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Interferon-Induced Revertants of *ras*-Transformed Cells: Resistance to Transformation by Specific Oncogenes and Retransformation by 5-Azacytidine

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Prolonged alpha/beta interferon (IFN-α/β) treatment of NIH 3T3 cells transformed by a long terminal repeat-activated Ha-*ras* proto-oncogene resulted in revertants that maintained a nontransformed phenotype long after IFN treatment had been discontinued. Cloned persistent revertants (PRs) produced large amounts of the *ras*-encoded p21 and were refractile to transformation by EJ*ras* DNA and by transforming retroviruses which carried the v-Ha-*ras*, v-Ki-*ras*, v-*abl*, or v-*fes* oncogene. Transient treatment either in vitro or in vivo with cytidine analogs that alter gene expression by inhibiting DNA methylation resulted in transformation of PR, but not of NIH 3T3, cells. The PR retransformants reverted again with IFN, suggesting that DNA methylation is involved in IFN-induced persistent reversion.

Abnormalities in *ras* expression have been observed in a wide variety of naturally occurring or induced tumors (6, 36, 41). The effect of interferon (IFN) on *ras* expression is of particular interest since IFNs have been shown to inhibit the growth of tumors in vivo (1) and to induce phenotypic reversion in some transformed, cultured cells (3, 5, 11, 20, 25). The mechanism of action of IFN in such systems is unknown.

The human c-Ha-*ras*1, a homolog of the transforming gene of Harvey murine sarcoma virus, can acquire transforming potential for NIH 3T3 cells by a point mutation (EJ*ras*) (39) or by transcriptional activation after ligation to a retroviral long terminal repeat (LTR) regulatory element (4). We have previously demonstrated that with prolonged treatment, mouse IFN-α/β induced phenotypic reversion in NIH 3T3 cells transformed by an LTR-activated human c-Ha-*ras*1 (clonal line RS485) (32, 34). The revertants retained the activated *ras*. In contrast, no such reversion was observed in repeated studies with IFN-treated RS504, an NIH 3T3 cell line transformed by the mutated EJ*ras* (33). Revertants of RS485 that were propagated in the continuous presence of 200 IU of IFN-α/β per ml were nontumorigenic (34) and produced four- to eightfold less *ras* mRNA and *ras*-encoded p21 than parental RS485 (32).

Our present studies showed that in contrast to the reversibility of cellular changes seen with short-term (several days) exposure to IFN (14, 20, 25, 30), the phenotypic reversion of RS485 established after 1 to 2 months of IFN treatment persisted long after treatment was discontinued. The persistent revertants (PRs) expressed high levels of p21, yet were not tumorigenic. While the PRs resisted retransformation by a variety of viral or cellular oncogenes, they were readily retransformed after exposure to DNA-demethylating drugs. The latter suggested possible mechanisms of the action of IFN.

MATERIALS AND METHODS

Cells and IFN. All cultures were originated by single-cell cloning and were propagated in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum. Mouse L-cell IFN-α/β (specific activity, 10^8 IU/mg) was a gift from M. Pauker, Medical College of Pennsylvania.

Azacytidine treatment. Logarithmically growing cells were treated with freshly prepared 3.0 μM 5-azacytidine (5AzaC) (Sigma Chemical Co., St. Louis, Mo.), 0.1 μM 5-aza-2′-deoxycytidine (5AzadC) (Sigma), or 3.0 μM 6-azacytidine (6AzaC) (P-L Biochemicals, Inc., Milwaukee, Wis.) at 24 and 48 h after plating. Cells were subcultured thereafter in the absence of the nucleosides and observed for phenotypic alterations. Oncogenic transformation was determined by loss of contact inhibition, growth in soft agar, or tumorigenicity in nude mice (34). For in vivo treatment, 4- to 5-week-old female athymic nude mice (Division of Cancer Treatment, NCI Animal Program, Frederick Cancer Research Facility, Frederick, Md.) were inoculated subcutaneously with 5×10^5 cells. Twenty-four hours later, 400 μg of freshly prepared 5AzadC in 200 μl of phosphate-buffered saline was administered intraperitoneally into each treated animal (20 mg/kg).

Analysis of *ras* DNA, RNA, and protein p21. Our modifications to the standard techniques used for Southern blot analysis of DNA (37) and immunoprecipitation of p21 (15) have been previously described (32). Northern blot analysis of cytoplasmic RNA was performed by standard procedures (26).

Transfection with cDNA and infection with retroviruses. The cDNAs for EJ*ras* (35) or pSV2neo (38) (100 ng each) were precipitated with calcium phosphate (18). Transfection of cells and selection of transformants were as previously described (33). For infection with retroviruses, 6×10^4 cells were seeded in 60-mm plates in the presence of 4 μg of Polybrene (Sigma) per ml. The next day the cells were infected (10) with virus (10^3 focus-forming units) and observed for focus formation at 5 to 7 days later.

RESULTS

Persistent reversion after IFN treatment. Like many other revertants of RS485 that were isolated after treatment with IFN-α/β, lines 4C3 and 4C8 (32, 34) remained nontransformed for over 6 months in the presence of 200 IU of IFN per ml. To evaluate whether IFN was required for the maintenance of the nontransformed phenotype, we discon-

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TABLE 1. Growth characteristics of PR cells

Cells	Cloning efficiency in agar (%)[a]	Tumorigenicity	
		Tumors/mice	Diam (cm)
NIH 3T3	0.01	0/6	
RS485	12.00	6/6	2.2 (±0.2)
PR4	<0.01	0/6	
PR5	0.01	1/5	0.3
PR10	ND[b]	0/3	
PRK	ND	0/3	
AzaC treatment in vitro[c]			
PR4/5AzaC	1.30	6/6	2.4 (±0.3)
RR4/5AzadC	6.50	3/3	2.6 (±0.3)
PR4/6AzaC	<0.01	0/3	
NIH 3T3/5AzaC	0.02	0/6	
NIH 3T3/5AzadC	0.01	0/6	
5AzadC treatment in vivo[d]			
PR4		3/3	0.8 (±0.3)
PR10		2/4	1.5
PRK		3/4	3.2 (±0.5)
NIH 3T3		0/4	

[a] For cloning efficiency 10^4 cells were plated in 0.36% agarose in 60-mm dishes, and colonies were scored 2 to 3 weeks later. Results are the average cloning efficiency determined from two dishes. To study tumorigenicity of cells, we injected 5×10^5 cells (subcutaneously) into nude (athymic) mice. The size of tumors was recorded 30 days after inoculation. Cells were considered nontumorigenic when no evidence of tumor formation was seen 2 months after injection.

[b] ND, Not determined.

[c] Cultured cells were treated with cytidine analogs (see Materials and Methods) and tested at the third passage after treatment.

[d] Twenty-four hours after inoculation of untreated cells, the animals were injected (intraperitoneally) with 5AzadC (20 mg/kg).

tinued treatment and observed the revertants for changes in morphology and *ras* expression. Three to four weeks after removal of IFN the revertant cultures developed a few foci of retransformed cells; however, more than 99% of the cell population remained contact inhibited. The latter, designated PRs, were isolated and cloned. Clonal lines PR4, PR5, PR10, and PRK (isolated from 4C3 after six to eight weekly passages in IFN-free medium) are representative of numerous other PRs having similar characteristics. The PRs did not grow in soft agar and were either nontumorigenic in nude mice (PR4, PR10, and PRK) or developed as a slow-growing tumor in one of five inoculated animals (PR5) (Table 1). This phenotype was stable in PRs for about 2 to 4 months after cloning.

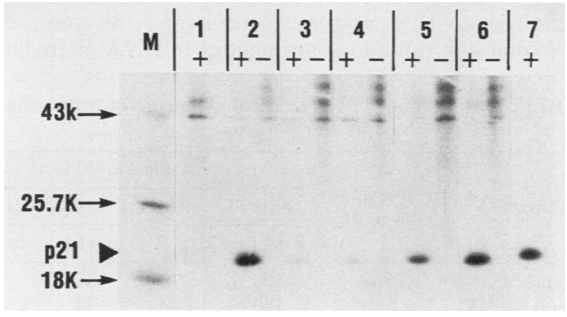

FIG. 1. Production of *ras*-encoded protein p21 in the presence or absence of IFN treatment. Cell extracts from [^{35}S]methionine-labeled cultures were immunoprecipitated with (+) or without (−) Y13-238 monoclonal antibody to p21 (15) and separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a discontinuous 8 to 20% gradient gel. Lanes: 1, NIH 3T3; 2, RS485; 3, 4C3 treated with 200 IU of IFN-α/β per ml; 4 and 5, 4C3 off IFN for 3 and 10 days, respectively; 6, PR4; 7, mutated p21 encoded by EJ*ras* (cell line RS504). M, Molecular size markers in daltons. K, $\times 10^3$.

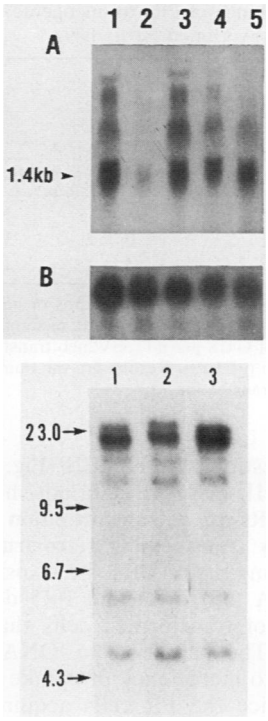

FIG. 2. Upper panel: Northern blot analysis of *ras*-specific mRNA in PR cells. Nick-translated [^{32}P]cDNA probes: (A) *Sac*I 3-kilobase fragment of human c-Ha-*ras*1; (B) *Bal*I 760-base-pair fragment of chicken actin (Oncor). Samples were 20 μg of cytoplasmic RNA from: lane 1, RS485; lane 2, 4C3 treated with 200 IU of IFN-α/β per ml; lane 3, PR4; lane 4, PR4/5AzadC retransformant; lane 5, PR10. Ha-*ras* mRNA was undetectable in NIH 3T3 cells. Lower panel: Southern blot analysis of c-Ha-*ras* DNA. Samples were 20 μg of *Hin*dIII-digested DNAs from: lane 1, RS485; lane 2, 4C3; and lane 3, PR4. Numbers on the left are size markers (kilobases).

PRs contained the transforming c-Ha-*ras* DNA and expressed high levels of *ras*-specific mRNA and p21. To test for *ras* expression, p21 was immunoprecipitated from ^{35}S-labeled cell extracts and resolved on sodium dodecyl sulfate-polyacrylamide gels. While there was a significant reduction in the levels of p21 in the revertant 4C3 in the presence of 200 IU of IFN per ml, removal of IFN from the medium resulted in a gradual elevation in p21 production (Fig. 1). Since the cloned PRs expressed high levels of p21, comparable to those in transformed RS485 cells (Fig. 1, lane 6), we investigated whether the nontransformed phenotype of PRs was associated with production of a defective p21 or with resistance to the transforming action of the protein. We found no difference in the electrophoretic mobility of p21 immunoprecipitated from PR or RS485 cells in contrast to the slower mobility of p21 encoded by the mutated EJ*ras* (Fig. 1, lanes 2 and 7). In addition, the RNA transcripts for *ras* in PRs did not differ in amount and size from those in RS485 cells (Fig. 2, upper panel). The comparable expression of *ras* in PRs and RS485 cells was in agreement with the similar restriction pattern of the transfected c-Ha-*ras* DNA in these

TABLE 2. Resistance of PRs to oncogenic transformation by cloned EJ*ras* DNA[a]

Cells	Oncogenic transformation by pEJras (no. of foci)		Biochemical transformation by pSV2neo (no. of G418-resistant colonies)	
	Expt 1	Expt 2	Expt 1	Expt 2
NIH 3T3	135	98	180	200
PR4	1	0	160	188
PR5	2	3	195	175

[a] Cells (10^5) were transfected (18) with pEJras or pSV2neo DNA (100 ng each), and transformants were selected for with medium containing 10% fetal calf serum and 400 μg of G418 per ml (pSV2neo transfection) or in medium with 5% fetal calf serum (pEJ*ras*). Results are the number of transformants recorded 21 days after transfection.

cell lines after digestion with *Hin*dIII (Fig. 2, lower panel), *Eco*RI, *Sma*I, *Hpa*II, or *Msp*I (data not shown).

Resistance of PRs to retransformation by transfecting pEJ*ras* DNA or by transforming retroviruses. To test the effect of transforming *ras* on PRs, we transfected them with cloned EJ*ras* DNA (35). PR4 and PR5 developed significantly fewer foci of transformed cells than did NIH 3T3 cultures (Table 2). The inability of *ras* DNA to transform PR cells was not due to inefficiency of uptake or expression of foreign DNAs, since the PR cells acquired resistance to G418 by transfection with pSV2neo DNA (38) (Table 2) and were readily transformed by cloned v-*mos* DNA (data not shown). These results suggested that the PR cells were refractory to transformation by EJ*ras*.

To examine further the resistance of PRs to oncogenic transformation, we infected cells with transforming retroviruses carrying the v-Ki-*ras*, v-Ha-*ras*, v-*abl*, v-*fes*, or v-*mos* oncogene. Except for infection with Moloney murine sarcoma virus (v-*mos*), the PR4 cells developed significantly fewer transformed foci than did NIH 3T3 cells (Table 3). When tested by an infectious center assay (10), PR4 cells infected with Moloney murine leukemia virus, like NIH 3T3 cells, produced viral particles with helper activity (data not shown), indicating that infection of PRs by retroviruses was not impaired. This, together with the transformation of PR4s by the v-*mos*-bearing retrovirus, strongly suggest that PRs were resistant to the transforming activity of the other retroviruses tested. The status of the v-*onc* DNAs and *onc* expression in this system are under further investigation.

Retransformation of PRs after treatment with azacytidine. The stable phenotype of PRs was acquired in conjunction with IFN, a nonmutagenic biological agent. One epigenetic mechanism associated with changes in gene expression and cell phenotype involves alterations in DNA methylation (8). To determine whether DNA methylation was involved in the phenomenon of persistent reversion, we treated PRs with the nucleosides 5AzaC and 5AzadC that inhibit enzymes which methylate cytosine residues of eucaryotic DNA (23, 24). 5AzaC can also induce alterations in chromosome morphology (23). In PR4, PR5, PR10, or PRK cultures treated twice during the log phase of growth with 5AzaC (3.0 μM) or 5AzadC (0.1 μM) and subsequently subcultured in their absence, over 50% of the cell population underwent a dramatic morphological alteration with loss of contact inhibition as early as three to six population doublings after treatment. Retransformed PR cultures developed colonies in soft agar and formed tumors in nude mice (Table 1). Preliminary studies suggested a significant and similar degree of demethylation in 5AzadC-treated PR or NIH 3T3 cells (V. Wilson and D. Samid, unpublished data), yet in contrast to PRs, the NIH 3T3 cells remained nontransformed 3 to 6 weeks after treatment with 5AzadC (Table 1), although 5AzaC-induced transformation has been reported in cultured C3H 10T(1/2) (21), CHEF/18 (19), and BHK (2) cells. The analog 6AzaC, which does not inhibit methylation (24), did not induce alterations in either NIH 3T3 cells or PRs.

To test whether 5AzadC would also induce tumorigenicity of PRs in vivo, we inoculated nude mice subcutaneously with G418-resistant PR4, PR10, PRK, or NIH 3T3 cells. The next day the animals were injected intraperitoneally with 20-mg/kg 5AzadC. Mice inoculated with PR cells developed tumors at the sites of cell inoculation 2 to 3 weeks after administration of 5AzadC, while those inoculated with NIH 3T3 cells did not (Table 1). Cultures established from the tumors were resistant to G418, indicating that the tumors originated from PRs.

Efficient reversion by IFN of azacytidine-transformed cells. Phenotypic reversion with varying efficiency was observed in four 5AzadC-induced retransformed lines of PR4 after 2 to 6 weeks of continuous treatment with 200 IU of IFN-α/β per ml. In PR4/5AzadC-2, the most responsive line, 30 to 50% of the cells reverted after only 2 weeks of treatment. Changes in phenotype by alternating treatments with IFN or with demethylating nucleosides support the hypothesis that DNA methylation is one of the mechanisms involved in the induction of a stable reversion by IFN.

Persistent reversion appears related to changes in genes other than *ras* because we found no indication that the LTR-*ras* sequences are hypermethylated in PR4 (data not shown). This was determined by using ^{32}P probes for either the LTR of Harvey murine sarcoma virus or the c-Ha-*ras*1 to analyze cellular DNA digested with *Msp*I or *Hpa*II, restriction enzymes that discriminate between methylated and unmethylated CG sequences (12). Furthermore, the high levels of *ras* mRNA in PR cells were not augmented after treatment with 5AzadC (Fig. 2).

DISCUSSION

One of the goals of cancer research is to identify means to arrest or reverse the transformed phenotype. It is intriguing therefore that a stable reversion and resistance to transformation by different oncogenes can be obtained with nontoxic, nonmutagenic biological agents such as IFNs. There is considerable evidence that methylation of DNA at specific regions is an epigenetic mechanism for controlling gene expression (8), and abnormalities in DNA methylation

TABLE 3. Resistance of PR cells to oncogenic transformation by retroviruses[a]

Retrovirus[b]	Viral oncogene	No. of transformed foci			
		NIH 3T3 cells		PR4 cells	
		Expt 1	Expt 2	Expt 1	Expt 2
Kirsten MuSV	Ki-*ras*	>200	70	36	7
Abelson MuLV	*abl*	180	>200	28	8
Harvey MuSV	Ha-*ras*	>200	>200	9	25
Fujinami SV	*fes*	160	>200	5	10
Moloney MuSV	*mos*	>200	190	160	110

[a] All virus preparations contained ectropic Moloney murine leukemia virus helper virus. NIH 3T3 and PR4 cells had similar sensitivity to virus infection, as determined by the infectious center assay (10) (data not shown). Results are the total number of foci in duplicate plates.

[b] MuSV, Murine sarcoma virus; SV, sarcoma virus; MuLV, murine leukemia virus.

have been implicated in carcinogenesis (12, 21, 22, 40). Since IFNs are known to up- or down-regulate the expression of a variety of genes (14, 30), it is likely that in inducing persistent reversion in *ras*-transformed RS485 cells, IFN has modulated the expression of a gene(s) that plays a role in cell transformation, such as a tumor suppressor(s) or cotransforming factors. As a result, elevation in *ras* expression in PRs no longer correlated with oncogenic transformation, as it normally does in NIH 3T3 cells (4, 29). DNA methylation could be a secondary event imprinting the altered gene activity with no further requirement for IFN (8). In such a case, demethylation should be sufficient for gene reactivation and subsequent oncogenic retransformation. The following results support this hypothesis. (i) PR cultures underwent rapid retransformation after transient treatment either in vitro or in vivo with 5AzadC, a nonmutagenic cytidine analog that inhibits DNA methylation (23, 24), while demethylation did not result in transformation of NIH 3T3 cells; (ii) the analog 6AzaC which does not affect methylation (24) did not alter the morphology of PRs; and (iii) PRs retransformed by 5AzadC reverted again with IFN treatment.

The PRs were refractory to transformation by RNA tumor viruses bearing the v-Ki-*ras*, v-Ha-*ras*, v-*abl*, or v-*fes* oncogene, yet were transformed by v-*mos*. In this respect, PR cells resembled mutagen-induced revertants of Ki-*ras*-transformed NIH 3T3 cells (27). The latter produced large amounts of p21 and suppressed tumorigenicity of other cells transformed by a variety of v-*onc* genes including *abl* and *fes*, but not *mos*. The PRs also resisted transformation by EJ*ras* DNA. In view of our previous finding that IFN did not cause reversion in NIH 3T3 cells transformed by the mutated EJ*ras* (cell line RS504) (33), it seems that induction of phenotypic reversion and acquisition of resistance to oncogenic transformation after IFN treatment involve different mechanisms. Cell-mediated resistance to transformation by *ras* has been observed in a variety of primary and established cultures (7, 9, 13, 17, 28, 31).

Retransformation of PRs after treatment with 5AzaC or 5AzadC may involve changes in a gene(s) other than *ras*, the products of which, while not sufficient to induce transformation of NIH 3T3 cells, may modulate or interact with the activated *ras* in PRs, thus restoring the conditions required for oncogenic transformation. Identification of such newly expressed gene products would be of major importance in understanding mechanisms of cell transformation. The ability of 5AzadC administered to animals to induce tumor progression in nontumorigenic cells expressing an activated *ras* (PRs) is of special interest, since demethylating drugs are being used in treatment of human cancer (16). The potential of such drugs to induce tumor progression of premalignant lesions should be studied.

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