Advances in Brief

A Phosphatidylinositol 3-Kinase Inhibitor Induces a Senescent-like Growth Arrest in Human Diploid Fibroblasts[1]

Maria Tresini, Madhumalti Mawal-Dewan, Vincent J. Cristofalo, and Christian Sell[2]

Center for Gerontological Research, Department of Pathology and Laboratory Medicine, MCP-Hahnemann School of Medicine, Allegheny University, Philadelphia, Pennsylvania 19129

Abstract

The signal transduction cascade initiated by the activation of phosphoinositide 3-kinase (PI-3 kinase) is implicated in mitogenic and antiapoptotic signaling generated by growth factors in a variety of cell types. We have examined the consequences of an inhibition of this pathway in human diploid fibroblasts. We find that a specific PI-3 kinase inhibitor (LY294002) causes growth arrest in these cells accompanied by changes in gene expression that are similar to those seen during cellular senescence. A second inhibitor, PD58029, which is specific for the mitogen-activated protein kinase kinase 1 (MEK-1), also induces a growth arrest but does not induce the same spectrum of gene expression. The pattern of gene expression in the presence the MEK-1 inhibitor is similar to that seen during growth arrest induced by serum starvation. The specific phenotypic changes seen following inhibition of PI-3 kinase are: an increase in β-galactosidase activity; a decrease in *EPC-1* gene expression; and a dramatic increase in collagenase gene expression. Thus, growth arrest with a PI-3 kinase inhibitor induces a senescent-like phenotype that is not seen when cells are growth arrested by either serum starvation or a MEK-1 inhibitor.

Introduction

Normal human fibroblast cells in culture undergo a limited number of population doublings. The life span of these cells is characterized by an initial period of vigorous growth, followed by a decline in proliferative potential and the establishment of a viable, nondividing population that is defined as replicative senescent cells (1, 2). In contrast, tumor-derived cells show an infinite life span when placed in culture, and the process whereby tumor cells escape cellular senescence is thought to be an integral step in their progression toward malignancy (3). Recently, it has been reported that the introduction of an oncogenic *ras* gene can induce cellular senescence in HDFs,[2] in marked contrast to its effects in immortal cells, which result in transformation (4). These results indicate that a rapid senescence program can be triggered in response to specific cellular insults, and senescence may represent a protective program similar to apoptosis. In fact, a strong parallel has been drawn between the activation of cellular senescence and the induction of an apoptotic response (5).

Polypeptide growth factors, in addition to their growth-promoting activity, serve as survival factors and prevent induction of apoptosis in target cells (6). The mitogenic, survival, and transforming activities of at least one of these growth factors, IGF-1, can be dissociated, as

defined by mutational analysis of the IGF-1 receptor (7). The survival signal generated by IGF-1 is mediated by the activation of PI-3 kinase (8). Activation of PI-3 kinase results in the production of phosphoinositol 3,4,5-trisphosphate and subsequent activation of the serine/threonine protein kinase AKT/PKB through a recently identified intermediate kinase, PKD (9). It has been shown that AKT mediates the survival signal generated by PI-3 kinase (10).

Here we report on the effects of an inhibitor of the PI-3 kinase (LY294002) in HDFs. Growth arrest induced by this inhibitor causes alterations in gene expression reminiscent of cellular senescence. This is in contrast to a growth arrest induced by either serum starvation or by inhibition of MEK-1 by a specific inhibitor, PD98059.

Materials and Methods

Cell Culture. Human WI-38 cells were maintained in 10% fetal bovine serum, supplemented with MEM (Life Technologies, Inc.) as described previously (11). All cultures were used prior to population doubling 35. All cultures were seeded at $1 \times 10^4/cm^2$ unless otherwise noted. Kinase inhibitors Ly294002 (Alexis, Inc.), which is specific for PI-3 kinase (12), and PD98059 (Alexis, Inc.), which is specific for mitogen-activated protein kinase (13), were dissolved in ethanol and DMSO, respectively, at 50 mM. Wortmanin (CalBiochem, Inc.) was dissolved in DMSO at 20 mM. Stock solutions were diluted as appropriate for experiments shown, and an appropriate volume of solvent was added to parallel cultures as a control. Cells were seeded in normal growth medium. After 24 h, cultures were treated with kinase inhibitors in the presence of normal growth medium containing 10% fetal bovine serum or placed in serum-free medium for the times indicated in the individual experiments. In our hands, the addition of PD98059 at 50 μM is sufficient to block serum-stimulated Erk activity by >70%, and LY29004 has a similar effect on serum-stimulated PI 3-kinase activity (data not shown).

RNA Isolation and Northern Blot Analysis. Total RNA was isolated following the guanidinium isothiocyanate method of Chomczynski and Sacchi (14). Northern analysis was carried out using 10 μg of total RNA in glyoxal agarose gels. Size-fractionated RNA was transferred to Nytran filters electrophoretically in 1× TAE. Probes were labeled by random priming using [^{32}P]dCTP in the presence of random hexamers and the klenow fragment of *Escherichia coli* DNA polymerase I. Probes used were full-length cDNA fragments of EPC-1, collagenase, and β-actin. Hybridizations were carried out using a solution of 7% SDS, 0.25 M NaPO₄, 0.1% NaPPi, and 2 mM EDTA. Washing was carried out under standard conditions at 65°C in 0.2% SSC, and 0.1% SDS for 20 min.

β-Galactosidase Activity. Staining for β-galactosidase activity was performed as described by Dimri *et al.* (15). Coverslips were placed into PBS containing 0.2% glutaraldehyde and 2% formaldehyde for 5 min. After three washes with PBS, cells were stained for 24 h in staining solution (150 mM NaCl, 2 mM MgCl₂, 5 mM K₃Fe(CN)₆, 5 mM K₄Fe(CN)₆, 40 mM citric acid, and 12 mM sodium phosphate, pH 6.0) containing 1 mg/ml 5-bromo-4-chloro-3-indolyl β-D-galactoside. Cells were then washed with PBS. β-Galactosidase activity is monitored visually by scoring blue precipitate in the cytoplasm.

[³H]Thymidine Labeling. Cells were plated onto coverslips and treated 24 h later with inhibitors at appropriate concentrations. [³H]Thymidine (10 μCi/ml) was added at that time, and cultures were incubated for an additional 24 h. Coverslips were then washed with PBS, twice with cold methanol, and air dried. When dry, coverslips were coated with Kodak NTB2 emulsion,
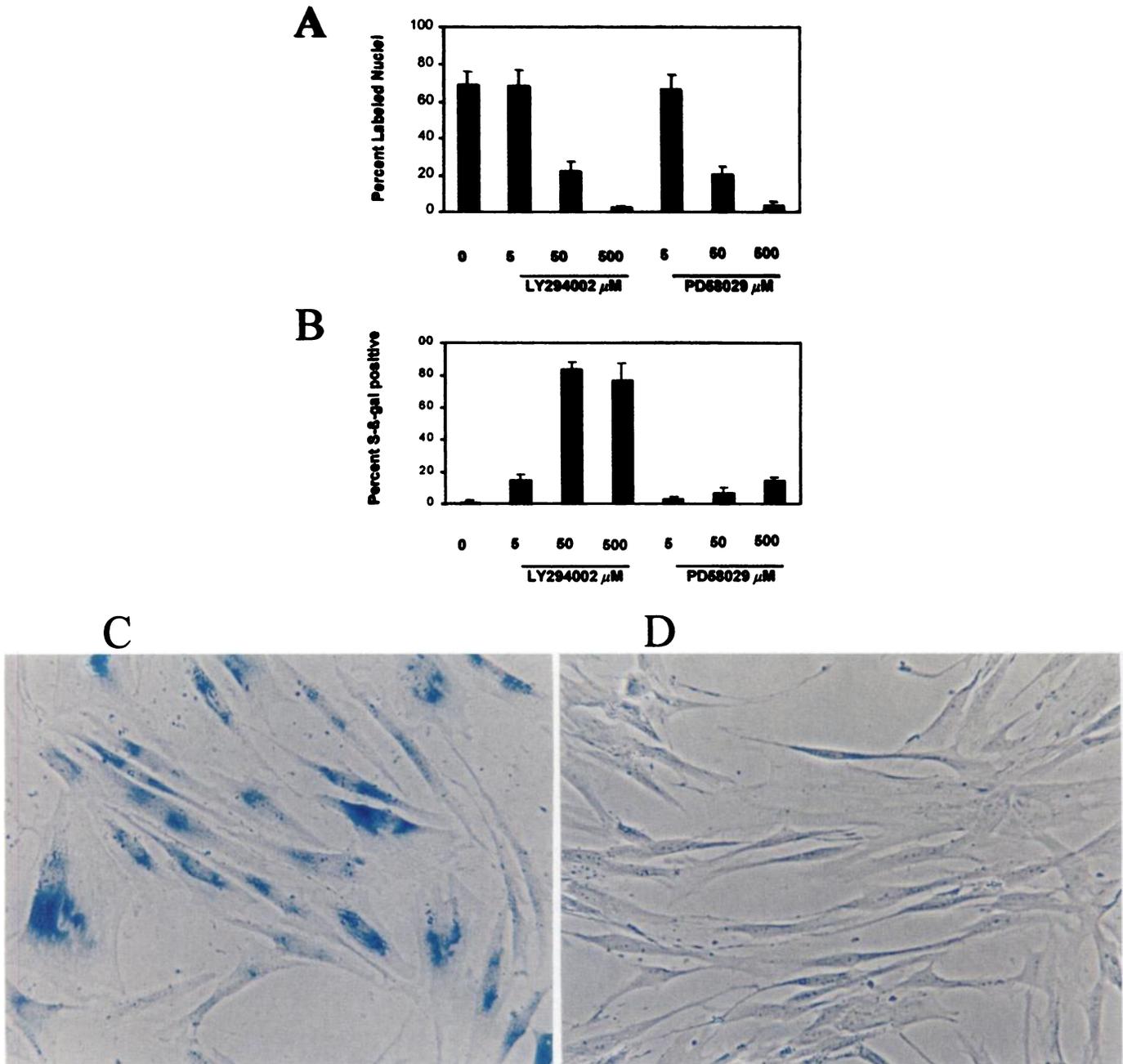
Fig. 1. Growth inhibition and induction f S-β-gal by LY294002 and PD58029. WI-38 cells were seeded at 1 × 10⁴/cm². After 24 h, cultures were placed in growth medium containing either LY294002 or PD58029 at the indicated concentrations. In A, the effect of these inhibitors on entry into S phase is shown. [³H]Thymidine (1 μCi/ml) was added at the same time as inhibitors. After a 24-h incubation, cultures were fixed and processed for autoradiography (see "Materials and Methods"). The results are presented as the percentage of cells in the culture that are positive for [³H]thymidine labeling, which represents the percentage of the population that has entered S phase during the labeling period. In B, the percentage of cells that stain positive for S-β-gal after 5 days of treatment with the indicated concentrations of inhibitors is shown. Cells were seeded as in A and allowed to incubate with or without inhibitors for 5 days. Cultures were then fixed and stained for S-β-gal, as described in "Materials and Methods." In C and D, photomicrographs of cultures treated with 50 μM LY294002 (C) or 50 μM PD58029 (D) are shown. Blue cells are those positive for S-β-gal.

exposed for 4 days at 40°C in complete darkness, and processed using Microdol X developer and Kodak rapid fix. Cells that have entered S phase and incorporated labeled thymidine can thus be visualized microscopically by the presence of silver grains superimposed upon the nucleus.

Results and Discussion

Early passage, proliferatively competent, WI-38 cells were growth arrested using two different methods. The first was the addition of an inhibitor of the PI 3-kinase enzyme (PI 3-K), LY294002, which is a specific inhibitor of PI 3-K at the concentrations used (12). The second method was treatment with an inhibitor specific for the mito-

gen-activated protein kinase kinase 1 (MEK-1) PD98059 (13). Both methods induce growth arrest measured by a decrease in the number of cells entering S phase, as seen in Fig. 1. Both kinase inhibitors induce growth arrest at 50 μM, which is within the range reported to specifically inhibit the respective enzymes in vivo (12, 13). Little cell death was observed at these concentrations; however, loss of viability was obvious at 10-fold higher concentrations. Concentrations of 50 μM were used for the remainder of these experiments. The aim of this study was to determine whether a blockade of the PI-3 kinase signal pathway would induce a growth arrest similar to that seen during cellular senescence. As a first estimation, cultures growth arrested in

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the manner described above were stained for pH 6, S-β-gal activity, which has been reported to be a marker for cellular senescence (15). As a control, WI-38 cells were serum deprived for 72 h to quiescence, a state not characterized by S-β-gal activity in WI-38 cells (Ref. 15; data not shown). Of the three growth-arrested states, only cultures treated with LY294002 contained a high percentage of cells positive for S-β-gal activity. The MEK-1 inhibitor PD58029 does not induce S-β-gal activity, even at 10-fold higher concentrations (500 μM; Fig. 1). Little or no staining is seen in serum-free medium (data not shown). A second inhibitor of the PI-3 kinase enzyme, wortminin, was also tested for the ability to induce S-β-gal activity in early passage WI-38 cells. Wortmanin, at 50 μM, also induced S-β-gal staining in 63% (SD, 7) of the population (data not shown).

To determine whether the addition of these kinase inhibitors irreversibly damages WI-38 cells, cultures treated for 5 days with either LY294002 or PD58029 (both at 50 μM) were trypsinized and reseeded at standard density. As seen in Fig. 2, WI-38 cells exposed to LY294002 or PD58029 proliferate as well as control cultures treated with serum-free medium for 5 days, indicating that the populations are fully viable and that the S-β-gal activity seen in the presence of LY294002 is not simply due to cell injury.

A number of changes in gene expression have been associated with cellular senescence (2). Among these is an increase in the expression of collagenase (2) and decreased expression of a gene known as early population doubling level cDNA 1 (*EPC-1*, also known as pigmented epithelial derived factor), which is a protein related to the serine protease inhibitor family (2). The *EPC-1* gene is a G$_0$-specific marker for human fibroblast cells, the expression of which is lost during senescence. We examined the expression of these two genes in WI-38 cultures treated with LY294002 or PD58029 compared to quiescent cultures in serum-free medium. The levels of *EPC-1* are dramatically reduced in WI-38 cultures treated with LY294002 compared to both serum-free conditions and PD58029 treatment (Fig. 3). In addition, the LY294002-treated cultures show an induction of the mRNA for collagenase relative to serum-free and PD58029 treatment. These results indicate that a blockade in the PI 3-kinase pathway specifically elicits a pattern of

Fig. 3. Analysis of gene expression in the presence of kinase inhibitors. WI-38 cells were seeded at standard density (1 × 10⁴/cm²) and treated, 24 h later, with LY294002, PD58029 (both at 50 μM), or serum-free medium. After a 5-day incubation, total RNA was harvested as described in "Materials and Methods." Steady-state mRNA levels of EPC-1, collagenase, and β-actin were examined by Northern blot analysis. *SF*, cultures treated with serum-free medium. *LY*, cultures treated with LY294002 at 50 μM. *PD*, cultures treated with PD58029 at 50 μM. The probes used are described in "Materials and Methods."

gene expression that is very similar to the pattern seen during cellular senescence.

Relatively few chemical treatments have been described that induce aspects of cellular senescence in HDF. Inhibitors of histone deacetylase (16), hydrogen peroxide (17), and ceramide (18) have recently been reported to induce some aspects of cellular senescence in early passage cells. Although some of the aspects of senescence are induced in each of these settings, none of the treatments described to date will induce the full senescent phenotype. This phenotype includes not only the typical G$_1$ growth arrest but also the altered pattern of gene expression typified by loss of *EPC-1* expression and increased expression of matrix-degrading enzymes such as collagenase. The temporal relationship between growth arrest and alterations such as loss of *EPC-1* expression is not yet clear, but one may be a prerequisite for the other. Among the changes that occur during senescence, one which may affect gene expression is alteration in the levels or activities of a number of transcription factors (19). It is possible that a perturbation of the PI-3 kinase pathway alters the levels of a specific subset of transcription factors to elicit a similar pattern of gene expression to that seen during senescence, for example the increased expression of collagenase.

The phenomenon of cellular senescence has been used as a model for organismic aging. The effects of alterations in PI 3-kinase associated here with cellular senescence may have a more global role in the intact organism and may play a role in longevity. For example, a homologue of the mammalian PI 3-kinase catalytic subunit is the gene responsible for the *age-1* mutant in *Caenorhabditis elegans* (20), which mediates longevity in this organism.

The identification of a specific biochemical pathway that controls certain aspects of cellular senescence provides a model to study the induction of cellular senescence and may give insight into the mechanisms controlling senescence. These mechanisms may have important implications in both cancer and aging.
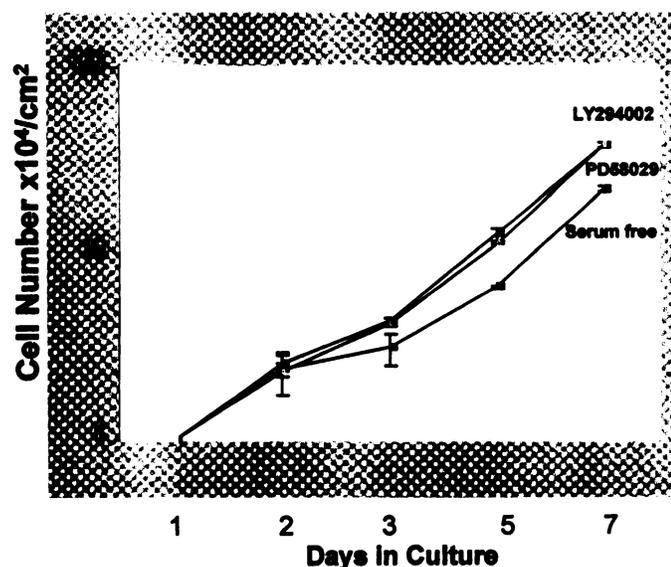

Fig. 2. Proliferation of WI-38 cultures released from kinase inhibitors. WI-3 cells were treated with either LY294002 (■), PD58029 (□), or serum-free medium (●) for a 5-day period. At this time, cultures were trypsinized and reseeded at 1 × 10⁴/cm² in growth medium containing 10% fetal bovine serum without inhibitors (day 1). Cell numbers were determined on subsequent days as indicated.
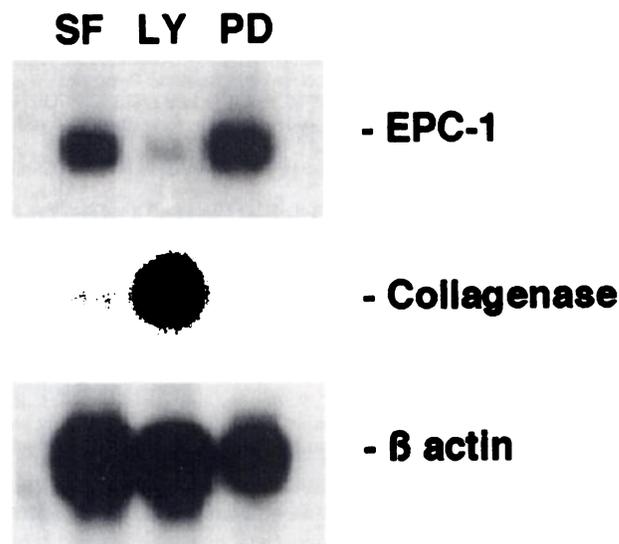
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