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DEVELOPMENT OF TRANSFORMED PHENOTYPE INDUCED BY A HUMAN ras ONCOGENE IS INHIBITED BY INTERFERON

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Mouse IFN inhibited the development of transformed foci in NIH 3T3 cultures transfected with the viral Ha-MuSV(ras) and Mo-MuSV(mos) oncogenes, or with the human bladder carcinoma ras EJ/T24 DNA. IFN treatment five or seven days after transfection was still effective in inhibiting the oncogenic transformation, but did not inhibit significantly the biochemical transformation induced by pSV2-neo or Ecogpt DNA, so that inhibition of ras-induced transformation was not a result of a general effect on the transfection process. Treatment with IFN did not alter the expression of ras EJ/T24 DNA after the transformed phenotype had been established. © 1985 Academic Press, Inc.

Interferons (IFNs) can inhibit virus, chemical, and radiation-induced oncogenic transformation both in animals (1) and in cultured cells (2). This antitumor action may result from IFN's antiviral activity, from its effect on the immune system, or from a direct inhibition of tumor cell multiplication (3). Since neoplastic transformation may sometimes be related to the abnormal expression of transforming genes, either viral (v-onc) or activated cellular oncogenes (c-onc) (4), it was of interest to investigate whether IFN can inhibit onc-induced transformation. Previous reports indicated that IFN treatment was associated with reversion in cells transformed by radiation (5), viruses (6), or human oncs (7), and with reduced synthesis of a specific v-onc (8) or c-onc mRNA (7,9) or protein product (7); however, there are no reports indicating that IFN treatment inhibits transformation induced by a transfected onc DNA.

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The transforming gene of Harvey murine sarcoma virus (Ha-MuSV) is designated v-Ha-*ras* (10-11). The human cellular homologue, c-Ha-*ras*1, is the proto-oncogene of the mutated *ras* isolated from two human bladder carcinoma cell lines (EJ/T24) ; both Ha-MuSV and human *ras* DNA activated either by upstream addition of a promoter, or by a point mutation can cause oncogenic transformation in cultured recipient cells (12-20). We studied the effect of IFN on *ras*-induced cell transformation in transfected cells, because *in vivo* daily injection of IFN delayed mortality in mice inoculated with Ha-MuSV (21). The gene transfer technique enabled us to study the effect of IFN on *ras*-induced cell transformation *in vitro*.

Materials and Methods. 2×10^5 NIH 3T3 cells were seeded in 35 mm plates in DMEM and 10% FCS. After 24 hrs pSV2-neo gene DNA (75 ng) (23), Ecogpt DNA (1 ug) (24), M1 Moloney murine sarcoma virus (Mo-MuSV) DNA (800 ng) (25), v-Ha-*ras* DNA (50 ng) (10, 11), or EJ/T24 *ras* DNA (50 ng) was calcium-precipitated (22,23) with 3 ug of carrier calf thymus DNA and added to subconfluent cultures. Control cultures were transfected with carrier DNA only. After 16-20 hrs cells were trypsinized, and replated into two 100 mm culture dishes in DMEM + 5% FCS; 48 hrs later the pSV2-neo transfected cells were fed with the same medium containing 400 ug/ml G418 where appropriate (23). Cells transfected with Ecogpt DNA were incubated in AMXT medium containing aminopterin (2 ug/ml), mycophenolic acid (25 ug/ml), xanthine (250 ug/ml) and thymidine (10 ug/ml) (25). Thereafter, the medium (with or without G418) or AMXT additions to the medium was replaced every four days. Mouse alpha/beta IFN (400 units/ml) with a specific activity of 10^8 units/mg protein was added to the medium one, three, five or seven days after transfection. Once added, the cells were kept in IFN with or without G418 or AMXT until the termination of the experiment 17 to 21 days after transfection, when they were methanol-fixed and stained with Giemsa stain. The number of G418 or AMXT resistant colonies was recorded in the case of cells transfected with pSV2-neo or Ecogpt respectively. With EJ/T24 *ras*, Mo-MuSV, or v-Ha-*ras* DNA transfection, we counted the number of oncogenically transformed foci. While one to four oncogenically-transformed foci were observed in cultures transfected with carrier, Ecogpt, or pSV2-neo DNA alone, only cells in cultures transfected with pSV2-neo or Ecogpt survived G418 or AMXT medium treatment respectively. In cotransfection experiments pSV2-neo and EJ/T24 DNA (75 ng each) were calcium coprecipitated with 3 ug of carrier calf thymus DNA and added to subconfluent NIH 3T3 cells. Results are reported as the number of oncogenically transformed colonies over the number of G418-resistant colonies per two 100 mm plates.

Results

Inhibitory Effect of IFN on transformation induced by transfection with Ha-MuSV, Mo-MuSV or EJ/T24 DNA. NIH 3T3 cells were transfected with cloned Ha-MuSV, Mo-MuSV, or EJ/T24 *ras* DNA using the calcium phosphate technique (13,22). Mouse L cell IFN (400 IU/ml) was added at various times after

Table 1 Effect of Interferon (IFN) Treatment on Oncogenic or Biochemical Transformation

IFN Treatment (days after transfection)	A Oncogenic Transformation by:			B Biochemical Transformation		C Co-transformation
	Ha-MuSV	EJ/T24 *ras*	Mo-MuSV	pSV_2-neo	Ecogpt	EJ-T24 *ras*/pSV_2 neo
Control (no IFN)	50(1)	75(1)	24(1)	182(2)	106(3)	156(1)/216(2)
1	3	8	1	100	-	7/75
3	-	11	1	159	103	9/140
5	-	10	-	183	-	20/188
7	6	14	-	-	-	32/191

All reported studies were repeated at least twice with similar results.

- not tested
(1) morphologically transformed foci
(2) G418-resistant colonies (neo^R)
(3) AMXT medium resistant (Ecogpt positive) colonies

transfection, then maintained in the medium throughout the experiment. IFN treatment inhibited the development of foci in *onc*-transfected cultures (Table 1A and Fig. 1); more than 80% (±10%) inhibition was observed when IFN was added to the medium as late as seven days after transfection. The inhibition of *onc*-induced neoplastic transformation was due to IFN because: (i) the mouse IFN preparation employed was highly purified; (ii) the effect was dose-dependent; (iii) antibody to mouse IFN neutralized the inhibitory effect of IFN; and, (iv) purified human recombinant IFN had no inhibitory activity on *onc*-induced transformation.

In order to examine whether IFN treatment impaired the uptake of *ras* DNA, the cells were treated with IFN 24 hrs after transfection and 5-10 days later either interferon was withdrawn or antibody to IFN was added to the medium. The cessation of IFN activity in both cases resulted in reappearance of foci of transformed cells, indicating the presence of EJ/T24 *ras* in the cells. Therefore, the inhibitory effect of IFN on the oncogeneic transformation was not an artifact of the transfection assay, but rather a more specific effect on the process of neoplastic transformation induced by human *ras* oncogene.

Effect of IFN on Transfection with pSV2-neo or Ecogpt DNA. Dubois *et al.* have

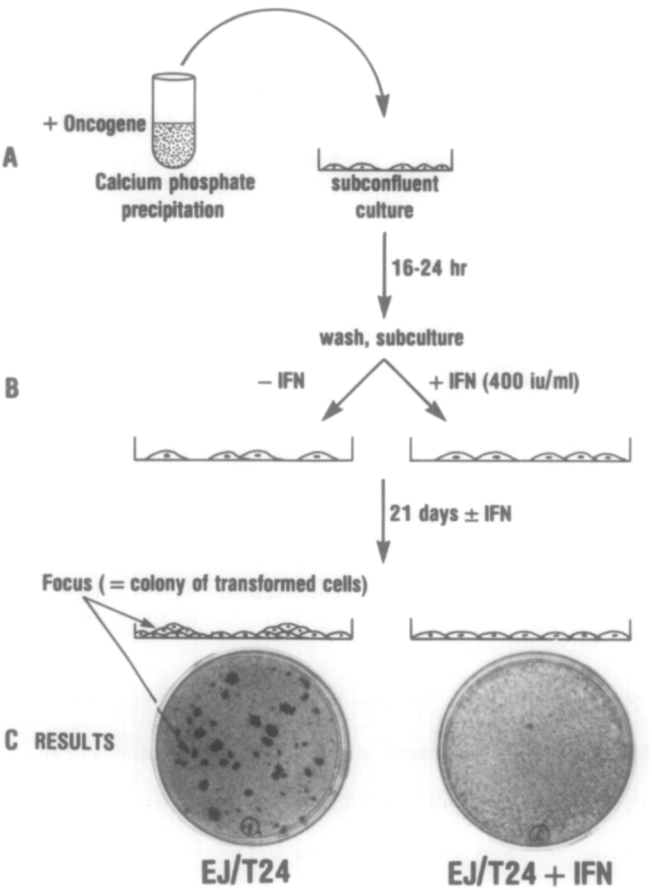

Figure 1: Inhibition of development of foci in ras transfected cells: NIH 3T3 cell were transfected with EJ/T24 c-Ha-ras1 DNA and treated with IFN. (see Materials and Methods).

reported that interferon (IFN) inhibited the biochemical transformation of mouse cells transfected with the herpes virus thymidine kinase or the Chinese hamster dehydrofolate reductase genes; however, expression of these genes after their stable integration into the cellular genome was not altered by IFN (26). In order to test whether IFN treatment was affecting the transfection assay itself, we tested whether IFN treatment inhibited biochemical transformation by transfected genes; we transfected NIH 3T3 cells with the cloned bacterial aminoglycoside phosphotransferase (neo) gene, pSV2-neo, that confers resistance to the antibiotic G418 (23), or with the E. Coli xanthine-guanine phosphoribosyl transferase (Ecogpt) gene, that confers resistance to AMXT medium (24). We then treated with IFN after transfection;

parallel studies were run with EJ/T24 ras DNA-transfected cells (Table 1A). Addition of 400 units/ml of IFN one day after transfection with neo resulted in 45% (±15%) inhibition of the formation of G418-resistant foci (Table 1B). IFN was much less effective, or ineffective in inhibiting neo-induced biochemical transformation when added three or five or more days after transfection with pSV2-neo; IFN added after 3 days also had no significant effect on Ecogpt-induced biochemical transformation (Table 1B). In contrast, we had observed significant inhibition of oncogenic transformation induced by EJ/T24 ras, Mo-MuSV, or Ha-MuSV DNA when IFN was added three to seven days after transfection (Table 1A).

We also performed cotransfection experiments, in which EJ/T24 ras and pSV2-neo DNA were coprecipitated and the cotransfected cells then treated with IFN (Table 1C). In these studies addition of IFN as late as five or seven days after transfection effectively inhibited EJ/T24 DNA-induced oncogenic transformation; however, significant inhibition of neo^r colony formation was observed only when IFN was added 24 hrs. after transfection, but not after 5-7 days. Taken together with the results of Dubois et al (26), our findings indicated that IFN treatment did not inhibit the process of transfection, if added five to seven days after transfecting DNA, since biochemical transformation induced by several DNAs was not affected by this treatment.

Lack of effect of IFN on NIH 3T3 cells transformed by the EJ/T24 human bladder carcinoma c-Ha-ras oncogene. One possible explanation for the results indicating that IFN treatment inhibited the appearance of foci of transformed NIH 3T3 cells after transfection with EJ/T24 ras DNA is that IFN treatment induced reversion in cells transformed by ras EJ/T24. Indeed, IFN treatment induced morphological and biochemical reversion in RS 485, an NIH 3T3 cell line transformed by an LTR-activated human c-Ha-ras, which the is proto-oncogene of EJ/T24 ras (7).

In order to test whether IFN treatment might induce reversion in EJ/T24-transformed NIH 3T3 cells, we assessed whether IFN-treated RS 504 underwent morphological reversion. RS 504 is an NIH 3T3 cell line that had

been transformed by transfection with EJ/T24 ras DNA. RS 504 cells contain several copies of the transfecting ras DNA, have a transformed phenotype in vitro, and are tumorigenic in nude mice. Unlike RS 485 in which IFN treatment with concentrations as low as 50 units/ml readily induced the emergence of revertant cells, prolonged treatment of RS 504 cultures with IFN concentrations as high as 500 units/ml in 10 experiments resulted in only three revertant cell lines. This was about the rate of spontaneous phenotypic reversion found in control cultures not treated with interferon. Southern blot analysis of the DNA of revertant RS 504 clonal line from both IFN-treated and control cultures showed that the transduced ras DNA was absent. This is in marked contrast to IFN-induced RS 485 revertants in which the exogenous transforming ras DNA was retained (7). These results indicated that IFN treatment did not induce reversion in EJ/T24 ras DNA-transformed NIH 3T3 cells.

Discussion

IFN treatment started one to seven days after transfection of NIH 3T3 cells with EJ/T24 ras, Ha-MuSV(v-ras) or Mo-MuSV(v-mos) DNA resulted in a marked decrease in the yield of morphologically transformed foci. This decrease was not the result of impaired uptake of DNA in the transfection assay since cessation of IFN treatment resulted in the development of neoplastically transformed foci. Furthermore, IFN did not inhibit biological changes induced by all transfected DNAs, because, IFN added five or seven days after transfection had no effect on the biochemical transformation induced by pSV2-neo or Ecogpt DNA. In addition IFN treatment did not induce reversion in EJ/T24 ras DNA-transformed cells. Therefore, it is likely that IFN treatment directly inhibited the malignant transformation of NIH 3T3 cells induced by the EJ/T24 ras, v-Ha-ras, or v-mos oncogenes.

The finding that IFN did not induce reversion in the EJ/T24 ras DNA-transformed cell line RS 504 is interesting, since IFN readily induced reversion in RS 485, an NIH-3T3 cell line transformed by an LTR-activated c-Ha-ras oncogene (7). These findings may be explained by the fact that the

oncogenicity of EJ/T24 ras DNA is due to a point mutation (12-20), while the oncogenicity of LTR-activated c-Ha-ras is probably related to increased production of the ras product, p21(4). IFN treatment significantly reduced the production of p21 in RS 485 revertant cell lines (7)

Cell lines producing regulatable amounts of onc-encoded protein provided the evidence that in some cases the cellular phenotype is dependent upon the dose of the transforming protein (27). The threshold amounts needed for neoplastic potential may be much lower for the mutated ras-encoded p21 produced in RS 504 cells as compared to that for the normal p21 (28, 29). Therefore, if a reduction in p21 levels was related to the phenotype reversion observed in IFN treated RS 485 cells, similar reduction may not have been sufficient to exert the same effect on EJ/T24 ras transformed cells.

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