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EXPRESSION OF THE TRANSFORMED PHENOTYPE AND TUMORIGENICITY IN SOMATIC CELL HYBRIDS

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SUMMARY

We have previously shown by examining the anchorage dependence, density-dependent inhibition of growth, LETS protein and microfilament bundles that the transformed phenotype of the parental tumours are suppressed in hybrids between rat embryo fibroblasts (REF) and mouse tumour cells (TA3B). Hybrids between TA3B and Syrian hamster sarcoma cells (BHK-B1) also show suppression. We now demonstrate that tumorigenicity in nude mice is also suppressed in TA3B × REF and B1 × TA3B hybrids. Tumours arise from the suppressed hybrids by the selective outgrowth of variants with properties different from the majority of cells inoculated. These tumour variants always had an altered cytoskeleton but 1 out of 14 cases retained anchorage-dependent growth. Selection in culture for anchorage-independent growth selected for tumorigenicity. The coordinate suppression of the transformed phenotype and tumorigenicity could be explained by postulating a pleiotropic control mechanism affecting both. However the occasional dissociation of tumorigenicity from anchorage dependence in the variants suggests that the targets for the control mechanism must be different.

INTRODUCTION

We have previously shown in hybrid cell lines between mouse TA3B tumour cells and normal rat embryo fibroblasts (REF) or between TA3B and Syrian hamster sarcoma cells (BHKBI) that a number of characteristics of tumour cells in culture are suppressed (Marshall & Dave, 1978; Marshall, Humphryes & Pollack, 1978). These characteristics termed the transformed phenotype include reduced levels of cell surface, large external transformation-sensitive protein (LETSP) (Hynes, 1976), alterations in the organization of the cytoskeleton (Pollack, Osborn & Weber, 1975), loss of density-dependent inhibition of growth (Stoker & Rubin, 1967), and anchorage-independent growth (Macpherson & Montagnier, 1964). However, the suppressed hybrids generate variants which reexpress the transformed phenotype, this subset of cells in the hybrid cell population can be isolated by virtue of their anchorage-independent growth. Thus, the hybrid cell lines consist of a mixture of cells, the major fraction consisting of cells in which the transformed phenotype is suppressed and a minor fraction of variants derived from the suppressed hybrids which express the transformed phenotype.

Preliminary studies showed that tumorigenicity was also suppressed in 2 hybrids

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(Marshall & Dave, 1978). We now study in detail the tumorigenicity of more hybrids in order to study further the expression of the transformed phenotype and tumorigenicity. We find the transformed phenotype and tumorigenicity coordinately suppressed in these hybrids. This provides novel material to examine the relationship between characters of the transformed phenotype and tumorigenicity in a particular cell line. If there is a close relationship between the transformed phenotype and tumorigenicity, selection in culture for variants which express the transformed phenotype should select for cells which do not show suppression of tumorigenicity. In addition, selection *in vivo* for variants which are tumorigenic should select for cells which express the transformed phenotype in culture.

MATERIALS AND METHODS

Most of the techniques and cell lines used in this present study have been previously described (Marshall & Dave, 1978; Marshall *et al.* 1978). Indirect immunofluorescence with antibody directed against chick gizzard myosin was performed as before except that the antibody was the gift of Dr U. Groeschel-Stewart (obtained through Dr L. M. Franks).

Tumorigenicity testing in nude mice

Single cell suspensions were prepared by removing cells from dishes with 0·125 % trypsin and 0·02 % EDTA. The cells were then washed twice with serum-free medium by centrifugation and resuspended in serum-free medium to estimate cell numbers with the aid of a haemocytometer and Coulter counter. Appropriate numbers of cells were then centrifuged and the pellet resuspended in a small volume of PBSA. Aliquots of this cell suspension were then subcutaneously injected into the rear flank of male nu/nu nude mice of unknown strain bred at the Imperial Cancer Research Fund. The mice were aged between 6 and 12 weeks at the time of injection. Cells were generally injected at doses of 10^6, 10^5 and 10^3 cells into 3 or more animals. The mice were observed twice weekly, the time taken for the formation of a tumour with a 0·5-cm diameter was recorded as the latent period. Mice which failed to form tumours were kept for up to 6 months.

Tissue cultures were initiated after dissection of the tumour followed by chopping into many fine fragments with crossed scalpels and curved scissors. Various dilutions of the tumour mince were then seeded into a series of 5-cm Petri dishes containing Dulbecco's modified Eagle's medium with 10 % foetal calf serum (Gibco, Paisley, Scotland). After 3–4 days growth at 37 °C, one or more of the dishes were usually confluent. The culture consisted primarily of tumour cells with some strongly adherent mouse cells, probably of macrophage origin. There were very few mouse connective tissue cells. Cells from the confluent dishes were then removed with trypsin/EDTA which left most of the adherent mouse cells on the dish. They were then subcultured at a 1:10 split ratio for one passage at which time frozen stocks were prepared. Cells for further experiments were then obtained from the frozen stocks, thawed and subcultured for 1–2 passages. In all tumour cell lines that were examined karyologically hybrid cells represented 100 % of the metaphase spreads.

RESULTS

Suppression of tumorigenicity in hybrids which show suppression of the transformed phenotype

Two TA3B × REF and four BI × TA3B hybrids which show suppression of the transformed phenotype (Marshall & Dave, 1978; Marshall *et al.* 1978) were tested for tumorigenicity in nude mice. Unlike the parental TA3B tumour cells no tumours

Table 1. *Tumour production in nude mice from hybrids which show suppression of the transformed phenotype in vitro*

Cell line	Expt no.	No. of cells inoculated						
			10^6		10^5		10^3	
		5×10^6: Take incidence*	Take incidence*	Latent period, days†	Take incidence*	Latent period, days†	Take incidence*	Latent period, days†
REF‡		0/4	—	—	—	—	—	—
TA3B‡		—	—	—	3/3	8	3/3	11
BI‡		—	—	—	3/3	20	3/3	20–30
TA3B × REF hybrids								
TREF 765‡		—	0/3	—	0/3	—	0/3	—
TREF 2/3.31		—	0/3	—	0/3	—	0/3	—
BI × TA3B hybrids								
BIT 2.1A	1096	—	3/3	42	3/3	70	—	—
BIT 3.4	1093	—	3/3	55	3/3	60	—	—
BIT 4.2‡	897/8	—	3/4	45	3/3	45	0/3	—
BIT 4.5	1055/6/7, 1072	—	5/5	40–60	4/6	60	0/3	—

* No. of mice with tumours/no. inoculated.
† Mean time (days) taken to produce a tumour approximately 0·5 cm in diameter.
‡ These results are from Marshall & Dave (1978).

were produced from either of the two TA3B × REF hybrids, each of which contained one genome from each parental cell. This suggests that both tumorigenicity and the transformed phenotype are very stably suppressed in these hybrids. It is ruled out that this result is merely an artifact of being a hybrid by the previous demonstration that a TA3B × REF hybrid which contained two TA3B genomes to one REF was highly tumorigenic and expressed the transformed phenotype in culture (Marshall & Dave, 1978).

Unlike the suppressed TA3B × REF hybrids, BI × TA3B hybrids which show suppression of the transformed phenotype do produce tumours in nude mice. However, these tumours arise after long latent periods, for example 10^5 TA3B cells produce a 0·5-cm tumour in 8 days, 10^5 BI in 20 days but for 10^5 cells of the hybrid cell lines the mean latent periods ranged from 45 to 70 days (Table 1). By analogy with the experiments of Harris and Klein (see Harris, 1971) these long latent periods suggest that tumours are arising from suppressed hybrids by the selective outgrowth of a subset of variant hybrids. It could be demonstrated that selection was operating by culturing tumours and testing their properties in culture. These results are described in detail below, suffice it to say that all tumours in culture had characteristics

Table 2. *Tumour production in nude mice of cells cultured from tumours derived from suppressed hybrids*

		No. of cells inoculated			
		10^6		10^5	
Original suppressed hybrid cell line	Tumour no.	Take incidence*	Latent period, days†	Take incidence*	Latent period, days†
BIT 2.1A	1096A	3/3	17	3/3	24
(42, 70)‡	1096C	3/3	19	3/3	30
BIT 3.4	1093L	3/3	20	3/3	30
(55, 60)‡					
BIT 4.2	898A	3/3	16	3/3	16
(45, 45)‡					
BIT 4.5	1056A	3/3	20	3/3	27
(50, 60)‡	1056B	3/3	15	3/3	15

* No. of mice with tumours/number inoculated.

† Mean time (days) taken to produce a tumour approximately 0·5 cm in diameter.

‡ Figures in parentheses are the mean latent periods, in days, for tumour formation for 10^6 and 10^5 cells respectively of the suppressed hybrids.

different to the majority of cells in the original hybrid cell lines. By testing the tumorigenicity of 5 cultures from tumours it could be shown that selection was operating for cells of high tumorigenicity. Table 2 shows that these cells gave tumours with 15- to 30-day latent periods, much shorter than the 45- to 70-day latent periods for the original hybrids. The original hybrid cell lines therefore consist of a majority of cells in which tumorigenicity is suppressed and a minority of cells of high tumorigenicity. Whether this subset of highly tumorigenic variants is identical to the subset of variants which express the transformed phenotype in culture is now investigated.

Variants selected in culture for expression of the transformed phenotype do not show suppression of tumorigenicity

If the tumorigenic variants are the same cells that express the transformed phenotype then selection for one property should invariably select for the other. Selection of anchorage-independent variants by isolating cells that could grow in soft agar at high efficiency should concomitantly select for highly tumorigenic cells. The results in Table 3 demonstrate that anchorage-independent variants are highly tumorigenic. Four such variants gave tumours with 21- to 30-day latent periods, much shorter than the original hybrids but similar to the variants selected *in vivo* for tumorigenicity (compare Table 3 with Table 2).

Table 3. *Tumour production in nude mice of variants selected for the ability to grow in soft agar*

		No. of cells inoculated			
		10^6		10^5	
Original suppressed hybrid cell line	Agar variant	Take incidence	Latent period, days	Take incidence	Latent period, days
BIT 2.1A (42, 70)*	2.1AAC8	3/3	21	2/2	30
BIT 3.4 (55, 60)*	3.4AC1	3/3	20	3/3	30
BIT 4.2 (45, 45)*	4.2AC2/3	—	—	4/4	30
BIT 4.5 (50, 60)*	4.5AC3/10	3/3	13	3/3	21

* Figures in parentheses are the mean latent periods, i.e. days, for tumour formation for 10^6 and 10^5 cells respectively of the suppressed hybrids.

Selection in vivo for hybrids which are tumorigenic selects for cells which express some but not necessarily all characteristics of the transformed phenotype

Fourteen tumours selected from 4 independent suppressed BI × TA3B hybrids were examined for the expression of the transformed phenotype in culture. Two characteristics were studied in detail. The organization of the cytoskeleton was investigated by staining the myosin of microfilament bundles by indirect immunofluorescence. Such an assay is for the morphological characteristics of the transformed phenotype and at least in certain systems shows a good correlation with both tumorigenicity and anchorage-independent growth (Pollack *et al.* 1975). Secondly, the cells were tested for anchorage-independent growth, a marker of abnormal growth control, by assaying their ability to grow in soft agar suspension (Macpherson & Montagnier, 1964).

Table 4 shows that all variants selected for tumour production had altered cytoskeletons compared to the original suppressed hybrids. These data provide unequivocal evidence that tumours arise from suppressed hybrids by the selective outgrowth of a minor subset of cells in the population. The alteration in the cyto-

skeleton ranged from almost complete absence of microfilament bundles to variants derived from BIT3.4 where microfilament bundles are present in a large fraction of cells. However the phenotype of these tumorigenic variants was clearly different from the suppressed hybrid BIT3.4 because fewer cells have bundles and the positive cells have less extensive arrays. This minimally altered cytoskeletal phenotype has also been seen in variants of BIT3.4 selected for growth in agar and in tumours derived from these variants (Marshall *et al.* 1978; unpublished results). These results suggest that an alteration rather than a complete disruption of the cytoskeleton is a

Table 4. *Expression of the transformed phenotype in vitro in variants from suppressed hybrids selected for tumorigenicity in nude mice*

Original suppressed hybrids			Tumour variants		
Cell line	CFE in agar (%)*	Cells with micro-filament bundles (%)†	Cell line	CFE in agar (%)*	Cells with micro-filament bundles (%)†
BIT 2.1A	1·3	70	1096A	3·9	5
			1096C	3·0	5
BIT 3.4	0·05	80	1093L	6·3	63
			1093R	1·5	48
BIT 4.2	0·1	31	897A	43	2
			897B	20	1
			898A	19	4
			898B	10	12
			898C	19	5
BIT 4.5	0·07	90	1056A	0·3	1
			1056B	28	12
			1055B	16·5	13
			1055C	6·4	< 1
			1072B	23	4

* Colony Forming Efficiency, percentage number of colonies in soft agar ⩾ 0·1 mm diameter/number of cells seeded.
† No. of cells with microfilament bundles/total number of cells counted.

sufficient condition for cells to grow in agar and for tumorigenicity. Furthermore the observation that the same distinctive phenotype is seen in cells selected either for tumorigenicity or for expression of the transformed phenotype is further evidence that it is the same variant derived from the suppressed hybrid which grows in nude mice and in agar.

Since all of the variants selected for tumorigenicity express a morphological characteristic of the transformed phenotype it might be expected that they would all express altered growth control. Table 4 shows that 13 out of the 14 variants grew in agar with high colony-forming efficiencies, demonstrating that selection for tumorigenicity also selected for growth in agar. However, variant 1056A selected from BIT4.5 had a colony-forming efficiency in agar comparable to BIT4.5 (Table 4).

Other tumours produced by BIT4.5 had greatly increased colony-forming efficiencies in agar. To confirm that 1056A was truly a tumorigenic variant it was cultured and then inoculated into nude mice. Table 2 shows that tumours arose with similar latent periods to that of the tumorigenic variants with anchorage-independent growth and shorter latent periods than for the suppressed hybrid BIT4.5. Furthermore 1056A could be shown to be a variant of BIT4.5 because it had an altered cytoskeleton. These results suggest that while selection for a tumorigenic variant generally selects for the variant which can grow in agar this is not the only type of highly tumorigenic variant that can be derived from a suppressed hybrid.

DISCUSSION

We have now shown that both tumorigenicity *in vivo* and the transformed phenotype in culture are coordinately suppressed in TA3B × REF and BI × TA3B hybrids. The suppressed hybrids generate variants which can be isolated by selection *in vivo* or in culture. Most of the variants are both highly tumorigenic and express all the characteristics of the transformed phenotype we have studied. However, the set of variants isolated *in vivo* by selection for tumorigenicity is not completely overlapping with the set of variants isolated in culture by selection for the transformed phenotype since a tumorigenic variant was isolated which was not anchorage-independent.

These experiments provide further information of the relationship between the transformed phenotype of tumour cells in culture and tumorigenicity *in vivo*. This question has been the subject of a number of previous investigations (e.g. Freedman & Shin, 1974), but previous studies have examined a range of cell lines of *different* origins. Given the heterogeneity of material involved (chemical, viral and spontaneous transformants) and the possibility of multiple mechanisms of neoplastic transformation it is not surprising that no characteristic in culture is a universal correlate of tumorigenicity (see Stiles *et al.* 1976). Furthermore we have previously shown that different tumours from the same organ have very different characteristics in culture (Marshall, Franks & Carbonnel, 1977). The present experiments with hybrid cells do not examine the question of a universal characteristic in culture that correlates with tumorigenicity but ask if in a *particular* tumour cell line there is an absolute correlation. Our data show that anchorage-independent growth, a characteristic most frequently correlated with tumorigenicity in a range of different cells, does not always correlate with tumorigenicity even for a particular cell line.

A plausible hypothesis to explain both the coordinate suppression of tumorigenicity and the transformed phenotype, and the observation that selection *in vivo* usually selects for anchorage-independent growth (Shin, Freedman, Risser & Pollack, 1975) would postulate that the transformed phenotype and tumorigenicity can be controlled by the same pleiotropic regulatory mechanism. But since anchorage-independent growth and tumorigenicity can be dissociated the molecules or 'targets' on which the control mechanism acts cannot be the same. Although an altered cytoskeleton always correlated with tumorigenicity in the hybrids studied, this does not necessarily mean that the 'targets' for tumorigenicity and an altered cytoskeleton are the same. It is

entirely possible that if more variants were characterized a dissociation would be found. Watt, Harris, Weber & Osborn (1978) working in a different hybrid cell system have already shown no correlation between the organization of the cytoskeleton and tumorigenicity.

The coordinate suppression of the transformed phenotype and tumorigenicity we have observed leads to hybrids with all the properties of normal mesenchymal cells we have tested. The original hybrids are non-tumorigenic and have density-dependent inhibition of growth, anchorage-dependent growth, extensive microfilament bundles and high levels of LETS protein (Marshall & Dave, 1978; Marshall *et al.* 1978). This coordinate suppression has also been found by Sager & Kovac (1978) with hamster cells. But even though this suppression can lead to a phenotype closely resembling a normal cell it is not known whether the phenotype of normal cells is the result of the same mechanism which leads to suppression in hybrids. This is an important question especially since there is evidence that hybrid cells can possess properties which are not shown by either parent (Stamatoglou & Marshall, 1978). Therefore we do not know whether neoplastic transformation is the result of perturbations in the mechanism of suppression.

Coordinate suppression of tumorigenicity and transformed phenotype has not been a universal observation in hybrid cells. Stanbridge & Wilkinson (1978) did not find suppression of any characteristics of the transformed phenotype in HeLa × fibroblast hybrids that were suppressed for tumorigenicity. Suppression of anchorage-independent growth and tumorigenicity was found in hybrids between SV40-transformed cells and 3T3 cells but there was no suppression of serum requirements (Howell & Sager, 1979). It is now becoming apparent that different degrees of suppression can be found ranging from a complete restoration of the normal phenotype to hybrids in which tumorigenicity is suppressed but the cells have some properties of the transformed phenotype. The analysis of these different degrees of suppression may shed some light on the mechanisms involved.

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