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Studies on Transformation Markers and Tumorigenicity in Segregant Clones from a Human Hybrid Line*

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Abstract—*Thirty segregant clones were back-selected in 8AG or 5BUdR media from a non-tumorigenic human intraspecific hybrid line (HeLa TK⁻ × fibroblasts HPRT⁻) displaying a high plasminogen activator (PA) level, a disorganized fibronectin (FN) matrix and anchorage-independence. These clones exhibited a widely modulated expression of the above markers concomitantly with different degrees of chromosome loss. Out of six representative segregant clones tested in nude mice, two were found to re-express tumorigenicity. No significant correlation was observed between PA or FN levels and anchorage-independence, as well as between these markers and tumorigenicity.*

INTRODUCTION

THE PROCESS of neoplastic transformation is accompanied by changes in cellular properties which can be studied *in vitro* [1–3]. Many attempts have been made in order to verify which of the changes are inherent in the transformed phenotype and can be correlated with tumorigenicity *in vivo* [4–11].

A useful approach to this problem is to analyse somatic cell hybrids, derived from the fusion of tumorigenic and normal cells (for reviews see [1, 12, 13]). The application of segregation methods to intraspecific hybrids provides a system for studying the relationship between different cell transformation traits and tumor-forming ability [14–17].

In recent years, among different transformation markers, special attention has been paid to the increased secretion of plasminogen activator (PA) [18–21] and to the decreased deposition of cell-surface fibronectin (FN) [22–25].

We have previously shown that human cell hybrids derived from the fusion of PA^+,FN^- HeLa cells and PA^-,FN^+ fibroblasts secrete PA at levels up to 100-fold higher than that of the PA^+ parent, express different patterns of FN-containing pericellular matrix, are able to grow in soft agar and are not tumorigenic [26]. In this report we used one of the above hybrid lines for correlation studies on *in vitro* transformation parameters and tumorigenicity. A series of secondary hybrid clones with reduced chromosome numbers, displaying different levels of these markers, was recovered. No significant correlation was found between PA or FN levels and colony formation in soft agar. Re-expression of tumorigenicity was observed in some segregant clones; none of the above transformation markers was found to show a clear relationship with tumor-forming ability.

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MATERIALS AND METHODS

Cell lines and culture conditions

The hybrid line, designated B5, used in this study was derived from fusion between thymidine kinase (TK)-deficient HeLa cells resistant to 20 μg/ml of 5-bromodeoxyuridine (BUdR) and human hypoxanthine phosphoribosyltransferase (HPRT)-deficient fibroblasts (GM1362), as reported by Larizza *et al.* [26].

8-Azaguanine (8AG)- or 5BUdR-resistant subclones selected from the B5 line were propagated in MEM medium supplemented with 10% calf serum (Gibco) containing the respective selecting analogue. The clones were transferred to normal medium 1 week before performing the assays. All

the cell lines used in this study were tested for mycoplasma contamination by Hoechst 33258 staining [27] and were found to be negative.

Selection of 8AG- or 5BudR-resistant clones

In order to isolate 8AG- or 5BUdR-resistant clones, 5×10^4 B5 hybrid cells were plated on 100-mm plastic Petri dishes (Nunc) in MEM medium containing 20 μg/ml of 8AG (Sigma) or 20 μg/ml of 5BUdR (Sigma). 8AG- or 5BUdR-resistant colonies were isolated after about 30 and 60 days of culture, respectively, by trypsinization *in situ* without selecting particular morphological types.

PA assay

Supernates from semiconfluent cell 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) [28]. The assays were performed with and without plasminogen (PG) (Kabo, 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 the PA levels were corrected for the number of cells and expressed as activity/cell/hr.

FN assay

Cell-surface FN was visualized by the indirect immunofluorescent method according to Vaheri *et al.* [29], as previously reported [26]. The density of the FN fibre network was scored on a scale of 0–6+, the latter being the pattern assigned to control fibroblasts. The percentage of FN^+ cells was also determined in each clone by counting at least 500 cells in confluent cultures.

Chromosome analysis

Chromosome preparations were made according to the standard procedures. Conventional Giemsa and quinacrine (Sigma) staining were used for chromosome counts and Y chromosome detection respectively. At least 30 metaphases were counted to determine chromosome number distribution.

Cloning efficiency in semisolid and liquid medium

Cells were tested for anchorage-independence in MEM containing 0.36% agar (Noble Agar, Difco) by the method of MacPherson and Montagnier [30]. Three replicate wells of a 24-well plate (Nunc), inoculated with 10^4 cells, were incubated in a humidified CO_2 incubator at 37°C with weekly feeding. 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 either with or without the analogues was determined in parallel by plating 10^3 cells in duplicate 60-mm Petri dishes (Nunc) and the number of colonies with more than 25 cells was scored after 7 days.

Tumorigenicity test and establishment of tumor cells in culture

The tumorigenicity test was performed as previously reported [26]. Developing tumors were excised surgically on day 30 after cell inoculation and used for histological examination and establishment of cells in culture. Cells were propagated in the presence of the analogue to which they were resistant before the *in vivo* transfer and characterized at the 4th culture passage.

Statistical analysis

Standard correlation analysis was applied [31].

RESULTS

Isolation and characterization of 8Ag- or 5BUdR-resistant clones

The non-tumorigenic B5 hybrid line, derived from the fusion of HeLa $5BUdR^R$ cells with $HPRT^-$ human fibroblasts, displayed a 50-fold increase in PA levels as compared to HeLa and altered FN pattern in respect to fibroblasts, as well as the ability to form colonies in soft agar [26]. This line, characterized by a highly scattered chromosome distribution, was back-selected in 8AG- or 5BUdR-containing media. The frequencies of $8AG^R$ and $5BUdR^R$ clones corrected for plating efficiency were 26.1×10^{-4} and 14×10^{-5} respectively. Fifteen AG^R and 15 $BUdR^R$ clones were characterized. Each of the $8AG^R$ and of the $5BUdR^R$ clones displayed the same plating efficiency in media with and without the respective selecting analogue (data not shown). No double-drug-resistant clones were detected.

After propagation to the mass population each isolated drug-resistant clone was concomitantly analysed for chromosome number distribution, PA activity, FN expression, growth in semisolid medium and tumor-forming ability.

Chromosome analyses

The results of chromosome analysis are reported in Fig. 1. As can be seen, each subclone displayed a reduction in modal chromosome number as compared to the B5 parental hybrid. The B5 line had a modal chromosome number of 91 and in the drug-resistant clones the mode ranged between 79 and 83 for $8AG^R$ and 69 and 84 for $5BUdR^R$ segregants. Clones with a higher chromosome loss can be seen in the $5BUdR^R$ series. The Y chromosome, present in the B5 line

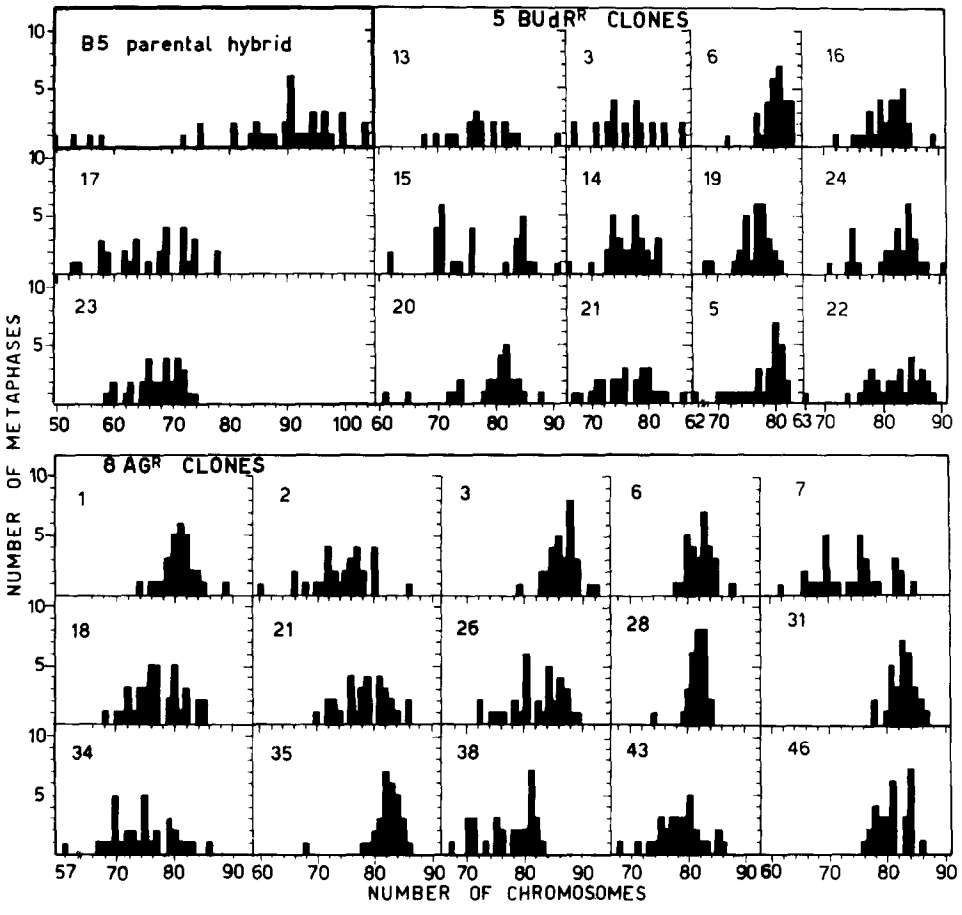

Fig. 1. Chromosome number distribution of the B5 parental hybrid and its 5BUdR and 8AG^R selected clones. (The chromosome number distribution in clone B5 5BUdR^R 18 was not determined.)

and contributed by the fibroblast parent, was always maintained in the segregants (data not shown).

PA activity

PA activity of the 8AG^R and of the 5BUdR^R segregants is reported in Table 1, 1st column. In both segregant series PA levels were found to vary considerably, ranging from the high levels of the B5 parental hybrid down to levels comparable to that of HeLa cells with a 50-fold decrease. On average, the 5BUdR^R segregants displayed a lower fibrinolytic activity than that of the 8AG^R clones.

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% [28]. A slight increase in PA activity (~15%) was observed in all the SDS-treated samples.

FN pattern

A broad spectrum of FN expression that deviated both qualitatively and quantitatively from that observed in the B5 parental hybrid was found in both segregant series, as reported in Table 1, 2nd column. Extreme phenotypes of fibroblasts (scored as ++++++) and of HeLa cells (scored as -) were not detected in any of the clones.

Anchorage-independence

The efficiencies of colony formation in soft agar of the 8AG^R and of the 5BUdR^R segregants are reported in Table 1, 4th column. Cloning efficiencies in semisolid medium were found to be either increased or decreased compared to the B5 hybrid. The increase in cloning efficiency was higher and more frequent in the 8AG^R series.

Statistical analysis

Correlation coefficients were calculated in the following comparisons: PA vs colony formation in soft agar; FN$^+$ cells vs colony formation in soft agar; PA vs FN$^+$ cells.

As far as FN expression is concerned, the percentages of FN$^+$ cells were used for statistical analysis. Indeed, there is a direct relationship between the qualitative and the quantitative criteria in the evaluation of FN content, as clones with an incomplete fibrillar network are characterized by a low percentage of FN-expressing cells (Table 1, 2nd and 3rd columns). The PA and FN levels are not significantly correlated either with

Table 1. Extent of expression of transformation markers in 8AG- or 5BUdR-resistant segregants

Cells	Secreted PA (Sigma Units/cell/hr $\times 10^{-11}$)	Pericellular FN pattern Score	% FN$^+$ cells	Colony formation in agar (%)
Parental cells				
GM1362	0.06	++++++	100	5×10^{-5}
HeLa 5BUdR^R	2.7	-	0	52.5
B5 hybrid line	113.0	++	19	12.2
8AG^R segregants				
1	5	++	23	32.5
2	94	++	15	0.9
3	85	+	1	15.3
6	13	+	2	0.6
7	10	++	14	7.4
18	21	+++	57	31.6
21	23	+++	36	5.8
26	47	++	13	7.5
28	67	+++	30	0.6
31	11	+++	44	32.5
34	6	+++	41	12.2
35	107	+++	46	20.7
38	53	++	8	0.7
43	22	+	11	27.7
46	60	++	18	28.9
5BUdR^R segregants				
3	11	+	7	9.2
5	3	++	28	7.4
6	27	+	6	9.4
13	3	++++	89	1.7
14	7	++	25	18.6
15	17	+++	55	1.1
16	2	++	28	5.4
17	11	+++	64	2.1
18	32	+	6	2.6
19	2	+	11	17.7
20	2	++	12	1.6
21	8	+++	33	3.3
22	2	++	24	3.5
23	7	+++	54	13.7
24	3	+++	58	10.6

the colony formation in soft agar or with each other (results not shown).

Tumorigenicity

Out of 6 segregant clones tested, 2 (AG^R 1 and BUdR^R 22) generated clearly detectable tumors 15 days after injection of 5×10^6 cells/animal. No tumors were detected after 5 months in any of the mice inoculated with the other segregants, as well as with the B5 parental line and normal fibroblasts. A histopathological study of tissues taken at autopsy confirmed the absence of neoplasia.

The properties of the tumorigenic and non-tumorigenic segregants are outlined in Table 2. Plating efficiencies in 8AG- or 5BUdR-containing media for the non-tumorigenic segregant clones as well as for the tumorigenic ones before and after *in vivo* transfer confirmed their resistance to their respective analogue. No quantitative differences in the expression of transformation markers were observed before and after *in vivo* transfer in the tumorigenic segregants.

A comparison of the expression levels of these markers between tumorigenic and non-tumorigenic segregants did not reveal any association between the *in vitro* characters and tumorigenicity.

DISCUSSION

Back-selection of the B5 human transformed non-tumorigenic hybrid line in media containing 8AG or 5BUdR proved to be an efficient means of recovering clones with reduced chromosome sets in which different transformation markers expressed by the parental hybrid could be modulated and tumorigenicity re-expressed. Indeed, the 8AG- and the 5BUdR-resistant clones exhibited an average chromosome loss equivalent to 10 and 15% of the original complement

Table 2. Comparison between tumorigenic and non-tumorigenic segregant clones

Characteristics	Tumorigenic segregants AG^R 1 Before *in vivo* transfer	AG^R 1 After *in vivo* transfer	BUdR^R 22 Before *in vivo* transfer	BUdR^R 22 After *in vivo* transfer	Non-tumorigenic segregants AG^R 31	BUdR^R 13	BUdR^R 15	BUdR^R 19
Chromosomes								
Mode	81	82	85	73	83	77	71	77
Range	74–89	71–85	63–89	68–80	78–87	68–91	62–91	68–81
Relative P.E.*	0.87	0.96	1.04	1.06	0.84	1.05	0.99	1.21
Colony formation in soft agar (%)	32.5	38.2	3.5	7.3	32.5	1.7	1.1	17.7
Secreted PA†	5	4	2	3	11	3	17	2
FN score	++	±	++	±	+++	++++	+++	+

*Expressed as the ratio of plating efficiency (P.E.) in medium containing the respective selecting analogue to that in medium without drug.

†Expressed as Sigma Units/cell/hr × 10^{-11}.

respectively. These results are in agreement with those reported by Sager and Kovac [14], Klinger [15] and Larizza *et al.* [32] in different intraspecific hybrid systems. All B5 segregants showed comparable plating efficiencies in media with or without the analogue, thus indicating homogeneity in the cell population of individual clones in the selective biochemical marker.

Since the intraspecific hybrid described here is not well suited for cytogenetic identification of the parental chromosomes, the relative chromosomal contribution of each parent to the segregation process could not be established, except with the Y chromosome, derived by the fibroblast parent and present in the B5 hybrid. This chromosome has been detected in all segregants, as also described by Stanbridge *et al.* [33].

A general feature of both 8AG- and 5BUdR-resistant segregants is the wide variability in the expression of the transformation markers analysed. Indeed, it was possible to isolate segregants which displayed a more than 50-fold difference in PA levels, an almost 90-fold difference in the percentage of FN$^+$ cells and a more than 50-fold difference in colony formation in soft agar. These differences cannot be ascribed to experimental variations, since all the FN assays were performed on confluent cultures [34] and the values of colony formation in semisolid medium were reproducible in successive platings. As far as PA activity is concerned, control experiments, in which PA levels as a function of cell density were measured, showed at most a 2-fold difference, which was far lower than those observed among different segregants [35].

No statistically significant correlations were found between PA or FN levels and colony formation in soft agar in either of the series. This lack of correlation also occurred when the values of colony formation in semisolid medium were corrected for plating efficiencies in liquid medium. Furthermore, no association was found between these markers and tumorigenicity. As to PA, the lack of any quantitative correlation between fibrinolytic activity and cloning efficiency in agar confirms similar results obtained in Chinese hamster cells [36] and in human osteosarcoma cells [4].

As far as FN expression is concerned, the results obtained are consistent with those of Der and Stanbridge [34], Kahn and Shin [8] and Klinger [15], who reported that reduction and alteration of FN deposition is generally associated with growth in semisolid medium, even if the *in vitro* level of FN is not a good correlate to anchorage-independence.

Since both the experimental design and the cell lines are similar to those described by Der and Stanbridge [37], it would be of interest to verify the presence of the 75 kd membrane phosphoprotein, a possible marker of *in vivo* growth potential, in our tumorigenic segregants.

In conclusion, the different transformation markers analysed were widely modulated but never dissociated in different clones originated by chromosome segregation from a common hybrid line. However, association of the above markers does not imply any correlation between their levels. Therefore, when assessing the transformed phenotype, the quantitative expression of one marker cannot be considered predictive for the others. Furthermore, none of the transformation markers analysed was found to show a clear relationship with tumor-forming ability.

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