Expression of c-fos and AP-1 Activity in Senescent Human Fibroblasts Is Not Sufficient for DNA Synthesis

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Abstract. Human fibroblasts have a limited replicative life span when maintained in culture after which they become unresponsive to treatment with mitogens, a phenomenon most commonly called senescence. Experiments indicating that serum does not induce expression of the c-fos proto-oncogene in senescent fibroblasts raised the issue of a potential central role for c-fos in the phenotype of sustained growth arrest. This was directly tested by microinjection of oncogenic c-Ha-ras protein into senescent fibroblasts. While ras injection was found to induce marked nuclear c-fos expression and functional AP-1 transcription activity, this did not lead to DNA synthesis. These results suggest that the senescence phenotype cannot be solely attributed to the absence of c-fos expression and that the proliferative block in these cells is either independent of AP-1 transcriptional activity, downstream of it, or involves multiple molecular mechanisms.

Essentially all eukaryotic cells demonstrate a finite capacity for cell division when serially passaged in vitro (Hayflick and Moorhead, 1961). This phenomenon, referred to as cellular senescence, is characterized by maintenance of viability and metabolism in the presence of sustained cell cycle arrest (reviewed in Goldstein, 1990; McCormick and Campisi, 1991). Because cultured fibroblasts taken from young individuals are capable of a greater number of population doublings than cells from older subjects, cellular senescence is considered to reflect and contribute to the aging process of the organism.

Recent studies have addressed the molecular basis for the block to proliferation in senescent cells. Comparison of the expression of cell cycle dependent genes in early passage and senescent fibroblasts reveals that growth arrest in the latter occurs near the GI/S boundary of the cell cycle just before the onset of DNA synthesis (Rittling et al., 1986). Senescent fibroblasts exhibit a highly attenuated growth response following addition of serum or a variety of purified growth factors despite the presence of normal numbers of growth factor receptors (Goldstein and Shmookler Reis, 1985).

In presenescent fibroblasts rendered quiescent by serum deprivation, stimulation by mitogenic growth factors results in the rapid expression of the proto-oncogenes c-fos and c-myc, and an increased expression of c-ras, before the onset of proliferation (Greenberg and Ziff, 1984; Muller et al., 1984; Lu et al., 1989). Expression of all three genes has been shown to be essential for the initiation of DNA synthesis (Holt et al., 1986; Heikkila et al., 1987; Mulcahy et al., 1985; Riabowol et al., 1988). Similar studies conducted using senescent human fibroblasts have shown that while c-myc and c-ras are expressed in response to mitogen treatment (Rittling et al., 1986), c-fos appears repressed as a result of a specific transcriptional block (Seshadri and Campisi, 1990). On the basis of this observation, it has been suggested that an inherent mechanistic difference exists between the responses of presenescent and senescent cells to treatment with serum, and that the lack of c-fos transcription may account for the failure of the latter to proliferate (Goldstein, 1990; McCormick and Campisi, 1991). Evidence for a central role for c-fos in the senescent phenotype is thus indirect and derived from the observation of a deficiency in expression of the proto-oncogene in senescent cells. A more direct test of this hypothesis is therefore important.

Here, we describe an experimental approach which allows a direct assessment of the potential role of c-fos in the proliferation of senescent fibroblasts. Through direct microinjection of human Ha-ras oncogene protein (T-24 ras) into senescent human fibroblasts, we find that c-fos is rapidly expressed. Moreover, by co-injection of a reporter gene construct containing an AP-1 regulated promoter, we determined that the c-fos protein expressed in these cells was likely to be active as the senescent cells injected with the ras protein efficiently expressed the AP-1 regulated reporter gene. The injected cells, however, failed to progress through S-phase of the cell cycle as detected by a lack of DNA synthesis. These results suggest that expression of c-fos, and active AP-1 transcription factor, is not sufficient to relieve the cell cycle block in senescent human fibroblasts.

The mammalian ras proto-oncogene family consists of three members, c-Ha-ras, c-Ki-ras, and c-N-ras, each of...
which can acquire oncogenic characteristics by point mutations at discrete locations in their coding sequences (Tabin et al., 1982), or by overexpression (Wigler et al., 1984). Microinjection of oncogenic ras protein into quiescent fibroblasts has been shown to result in changes in cell morphology (Feramisco et al., 1984), induction of c-fos expression (Stacey et al., 1987), and initiation of DNA synthesis. In contrast, senescent human fibroblasts injected with oncogenic c-Ha-ras expression vectors (Lumpkin et al., 1986) fail to synthesize DNA. Transformation of cycling or quiescent fibroblasts by microinjected Ha-ras proteins proceeds temporally, with stimulation of membrane ruffling and pinocytosis within 30-90 min postinjection, induction of c-fos expression within 1-2 h, and increased DNA synthesis within 24 h. We sought to determine which identifiable components of the oncogenic ras proliferative program were active in senescent human fibroblasts.

Materials and Methods

Cell Culture

Human fetal lung fibroblasts (IMR-90 cells) were maintained at 37°C in a humidified incubator under a 5% CO2 atmosphere in DME/high glucose (Gibco Laboratories, Grand Island, NY) supplemented with 10% FBS (Gemini Bioproducts, Inc., Calabasas, CA) and penicillin/streptomycin. Some tumor cells were generated from late passage human fetal lung fibroblasts, IMR-90 cells, which became nonproliferative after 47-55 population doublings during serial passage in vitro (Adler et al., 1991). Typically 5-7% of these cells were capable of DNA synthesis as compared to ~5% of early passage cells defined by <20 population doublings. An additional operational criterion for senescence was that the cells could not grow to confluence 4 wk after their final passage. Cells were grown initially on plastic dishes and were then prepared for microinjection by trypsinization and centrifugation. The following samples and concentrations were used in microinjection experiments: oncogenic T-24 ras protein at a final concentration of ~1.5 mg/ml, resulting in the introduction of 1010-1010 molecules per cytoplasmically injected cell. The ras protein was purified from an Escherichia coli expression vector system (Gross et al., 1985) to near homogeneity as in Feramisco et al. (1984). Inert carrier IgG (Sigma Chemical Co., St. Louis, MO) was coinjected with all samples at a concentration of 2 mg/ml to allow the definitive identification of all microinjected cells. The coinjected IgG was diluted in a solution of 0.5% NP-40 in PBS containing 1,000 units/ml DNase I (Sigma Chemical Co.), 10 mM MgCl2, and 5 mg/ml BSA. Staining was visualized using rhodamine-conjugated donkey anti-rat IgG (Jackson Immunoresearch Laboratories, Inc., West Grove, PA). Microinjected cells were detected by staining with a fluorescein isothiocyanate-conjugated horse anti-rabbit IgG (Cappel Laboratories, Cochranville, PA). Expression of c-fos protein was detected by staining with a polyclonal antibody to human c-fos (Oncogene Science, Inc., Manhasset, NY) followed by sequential use of biotinylated horse anti-rabbit IgG (Vector Laboratories, Burlingame, CA) and streptavidin-conjugated Texas red (Amersham Corp.). The anti-fos antibody is specific for c-fos and other fos family members.

In experiments measuring transcriptional activity from microinjected reporter plasmids, nuclei of IMR-90 cells were injected with mixtures of carrier IgG and the appropriate expression vector either in the absence or presence of T-24 ras protein. To test for the presence of AP-1 (fos dependent) transcriptional activity, the 4XCRE/lacZ construct (Meinkoth et al., 1990) was used. As a control, the 5XCRE/lacZ expression vector was microinjected parallel each 6 h after injection, cells were fixed and stained overnight with the chromogenic substrate 5-bromo-4-chloro-3-indolyl-β-D-galactoside (Xgal) to detect β-galactosidase expression. To ensure the activity of the vector, duplicate injections of the 5XCRE/lacZ plasmid were performed, and the cells were treated with 1 mM 8-bromo-cAMP and 1 mM isobutylmethylxanthine for 3 h before staining. In all cases, injected cells were detected by staining with fluorescein isothiocyanate-conjugated rabbit anti-mouse IgG (Cappel Laboratories). All injection experiments described were performed a minimum of three separate times. Cells were viewed and results analyzed on an Axioskop epifluorescence microscope (Carl Zeiss) equipped with fluorescein and rhodamine fluorescence filters. Photomicrographs were taken on Technical Pan 100 film (phase contrast) or T-Max 400 film for fluorescence (Eastman Kodak Co., Rochester, NY).

Results

ras-Induced Morphological Changes

Microinjection of T-24 ras protein into senescent fibroblasts resulted in membrane-associated morphological changes within a short period of time, which became obvious with immunohistochemical staining within 2-4 h (Fig. 1). Increased membrane ruffling, closely associated with pinocytosis, has been shown previously to be an integral part of the ras-induced proliferative process (Bar-Sagi and Feramisco, 1986). Similar cell surface ruffling and fluid-phase pinocytosis, although less distinct and sustained, occur before the mitogenic effect of serum or purified growth factors upon cells maintained in serum-free medium (Myrdal and Auersberg, 1986). The majority of ras protein-injected cells exhibited grossly observable cytoplasmatic vacuolation. Senescent cells which were injected with carrier IgG alone for comparison purposes did not exhibit observable effects from the microinjection process (Fig. 1). These cell surface changes seen in senescent fibroblasts injected with T-24 ras protein provided evidence that at least some of the early

Staining and Fluorescence Microscopy

To examine morphological effects of oncogenic ras protein microinjection, early passage and senescent IMR-90 cells were injected with either murine IgG alone or with IgG and T-24 ras. After 3 h of incubation at 37°C, the cells were fixed for 30 min in 95% ethanol, 5% acetic acid and then stained to visualize the distribution of coinjected IgG. Microinjected murine IgG was detected using HRP-conjugated rabbit anti-mouse IgG (Amersham Corp., Arlington Heights, IL) followed by 3,3′-diaminobenzidine tetrahydrochloride as substrate. Coverslips were permanently mounted on glass microscope slides for analysis.

To quantitate DNA synthesis in injected cells, a solution of 5-bromo-2′-deoxyuridine (BrdU) (Amersham Corp., cell proliferation kit) was added to the culture medium for 24 h after microinjection. After labeling, coverslips were washed in PBS, and the cells were then fixed in 3.7% formaldehyde in PBS and incubated with a monoclonal rat antibody against BrdU (Accurate Chemical and Scientific Corp., Westbury, NY). The antibody was diluted in a solution of 0.5% NP-40 in PBS containing 1,000 units/ml of DNase I (Sigma Chemical Co.), 10 mM MgCl2, and 5 mg/ml BSA. Staining was visualized using rhodamine-conjugated donkey anti-rat IgG (Jackson Immunoresearch Laboratories, Inc., West Grove, PA). Expression of c-fos protein was detected by staining with a polyclonal antibody to human c-fos (Oncogene Science, Inc., Manhasset, NY) followed by sequential use of biotinylated horse anti-rabbit IgG (Vector Laboratories, Burlingame, CA) and streptavidin-conjugated Texas red (Amersham Corp.). The anti-fos antibody is specific for c-fos and other fos family members.

1. Abbreviations used in this paper: BrdU, 5-bromo-2′-deoxyuridine; Xgal, 5-bromo-4-chloro-3-indolyl-β-D-galactoside.
jected cells were processed for the incorporation of the thymidine analog BrdU to identify cells which entered S phase of the cell cycle. The results demonstrated (Table I) that the majority of the serum-starved early passage fibroblasts synthesized DNA after ras injection whereas only a rare cell in senescent populations was found to incorporate BrdU. Unexpectedly, injected ras protein induced a strong positive signal for c-fos in the majority of nuclei in both presenescent and senescent fibroblasts (Fig. 2). Because the majority of ras-injected senescent cells expressed c-fos protein while few cells synthesized DNA under the same conditions, it appears clear that expression of c-fos in these cells was not sufficient to stimulate DNA synthesis.

Table I. c-fos Expression and DNA Synthesis as a Result of Oncogenic H-ras Microinjection in IMR-90 Cells

| Protein injected | c-fos expression | BrdU incorporation |
|------------------|------------------|--------------------|
|                  | Presenescent     | Senescent          | Presenescent | Senescent |
| T-24 ras         | 71 (92/129)*     | 62 (95/154)        | 77 (85/111) | 5 (7/127) |
| IgG alone        | 10* (14/136)     | 10 (12/117)        | 3 (4/122)   | 2 (2/99)  |

* Numbers in parenthesis indicate total numbers of cells scored positive for c-fos expression or DNA synthesis relative to total number of cells injected.
† Microinjection of control protein resulted in weak nuclear c-fos staining in a small percentage of cells. This staining was not as intense as that seen when ras protein was injected.

components of the proliferative process were intact, and that the block in proliferation was therefore either independent of these phenotypic changes or downstream of them. In addition, the morphological changes demonstrated the biological activity of the ras protein in this cell line. This observation raised the question of whether ras-mediated induction of c-fos expression, observed in quiescent presenescent fibroblasts, also occurs in senescent cells.

ras-Induced fos Protein Expression

To evaluate the expression of c-fos after the injection of oncogenic ras protein, senescent fibroblasts were injected with ras protein and subsequently were stained simultaneously for the presence of coinjected carrier IgG and for the presence of c-fos protein in the nucleus of injected cells. The use of two different fluorescent probes allowed simultaneous detection of injected cells and c-fos expression in those individual cells (Fig. 2). In addition, a parallel set of microinjected cells were processed for the incorporation of the

Figure 1. Morphological changes occur in senescent human fibroblasts as a result of oncogenic H-ras microinjection. Subconfluent IMR-90 cells were cultured on glass coverslips and microinjected with either T-24 ras protein (b) (containing carrier IgG for detection purposes) or with IgG alone (a). 3 h after injection, the cells were fixed and stained to visualize the distribution of injected IgG. Phase contrast, scale bar, 25 μm.
Figure 2. Expression of c-fos in ras-injected senescent fibroblasts in the absence of DNA synthesis. Microinjection was carried out as in Fig. 1. Shown are phase contrast (a–e) and the corresponding fluorescence photomicrographs which demonstrate staining for microinjected protein (f–j), incorporation of BrdU (k, l) or expression of c-fos (m–o). Microinjection of ras induces DNA synthesis in presenescent fibroblasts (a, f, k) but not in senescent fibroblasts (b, g, l). Microinjection of ras results in c-fos expression regardless of whether the cells are presenescent (c, h, m) or senescent (d, i, n). Senescent cells do not express c-fos in response to carrier IgG injection (e, j, o). Scale bar, 25 μm.
contrast, neither type of cell expressed the marker enzyme when the CRE-lacZ reporter construct was coinjected with ras protein. The activity of the CRE-lacZ plasmid was confirmed by duplicate microinjections followed by treatment of the cells with 8-bromo-cAMP and isobutylmethylxanthine (Table II). These results suggested that while senescent cells injected with the ras protein show enhanced AP-1 dependent gene expression, not all types of transcription factors (i.e., cAMP response element binding protein) are thus activated. The results document the presence in senescent cells of active AP-1 transcriptional activity specific for a TRE-containing regulatory element in response to oncopgenic ras protein microinjection. Thus, while this activity has recently been shown to be decreased in mitogen-stimulated human fibroblasts as the cells age (Riabowol et al., 1992), injection of ras protein appears to generate a sufficient amount of active AP-1 to give rise to reporter activity.

**Discussion**

Evidence for a causative role of c-fos in the senescent phenotype has been, to date, the observation of a deficit of its expression in senescent cells. Considering the potential importance of this possibility, we sought a direct experimental approach to test the role of c-fos by attempting to induce the expression of the protein in an intact cellular context. Using an assay which permits examination of effects upon individual cells, we have investigated the role of c-fos in senescent cell growth control. Expression of the c-fos proto-oncogene is an essential prerequisite for the initiation of DNA synthesis in quiescent fibroblasts stimulated with serum. Senescent human fibroblasts do not express c-fos, however, in response to serum treatment (Sheshadri and Campisi, 1990), despite the observation that these cells appear to express and regulate a number of other genes in a manner typical of fibroblasts. If the observed repression of c-fos expression were the primary defect in senescent cells, one would anticipate that its expression would restore the proliferative potential of the cells, particularly in the presence of a strong replicative signal such as oncopgenic ras. The present results, expression of c-fos protein in the absence of DNA synthesis, appear to indicate that this is not the case. Recent results obtained by others (Surmacz et al., submitted for publication) lead to a similar conclusion. Moreover, the present results appear to indicate that while senescent cells are capable of enhanced expression of indicator constructs regulated by AP-1 promoter sequences when they are coinjected with oncopgenic ras protein, this increased activity, presumably a downstream effect of increased c-fos expression (Schonthal et al., 1988) is not sufficient to alleviate the block in the cell cycle of senescent cells. Thus, c-fos expression is likely to be necessary but not sufficient for the proliferation of these cells (Surmacz et al., submitted for publication).

These results support the view that senescent cells do not fail to proliferate because of an inherent inability to transduce these early stimulatory signals for growth. Senescent cells possess apparently normal growth factor receptors (Goldstein and Shmookler Reis, 1985). The generation of transcriptionally active AP-1 following injection of oncopgenic ras protein into senescent cells indicates that at least some of the intracellular signaling pathways are operative. It is still possible that the arrest to growth in senescent cells is due to the selective repression of one or several normal responses to signals for growth. Microinjection of oncopgenic ras protein apparently provides a positive signal of sufficient intensity to overcome the repression of c-fos. This result is of interest in and of itself in view of the general lack of induction of c-fos expression by serum. One possible explanation for this is that injected ras oncogene proteins, which cause an exaggerated ruffling response in comparison to the response seen for serum treatment, overcome a putative block in the normal signaling process associated with the growth factor receptors in the plasma membrane. As it appears that purified mitogens may also induce c-fos expression to some extent in senescent cells (Paulsson et al., 1986), this possibility seems plausible.

Another potential underlying mechanism in senescence involves the regulation of the retinoblastoma susceptibility gene product (Rb). The Rb protein is thought to function in a growth-inhibitory manner in normal cells. It is found in a hypophosphorylated state in quiescent cells, and it is thought that traversal of the G1/S cell cycle boundary, allowing DNA synthesis to occur, is dependent upon the hyperphosphorylation of the protein (Mihara et al., 1989). In senescent cells, this phosphorylation does not occur (Stein et al., 1990). Additionally, SV40 T antigen, which preferentially binds to and is thought to inactivate the hypophosphorylated form of the Rb protein, also induces DNA synthesis when introduced into senescent cells (Wright et al., 1989; Radna et al., 1989) and induces c-fos expression (Surmacz et al. submitted for publication). In the S' promoter region of the c-fos gene, a negative-acting Rb response element has recently been identified (Robbins et al., 1990). It is not currently known whether the phosphorylation state of the Rb protein has any effect upon its ability to influence the expression of the c-fos gene, or whether such a mechanism is operative during senescence. We are currently in the process of evaluating the regulation of the Rb protein in response to microinjection of oncopgenic ras protein. It is clear, however, from results presented here that if in fact the phosphorylation state of the Rb protein is a determining factor in the lack of progression from G1 to S phase in senescent cells, it appears not to be the mechanism responsible for repression of c-fos expression. Because microinjection of ras protein affects c-fos protein expression but not DNA synthesis, the two
events appear to be independently regulated. Events downstream or parallel to c-fos expression deserve particular attention in future efforts to explain the molecular nature of the senescent state.

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Figure 3: Microinjection of oncogenic H-ras results in functional AP-1 activity in presenescence and senescent human fibroblasts. Nuclei of presenescence (a, c, e) or senescent (b, d, f) IMR-90 cells were injected with a mixture of carrier IgG and 4X TRE/lacZ expression vector (Meinkoth et al., 1990) either in the absence (a-d) or presence (e, f) of added T-24 ras protein. 6 h later, all cells were fixed and stained with the chromogenic substrate Xgal to detect β galactosidase expression and also stained for injected protein. Fluorescence photomicrographs demonstrate which of the cells in c and d have been microinjected; the corresponding phase contrast pictures demonstrate the ras-dependence of β galactosidase expression (the lack of blue staining in c and d) and the presence of AP-1 transcriptional activity in cells which have been injected with ras protein, regardless of the age of the cells.

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