Journal of Cellular Biochemistry 34:23–30 (1987)
Growth Factors, Tumor Promoters, and Cancer Genes 111–118

The Ha-ras-Induced Transformed Phenotype of Rat-1 Cells can be Suppressed in Hybrids With Rat Embryonic Fibroblasts

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Somatic cell hybrids were isolated from fusions of diploid embryonic rat fibroblasts with transformed Rat-1 cells which contained 4 to 5 copies of the transforming human Ha-ras 1 gene. In contrast to their transformed parental cells four hybrid clones showed normal morphology, long latency periods of tumorigenicity in newborn rats, anchorage requirement of proliferation, and an eightfold-reduced amount of secreted transforming growth factor activity. Thus these hybrids are called suppressed with regard to expression of the Ha-ras-induced transformed phenotype. Tumorigenic derivatives of the suppressed hybrids that had segregated chromosomes were isolated. Since two of the tumorigenic hybrid clones showed the similar low level of secreted transforming growth factors as the suppressed hybrids, decreased production of transforming growth factor activity is unlikely to be a sufficient criterion for suppression of malignancy. Whereas one of the suppressed hybrids expressed the transforming gene product p21 at a level similar to that of the transformed parental cells, other suppressed hybrids expressed less p21. This suggests that the suppressed phenotype can be regulated at the posttranslational level of p21 but that additional controls of expression of p21 are likely to exist. DNA of the suppressed hybrids transformed Rat-1 cells to proliferation in the presence of semisolid agar. Thus the activated human Ha-ras gene in the suppressed hybrids retained its biological activity even though it did not transform these cells to tumorigenicity.

Key words: cellular hybrids, tumor suppression, Harvey-ras oncogene

It is now well documented that the ability of transformed cells to form tumors can be suppressed in cell hybrids [for review, see 1]. This suppressed phenotype of hybrid cells is counterselected by the appearance of tumorigenic segregrants which

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Received June 6, revised and accepted November 10, 1986.

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have lost chromosomes of the normal parent. Several groups have reported that the presence of single chromosomes [2–4] or of two and more chromosomes of the normal parental cells is required for suppression of malignancy in somatic cell hybrids [5–8]. Among the tumorigenic cell lines for which suppression by somatic cell hybridization has been demonstrated are several virally transformed cells [9–12] and a human fibrosarcoma cell line containing an activated N-ras gene [8]. In none of these tumorigenic cell lines, however, is it known whether or not the tumorigenicity is due to a single gene mutation. If this is the case the mechanism of suppression may be less complicated than in a cell line that harbors different lesions which contribute to the expression of malignancy. Immortalized, nontumorigenic cells can be transformed to tumorigenicity by transfection with activated human ras genes [13–15]. In this case tumorigenicity appears to be dependent on the presence and expression of an activated ras gene which carries a single nucleotide mutation. We wished to study suppression of tumorigenicity in hybrids of established Rat-1 cells which were transformed with the activated human Ha-ras 1 gene from EJ bladder carcinoma cells. While our work was in progress Craig and Sager [16] published results of a similar approach, ie, the fusion of a Ha-ras transformed Chinese hamster cell line with nontransformed ("normal") established Chinese hamster cells. Similarly to these authors, we found stable suppression of the Ha-ras-induced transformed phenotype. If this effect is indeed due to the presence of a single suppressor gene ("antioncogene") it may eventually be characterized by DNA-mediated transfer rather than somatic cell hybridization.

MATERIALS AND METHODS

Cells and Cell Hybridization

Rat 208F cells are derived from Rat-1 cells and defective for hypoxanthine phosphoribosyl transferase activity [17]. The transformed parental cells FE6 and FE8 cells were isolated after transfection of 208F cells with the plasmid pEJ containing the 6.6-kb DNA fragment of the mutated human Ha-ras 1 gene [18]. Furthermore, FE6 and FE8 cells are resistant to the antibiotic G418 due to cotransfection with the pSV2 neoplasmid [19]. Rat embryonic fibroblasts (REFs) were prepared from BDIX rats and used for cell hybridization at the sixth passage. For cell fusions with polyethylene glycol 1500 a 20-fold excess of normal rat fibroblasts over transformed FE6 or FE8 cells was used. Hybrid cells were grown in Dulbecco modified Eagle medium containing 10% fetal bovine serum and selected in the presence of G418 (400 μg/ml) and HAT (hypoxanthine, aminopterine, thymidine) [20,21]. Hybrid clones FER5 and FER9 were derived from fusion of FE6 and REF cells; the other clones were derived from fusion of FE8 and REF cells. The original number of hybrid clones is not exactly known due to cross-feeding effects under these conditions of selection. Out of 18 isolated proliferating hybrids only the four hybrid clones described in this paper showed flat morphology and were analyzed for their DNA content by using an ICP11 pulse cytophotometer. All hybrids were found to have a tetraploid DNA content except hybrid FER5, which contained a near-triploid amount of DNA. These results were confirmed by karyotype analyses of hybrids FER1 and FER5.

Southern Blot Hybridization

BamHI-digested DNA of parental and hybrid cells was electrophoresed in agarose gels, transferred to nylon filters, and hybridized following standard condi-

tions [22] to the 2.9-kb Sac I fragment of pEJ, which had been labelled by nick translation [23].

Immunoprecipitation

Cells were metabolically labelled with ^{35}S methionine. Cell lysates were subjected to a standard protocol of immunoprecipitation [24] by using the rat monoclonal antibody Y13-238 (Oncogene Science), which recognizes the p21 product of the Ha-ras gene. The immunocomplexes were electrophoresed and autoradiographed. The amounts of p21 in different cells were compared by densitometric analysis of the autoradiographs. For determination of p21 half-lifetime the cells were pulse labelled for 16 hr with ^{35}S methionine followed by different chase periods in the presence of unlabelled methionine [25].

Growth Factor Analysis

Binding assays with ^{125}I-epidermal growth factor (EGF, Amersham) were performed on subconfluent monolayers of parental and hybrid cells following the published procedure [26]. For detection of secreted growth factor activity conditioned media of the different cell lines were centrifuged at 100,000g [27] and tested with normal rat kidney (NRK) cells for induction of anchorage-independent growth [28].

Tumorigenicity

Cells (1×10^6) of each cell line were injected subcutaneously into the back of newborn BDIX rats. Tumors were scored positive when they had reached a size of about 0.5 cm^3.

DNA Transfection

Calcium-phosphate-mediated transfections with DNA from cultured hybrid cells together with pSV2 neo-DNA were performed by using 208F or NIH 3T3 recipient cells [29]. G418-resistant colonies were selected and transferred to standard medium containing 0.15% agar (Difco). Colonies in semisolid agar were counted after 3 wk.

RESULTS

Four somatic cell hybrids of FE6 or FE8 cells with rat embryonic fibroblasts were isolated that exhibited flat morphology and saturation densities of proliferation on plastic surfaces similar to their normal parental cells. Three of these hybrids had a tetraploid karyotype (average number of chromosomes, 82) and one (FER5) had a near-triploid DNA content. Several transformation parameters of the parental and hybrid cells are summarized in Table I. The hybrid cells show saturation densities similar to rat embryonic fibroblasts, ie, one order of magnitude lower than the transformed parental cells. Furthermore, all hybrid cells formed colonies in semisolid agar at frequencies three orders of magnitude lower than the transformed parental cells. When tumorigenicity of parental cells and hybrid cells was compared (Table I) it turned out that all hybrids eventually formed tumors after injection into newborn BDIX rats. The latency period of tumor formation, however, was widely different between the transformed parental cells and their hybrid derivatives. Whereas the transformed parental cells formed progressively growing tumors, palpable already after 4 days, the hybrid clones formed slowly growing tumors only after five- to

TABLE I. Transformation Parameters

Cells	Saturation density[a]	Colony formation in semisolid agar (%)[b]	No. of tumors/ no. of injections[c]	Latency period (days)
Parental cells				
FE6	1×10^7	66	7/7	4
FE8	1×10^7	31	8/8	4
REF	1.1×10^6	—	—	—
Suppressed hybrids				
FER1	0.5×10^6	0.01	1/6	33
FER5	1×10^6	0.01	6/6	26
FER8	0.5×10^6	0.01	5/5	26
FER9	0.5×10^6	0.03	5/5	19

[a]Number of attached growing cells per 25 cm^2.
[b]Cells (10^2–10^4) were plated into medium containing 0.15% (w/v) Difco Noble Agar.
[c]Per site 10^6 cells were injected. Tumors were scored positive when they reached a size of about 0.5 cm^3.

TABLE II. Expression of p21 in Hybrids and Parental Cell Lines

Cells	Copy no. of human Ha-ras 1[a]	Expression of p21[b]	Doubling time (hr)	Half-life of p21[d] (hr)
Parental cells				
FE6, FE8	4–5	+	14	18
REF	–	–	20	ND
Suppressed hybrids				
FER1	4–5	+	31	37.5
FER8	4–5	+	31	ND
FER9	4–5	+	31	ND
FER5	4–5	+	24	23
Tumorigenic hybrid				
FER5T[c]	ND[e]	+	16	16

[a]Determined by comparison of Southern blot signals of DNA from cells with known Ha-ras 1 copy numbers.
[b]Ha-ras 1 gene product analyzed by immunoprecipitation.
[c]Cells, isolated from a tumor explant, isolated 5 wk after an injection of 1×10^6 cells in newborn BDIX rat.
[d]Determined by immunoprecipitation of pulse/chase-labelled p21.
[e]ND, not determined.

eightfold-longer latency periods. In comparison with the original hybrid cells, loss of chromosomes was noticed in tumorigenic hybrid derivatives which had been reestablished in culture for karyotype and biochemical analysis: Modal numbers of 54 chromosomes were found in FER5T and FER8T cells.

Southern blot analysis of DNA from hybrid clones showed that the human Ha-ras 1 gene (6.6-kb BamHI fragment) was present as 4 to 5 copies per genome (Table II). Immunoprecipitations with the rat monoclonal antibody revealed expression of p21 in all hybrid cell lines and in the transformed parental cells. The detailed quantitative comparison of p21 levels showed that only the suppressed FER5 hybrid contained about as much p21 as the transformed parental cells. The other hybrid cells, FER1, FER8, and FER9, contained 27, 11, and 24%, respectively, of the amount of p21 in the transformed parental cells. The reduction of the amount of p21 in these hybrid cells does not appear to be crucial for suppression of malignancy since the

transformed phenotype is suppressed in these cells to a similar extent as in FER5 hybrid cells (Table I).

The doubling times of the suppressed hybrids FER1, FER8, and FER9 were significantly longer (ie, 31 hr) than that of the diploid rat embryonic fibroblasts (20 hr) or that of the hybrid FER5 (23 hr). The half life of p21 was compared in several hybrid and parental cells (Table II). In the parental FE6 cells as well as in FER5T tumor-forming hybrid cells half-lifetimes of 18 hr and 16 hr were determined. This is in line with previous results to the effect that the half-lifetime of p21 was 18 hr in cells transformed with Harvey sarcoma virus [25]. In the FER1 hybrid cells we measured a half-lifetime of 37.5 hr. This is about twice as long as found in the transformed parental cells FE6. Table II indicates that the half-lifetimes of p21 in the different hybrid and parental cells appear to be proportional to the doubling times of these cells. Thus we think it unlikely that an extension of the half-lifetime of the transforming gene product p21 is significant for suppression of malignancy in the hybrid cells.

Suppressed hybrids and tumorigenic derivatives were also compared with regard to their binding of ^{125}I EGF (Table III). TGFα competitively inhibits binding of EGF to the EGF receptor [30]. The suppressed hybrids showed a fourfold increase of free EGF receptors compared to the tumor-forming hybrids. Relatively few free EGF receptors were found on tumorigenic hybrid cells as well as on cells of the transformed parental cell line FE6. This agrees with results previously reported [31,32]—that ras-transformed cell lines showed reduced EGF binding. This can be due to occupation of EGF receptors by TGFα secreted by the transformed cells or due to disappearance of EGF receptors from the cell surface. In order to decide between these two possibilities we measured the transforming growth factor activity secreted

TABLE III. Binding of EGF to Free EGF Receptor and Transforming Growth Factor Activity Secreted by Hybrids and Parental Cells

	Binding of ^{125}I EGF[a] (%)	Cloning efficiency of NRK cells in semisolid agar[b] (%)
Parental cells		
REF	100	1
Rat 1-208F	100	1
FE6	22	17
Suppressed hybrid cells		
FER1	50	2
FER5	42	2
FER8	72	3
FER9	52	2
Tumorigenic hybrid cells		
FER5T	10	2
FER8T	0	20
FER9T	14	1

[a]Analyzed with 10^6 cells. The standard deviations of binding data were within 5% of the mean values for three independent experiments.
[b]NRK cells (2×10^3) were cultivated in the presence of 100 μg protein from conditioned medium per ml of culture medium containing 3% fetal bovine serum in semisolid agar. Note that both TGFα and EGF or TGFβ must be present in order to induce the formation of large colonies of NRK cells in semisolid agar [33]. The standard deviations of cloning deficiency data were within 2% of the mean values for three independent experiments.

into the culture medium. Transforming growth factors can induce anchorage-independent proliferation of mammalian cells in culture [33]. Table III shows that the suppressed hybrids, like the normal parental REF cells, secreted virtually no transforming growth factor activity as measured by proliferation of normal rat kidney (NRK) cells in the presence of semisolid agar. In contrast, the tumorigenic hybrid derivative FER8T secreted much more transforming growth factor activity, similar to the amount secreted by the transforming parental cell line FE6. However, the data of Table III also indicate that two of the tumorigenic hybrid derivatives, FER5T and FER9T, produced about as little transforming growth factor activity as the corresponding suppressed hybrid cells. Thus we conclude that the lack of secretion of transforming growth factor activity is not a sufficient criterion for suppression of malignancy in hybrid cells.

DISCUSSION

At the present state of tumor research two different observations appear to contradict each other. On the one hand, DNA transfections with transforming oncogenes isolated from tumor cells or retroviruses suggest that tumorigenesis appears to be due to dominantly acting genes. On the other hand, analysis of large numbers of somatic cell hybrids led to the conclusion that the tumorigenicity of transformed cells can be suppressed in somatic hybrids with normal cells. We have shown in this paper that even the malignant phenotype of transformed Rat-1 208F cells which is caused by the transfected Ha-ras gene from human bladder carcinoma cells can be suppressed in somatic cell hybrids with normal rat embryonic fibroblasts. The same activated Ha-ras gene appears to transform Rat-1 208F cells in a dominant fashion. In order to solve this dilemma one has to recall that neoplastic transformation of diploid rat cells by transfection with isolated oncogenes requires that the Ha-ras oncogene be under control of a strong promotor or that it cooperate with another cotransfected oncogene [34,35]. Furthermore, transformed colonies were selected via expression of a cotransfected resistance gene. Apparently in addition to the uptake and expression of the oncogene one or more further steps are required before a diploid rat fibroblast becomes tumorigenic.

It has been suggested [1,36] that certain gene loci ("suppressor genes" or "antioncogenes") need to be inhibited, inactivated, or deleted before a transforming gene can convert a normal recipient cell to a malignant one. In this context it is interesting that tumorigenic derivatives from Syrian hamster embryonic cells transformed by v-Ha-ras and v-myc oncogenes had consistently lost one copy of chromosome 15 [37]. The extent of suppression of the transformed phenotype differs between human-human and rodent-rodent somatic cell hybrids. Hybrids of human tumorigenic HeLa cells with normal human fibroblasts appear to be suppressed only in their tumorigenicity as measured after injection into nude mice [5,6]. In contrast, hybrids of tumorigenic and normal rodent cells [1,7] are also suppressed with regard to several additional transformation parameters (for example: morphology, requirement for growth factors, colony formation in semisolid agar, etc). These differences between human-human and rodent-rodent hybrids may be caused by different specificities of the products of the putative suppressor genes in the two experimental systems.

The mechanism of suppression of tumorigenicity is not completely understood at present. In several of the suppressed Chinese hamster hybrids [16] the Ha-ras product p21 was expressed to about the same level as in the corresponding transformed Chinese hamster parental cell lines, which suggests posttranslational control of suppression of tumorigenicity. Only one of the suppressed hybrids characterized in this paper showed the same level of p21 expression as the transformed parental cells and a tumorigenic hybrid derivative. This confirms that the phenotype of Ha-ras-transformed cells can be regulated at the posttranslational level of p21, but additional controls of expression of p21 are likely to exist. Apparently oncogene-induced tumorigenicity can be suppressed at several levels of oncogene expression. For example, flat, nontumorigenic revertants of Kirsten sarcoma virus–transformed cells contain elevated amounts of the p21 gene product and can be retransformed by Moloney murine sarcoma virus [38]. Furthermore, it has been shown in hybrids of Rous sarcoma virus–transformed cells and nontransformed cells that suppression of neoplastic transformation occurred at the level of transcription of the oncogene product pp60src [10,39]. In order to dissect the different molecular mechanisms of the suppression of tumorigenicity, cloning and reexpression of the putative suppressor genes in tumorigenic cells are required.

ACKNOWLEDGMENTS

This work was supported by the Deutsche Forschungsgemeinschaft (SFB 102/A6 and Wi 270/13) and the Fonds der Chemischen Industrie.

REFERENCES

1. Sager R: Adv Cancer Res 44:43, 1985.
2. Evans EP, Burtenshaw MD, Brown BB, Mennion R, Harris H: J Cell Sci 56:113, 1982.
3. Marshall CJ, Kitchin R, Sager R: Somatic Cell Genet 8:709, 1982.
4. Stoler A, Bouck N: Natl Acad Sci USA 82:570, 1985.
5. Stanbridge EJ, Der CJ, Doersen CJ, Nishimi RY, Peehl DM, Weissman BE, Wilkonson JE: Science 215:252, 1982.
6. Klinger HP, Shows TB: J Natl Cancer Inst 71:554, 1983.
7. Schäfer R, Hoffmann H, Willecke K: Cancer Res 43:2240, 1983.
8. Benedict WF, Weissman BE, Mark C, Stanbridge EJ: Cancer Res 44:3471, 1984.
9. Sager R, Anisowicz A, Howell N: Cell 23:41, 1981.
10. Dyson PJ, Quade K, Wyke JA: Cell 30:491, 1982.
11. Howell N: Cytogenet Cell Genet 34:215, 1982.
12. Noda M, Selinger Z, Scolnick EM, Bassin RM: Proc Natl Acad Sci USA 80:5602, 1983.
13. Reddy EP, Reynolds RK, Santos E, Barbacid M: Nature 300:144, 1982.
14. Tabin CJ, Bradley SM, Bargmann CJ, Weinberg RA, Papageorg AG, Scolnick EM, Dhar R, Lowy DR, Chang EH: Nature 300:143, 1982.
15. Taparowsky E, Suard Y, Fasano O, Shimizu K, Goldfarb M, Wigler M: Nature 300: 762, 1982.
16. Craig RW, Sager R: Proc Natl Acad Sci USA 82:2062, 1985.
17. Quade K: Virology 98:461, 1979.
18. Shih CS, Padhy LC, Murray M, Weinberg RA: Nature 290:61, 1981.
19. Southern PJ, Berg P: J Mol Appl Genet 1:327, 1982.
20. Littlefield JW: Science 145:709, 1964.
21. Calvo Riera F, Blam SB, Teng NNH, Kaplan HS: Somatic Cell Mol Genet 10:123, 1983.
22. Wahl GM, Stern M, Stark GR: Proc Natl Acad Sci USA 76:3683, 1979.
23. Rigby PW, Dieckmann M, Rhodes C, Berg P: J Mol Biol 113:237, 1977.
24. Furth ME, Davis LJ, Fleurdelys B, Scolnick EM: J Virol 43:294, 1982.

25. Ulsh LS, Shih TY: Mol Cell Biol 4:1647, 1984.
26. Todaro GJ, De Larco JE, Cohen S: Nature 264:5258, 1976.
27. Todaro GJ, Tryling C, De Larco JE: Proc Natl Acad Sci USA 77:5258, 1980.
28. De Larco JE, Todaro GJ: Proc Natl Acad Sci USA 75:4001, 1978.
29. Wigler M, Pelliger A, Silverstein S, Axel R: Cell 14:725, 1978.
30. Derynck R, Roberts AB, Eaton DM, Winkler ME, Goeddel DV: In Feramisco J, Ozanne B, Stiles C (eds): "Cancer Cells 3." Cold Spring Harbor, NY: Cold Spring Harbor Laboratory, 1985, pp 79-86.
31. Kamata T, Feramisco JR: In Levine AJ, Van de Woude GF, Topp WC, Watson JD (ed): "Cancer Cells 1." Cold Spring Harbor, NY: Cold Spring Harbor Laboratory, 1984, pp 11-16.
32. Stern DF, Roberts AB, Roche NS, Sporn MB, Weinberg RA: Mol Cell Biol 6:870, 1986.
33. Anzano MA, Roberts AB, Smith JM, Sporn MB, De Larco JE: Proc Natl Acad Sci USA 80:6264, 1983.
34. Land H, Parada LF, Weinberg RA: Nature 304:596, 1983.
35. Spandidos DA, Wilkie NM: Nature 310:469, 1984.
36. Knudson AG: Cancer Res 45:1437, 1985.
37. Osmimura M, Gilmer TM, Barrett JC: Nature 316:636, 1985.
38. Noda M, Selinger Z, Scolnick EM, Bassin RH: Proc Natl Acad Sci USA 80:5602, 1983.
39. Chiswell DJ, Enrietto PJ, Evans S, Quade K, Wyke JA: Virology 116:428, 1982.