all hybrids is their dominant expression of PA activity at much higher levels than those of the PA^+ HeLa parent. This cannot be ascribed to a decrease in the secretion of fibrinolysis inhibitors by the hybrid cells since the levels of these inhibitors were found to be equally low in both the parental and hybrid lines.

Activation of the PA gene has previously been reported in interspecific hybrid lines [25] and in one murine intraspecific lymphoma × fibroblast hybrid clone [5]. Although it is not easy to envisage the mechanism responsible for activation in these different systems, it is worth mentioning that PA synthesis is prone to modulation by a wide variety of agents [26–28]. The persistence of high PA levels in hybrids tested a few passages before the cessation of their *in vitro* lifespan suggests that the enzyme is not sufficient to prevent exhaustion of growth *in vitro*. Furthermore, since the hybrids displayed a PA activity 20 to 100-fold higher and a cloning efficiency in semisolid medium 2 to 20-fold lower as compared to the HeLa parent, no correlation between these two parameters can be inferred. These results are in agreement with those obtained by Barrett *et al.* [29].

As far as FN expression is concerned, the results we obtained are consistent with the observations of Der and Stanbridge [30], who pointed to alteration and discontinuity of the fibrillar network in hybrids between normal and transformed cells. However, FN expression in hybrids is not suppressed by the trans-

formed genotype, as observed by Laurila *et al.* [31] in heterokaryons between normal and malignant cells.

The data presented also confirm, as previously reported [32], that a pericellular matrix completely devoid of FN is not a prerequisite for growth in soft agar.

Furthermore, the results obtained rule out the possibility of a positive correlation between the expression of surface-associated FN and secreted PA levels.

The suppression of tumorigenicity, observed in two hybrid lines, has previously been reported in similar intraspecific hybrid systems [7. 9, 33], but in all cases the non-tumorigenic hybrids contained a nearly complete chromosome complement and tumorigenic isolates could be recovered by chromosome segregation. The fact that our two hybrids remained non-tumorigenic even after marked chromosome segregation suggests that the quality of the chromosomes lost is more important than their actual number, as also pointed out by Klinger [9].

In any case, the suppression of tumorigenicity observed in these two hybrid lines indicates that *in vitro* transformation markers segregate independently of tumorigenicity. This finding provides further evidence for the hypothesis of dissociation between the transformed and the malignant phenotype [7, 33].

In conclusion, hybrids produced from HeLa cells and normal fibroblasts may or may not express finite or indefinite lifespan characteristics, depending on the particular parental chromosome combinations. In hybrids surviving in culture, enhanced PA activity and reduced FN content are generally associated with anchorage independence, whereas tumorforming capability is suppressed.

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