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Mitogenic Effects of the Proto-Oncogene and Oncogene Forms of c-H-*ras* DNA in Human Diploid Fibroblasts

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Nuclear microinjection of c-H-*ras* DNA induced DNA synthesis in reversibly nonproliferating quiescent human cells. The proto-oncogene and oncogene forms were equally effective inducers. In contrast, c-H-*ras* DNA either alone or in combination with the adenovirus E1A gene did not cause terminally nondividing senescent cells to synthesize DNA.

Normal human cells exhibit a limited capacity for division (senescence) in vitro, in contrast to tumor-derived and virus- or carcinogen-transformed cells, which can divide indefinitely (immortal) (11, 12). A number of recent studies have suggested that senescent cells produce a membrane-associated protein inhibitor(s) of the initiation of DNA synthesis that is not present in young proliferating cells; production of this protein inhibitor(s) might be involved in senescence (4, 7, 23). Cell hybrid studies have shown that the phenotype of senescence is dominant over that of cellular immortality (24, 26). Therefore, an altered regulation of the expression of the senescence inhibitor protein(s) or modifications of the protein(s) itself might result in cellular immortality. Cellular proto-oncogene and oncogene proteins have been clearly demonstrated to be positive regulators of cell growth and are potential candidates for initiators of DNA synthesis that could act antagonistically to the inhibitor(s) in senescent cells (2, 13). Since the H-*ras* product acts at the membrane of cells, as does the putative senescence protein(s) (29, 36), we tested the ability of cloned c-H-*ras* DNAs to functionally reverse inhibitor activity after microinjection into senescent cells. Normal young cells that have been made nonproliferating (quiescent) by the reduction of serum growth factors also produce a membrane-associated protein inhibitor(s) of the initiation of DNA synthesis (3, 23). Therefore, the effect of microinjected c-H-*ras* DNA in these cells was tested as well.

Two human cell lines were used as recipients of the microinjected DNA in this study. CSC303 is a diploid fibroblast line which was derived from neonatal foreskin and has a total in vitro life span of 80 population doublings (25). CSC301 is a diploid fibroblast line which was derived from fetal lung and has a total in vitro life span of 100 population doublings (8). Cultures were considered to be senescent when they did not reach confluence within 4 weeks after subculturing and had a labeling index of <1%. Quiescent cells were obtained from cultures having at least 50 population doublings remaining.

For microinjection, the cells were plated at a density of 5×10^3 per 35-mm dish onto glass cover slips (22-mm square) into which 3-mm-diameter circles had been etched with a diamond pencil; the cells were allowed to attach in Eagle minimal essential medium plus 10% fetal bovine serum. Senescent cells and cells made quiescent by contact inhibition were maintained in this medium for the duration of the experiment. The cells to be made quiescent by serum deprivation were washed with buffered saline 24 h after being plated and were maintained for at least 10 days in Eagle minimal essential medium plus 0.5% fetal bovine serum (6). Microinjection was performed as described in detail previously (19). At least 200 cells were microinjected per experiment. Tritiated thymidine (0.1 μCi/ml) was then added to the cells. The cells were incubated for appropriate intervals (48 h for senescent cells, 30 h for quiescent cells) and then processed for autoradiography (31). For analysis, cells within the circle (injected) and peripheral to the circle (uninjected) were examined. Within each category the cells whose nuclei contained >10 silver grains were counted as labeled. All microinjected cells and at least 300 uninjected cells were analyzed. The results are the average of two or more independent experiments.

Initially, the effect of the oncogene form of c-H-*ras* DNA was examined. Cloned DNA isolated from the bladder carcinoma cell line EJ (30) (kindly provided by R. Weinberg, Massachusetts Institute of Technology) was injected into quiescent and senescent cells derived from the CSC303 cell line. Cells made quiescent either by maintenance in low serum or by growth to a high density were stimulated to enter DNA synthesis (Table 1). Senescent cells, however, did not respond to the c-H-*ras*(EJ) DNA by synthesizing DNA. Both senescent and quiescent cells expressed the p21 product of the DNA at very low levels, as detected by immunofluorescence staining with antibody Y13-259 against p21 (a kind gift from E. Scolnick and R. B. Stein, Merck Sharp & Dohme). Control injections of pBR322, the plasmid in which c-H-*ras*(EJ) DNA was cloned, did not increase the percentage of labeled nuclei over background (Table 1), indicating that the microinjection procedure itself did not stimulate the cells.

Feramisco et al. (9) have reported that microinjection of the p21 protein product of oncogenic c-H-*ras*(T24) failed to stimulate DNA synthesis in a human fibroblast cell line (H8). In contrast, Mulcahy et al. (20) demonstrated that injection of anti-p21 antibody into MRC5 cells (diploid human lung fibroblasts) resulted in a decrease in the number of cells synthesizing DNA during the time period observed, results more in agreement with our observations. Due to the apparent discrepancy with the report of Feramisco et al. (9), we examined the effect of c-H-*ras*(T24) DNA in our system. Another human cell line, CSC301, also was included to

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TABLE 1. Effect of microinjected c-H-*ras*(EJ) DNA on DNA synthesis in human diploid fibroblasts

Material microinjected (mg/ml)	Cells injected	% Serum used	% Labeled nuclei in:	
			Injected cells	Uninjected controls
pBR322 (1.0)	Quiescent CSC303	0.5	7	8
c-H-*ras*(EJ) (1.0)	Quiescent CSC303	0.5	80	11
	Quiescent CSC303[a]	10	50	10
	Senescent CSC303	10	<1	<1

[a] High-density, contact-inhibited cells.

determine whether *ras* functions varied with the cell line used. In addition, both proto-oncogene and oncogene forms of c-H-*ras* DNA were compared to determine if the activated form of c-H-*ras* was more mitogenic in human cells. Cloned DNAs for the proto-oncogene (pP3) and oncogene (pT24) forms of c-H-*ras* were kindly provided by M. Wigler, Cold Spring Harbor Laboratory (34).

The results of these injections are presented in Table 2. Oncogenic c-H-*ras*(T24) DNA was effective in stimulating DNA synthesis in both quiescent CSC303 and quiescent CSC301 cells. Therefore, neither the cell line used nor the source of c-H-*ras* DNA affected our general observations. As senescent cells were refractory to the mitogenic effects of c-H-*ras*, it is possible that Feramisco et al. (9) used cells at or near the end of their in vitro life span. Both we and Mulcahy et al. (20) worked with cells having a labeling index of >50% (i.e., cells early in their in vitro life span). Future studies will be necessary to determine the point in their in vitro life span at which human cells become refractory to the stimulatory effect of c-H-*ras* DNA.

Both the proto-oncogene and oncogene forms of c-H-*ras* DNA were equally efficient in stimulating DNA synthesis in quiescent human cells (Table 2). A maximal response was obtained with 0.3 mg of DNA per ml, which corresponds to approximately 170 copies of c-H-*ras* injected per cell, if one assumes a microinjection volume of 5×10^{-11} ml per cell. DNA synthesis stimulation was slightly reduced when 3 mg of DNA per ml was injected, possibly reflecting some toxicity because of the introduction of such large amounts of DNA into the cells. Both activities diluted out at equivalent concentrations; no stimulation was observed when either plasmid was injected at a DNA concentration of 0.003 mg/ml. One possible explanation for the equivalent activities of the proto-oncogene and oncogene forms of c-H-*ras* DNA is that pP3 DNA is expressed better in human cells than is pT24. This could not be tested directly and quantitatively because human cells are not transfectable. However, following microinjection of either plasmid into human cells, a few cells in each system became weakly positive for *ras* protein by immunofluorescence. Further, both DNAs were expressed to similar degrees (based on immunofluorescence) in transient assays following transfection of NIH 3T3 cells. Therefore, it seems unlikely that the two plasmids are inherently expressed to markedly different degrees.

It is known that the oncogene form (T24) of the *ras* product has a reduced GTPase activity relative to the proto-oncogene form (P3) (33). This reduction in enzyme activity correlates with the ability of the p21(T24) protein to stimulate DNA synthesis and to induce morphological changes in rodent (REF 52 or NIH 3T3) cells at concentrations at which the normal p21(P3) protein has no effect (9). However, the cells used in this study responded equally well to the proto-oncogene and oncogene c-H-*ras* DNAs, indicating that the mitogenic activities of the products in human fibroblasts do not correlate with the levels of GTPase activity. Recent observations by Lacal et al. (15) have suggested that the transforming activity of *ras* proteins can occur by mechanism(s) other than reduction in GTPase activity.

Other laboratories have demonstrated the need for two oncogenes working in concert to convert primary rodent cells to tumorigenic cells. Oncogenes that have been shown to complement H-*ras* in this process are polyomavirus T antigen, the E1A region of the adenovirus genome, and c-*myc* (16, 21, 27). We tested the hypothesis that a combination of complementary oncogenes may be necessary to stimulate DNA synthesis in senescent cells. A plasmid carrying the adenovirus E1A gene (pAd12E1A, kindly provided by R. Bernards [1]) was coinjected with the oncogene form of c-H-*ras* (pT24) into senescent cells (Table 2). The cells did not incorporate tritiated thymidine, indicating that senescent normal human cells do not respond to the mitogenic effects of a combination of oncogenes that is able to permit transformation and tumorigenic progression in primary rodent cells.

This lack of response of senescent cells to microinjected oncogenes is not due to an increased sensitivity to microinjection or to an absolute inability of the cells to synthesize DNA following any stimulus. Senescent cells can

TABLE 2. Comparison of the ability of the proto-oncogene and oncogene forms of c-H-*ras* to induce DNA synthesis in human fibroblasts

DNA microinjected (mg/ml)	Cells injected[a]	% Labeled nuclei in:	
		Injected cells	Uninjected controls
pT24			
0.3	Quiescent CSC301	42	11
3.0	Quiescent CSC303	39	2
0.3	Quiescent CSC303	55	5
0.03	Quiescent CSC303	28	4
0.003	Quiescent CSC303	5	4
pP3			
0.3	Quiescent CSC301	51	10
3.5	Quiescent CSC303	52	3
0.3	Quiescent CSC303	60	12
0.03	Quiescent CSC303	33	4
0.003	Quiescent CSC303	1	1
pT24 + pAd12E1A (1.5 + 1.5)	Senescent CSC303	<1	<1
pBSV-1 (0.07)	Senescent CSC303	12	<1

[a] Cells were made quiescent by serum deprivation.

be induced to synthesize DNA following infection with the DNA tumor virus simian virus 40 (SV40) or cytomegalovirus (10, 14, 35) and following fusion to immortal human cells known to contain SV40, adenovirus, or papillomavirus sequences (22, 32). To determine if we could induce DNA synthesis in senescent cells, we microinjected a plasmid containing the entire SV40 genome (pBSV-1) (17) into senescent-cell nuclei. At 24 h after microinjection, 20% of the microinjected cells stained positively for SV40 T antigen by immunofluorescence. A total of 12% of the injected cells (60% of T-antigen-positive cells) synthesized DNA (Table 2). Senescent cells infected with 10 PFU of SV40 per cell responded comparably.

The lack of response of senescent cells to microinjected oncogenes could be due to the fact that high-abundance antiproliferative mRNAs are present in these cells (18). These mRNAs probably code for a protein inhibitor(s) of DNA synthesis that is expressed in senescent cells (4, 7, 23). Sager and co-workers (5, 28) have proposed the existence of anti-oncogenes in normal cells that suppress the action of the oncogene protein products. We consider the senescent-cell inhibitor to be a prime candidate for an anti-oncogene.

These results emphasize the marked difference in response to the introduction of exogenous DNA between human and rodent cells. This study clearly shows that human cells are highly refractory to changes in the mechanisms that limit their in vitro life span. This is in agreement with the finding that the frequency of immortalization of human cells in culture is very low.

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