Ras Proteins Induce Senescence by Altering the Intracellular Levels of Reactive Oxygen Species*

Andrew C. Lee‡‡, Brett E. Fensterer‡, Hideki Ito‡, Kazuyo Takeda‡, Nancy S. Bae†, Tazuko Hirai‡, Zu-Xi Yu†, Victor J. Ferrans‡‡, Bruce H. Howard‡‡, and Toren Finkel‡‡

From the ‡Cardiology Branch and the §Pathology Section, NHLBI and the ¶Laboratory of Molecular Growth Regulation, NICHD, National Institutes of Health, Bethesda, Maryland 20892

**To whom correspondence should be addressed: Cardiology Branch, NHLBI, National Institutes of Health, 10 Center Dr., Bethesda, MD 20892-1650.
Tel.: 301-402-4081; Fax: 301-402-0888; E-mail: finkel@gwgate.nhlbi.nih.gov.

‡Supported by the Howard Hughes Medical Institute National Institutes of Health Research Scholars program.

*The abbreviations used are: ROS, reactive oxygen species; β-gal, β-galactosidase; DCF, dichlorodihydrofluorescein diacetate; FACS, fluorescence-activated cell sorter; MnTMPyP, Mn(111) tetrakis 1-methyl 4-pyridyl porphyrin pentachloride; NAC, N-acetylcysteine; SOD, superoxide dismutase; DHR123, dihydrorhodamine 123; IL, interleukin.

The Journal of Biological Chemistry Vol. 274, No. 12, Issue of March 19, pp. 7936–7940, 1999
Printed in U.S.A.

Human diploid fibroblasts eventually lose the capacity to replicate in culture and enter a viable but nonproliferative state of senescence. Recently, it has been demonstrated that retroviral-mediated gene transfer into primary fibroblasts of an activated ras gene (V12ras) rapidly accelerates development of the senescent phenotype. Using this in vitro system, we have sought to define the mediators of Ras-induced senescence. We demonstrate that expression of V12Ras results in an increase in intracellular and in particular, mitochondrial reactive oxygen species. The ability of V12Ras to induce growth arrest and senescence is shown to be partially inhibited by coexpression of an activated rac1 gene. A more dramatic rescue of V12Ras-expressing cells is demonstrated when the cells are placed in a low oxygen environment, a condition in which reactive oxygen species production is inhibited. In addition, in a 1% oxygen environment, Ras is unable to trigger an increase in the level of the cyclin-dependent kinase inhibitor p21 or to activate the senescent program. Under normoxic (20% O2) conditions, the V12Ras senescent phenotype is demonstrated to be unaffected by scavengers of superoxide but rescued by scavengers of hydrogen peroxide. These results suggest that in normal diploid cells, Ras proteins regulate oxidant production and that a rise in intracellular H2O2 represents a critical signal mediating replicative senescence.

Numerous studies suggest that ROS may also participate in aging (11–14). On a cellular level, replicative senescence is perhaps the most widely studied model of organismal aging. Consistent with a role for ROS in senescence, examination of cells in culture suggest that older cells have higher levels of ROS than younger ones (15). In addition, cells treated with antioxidants or grown in conditions of low oxygen appear to have a prolongation of life span (16, 17). Finally, treatment of primary fibroblasts with a sublethal concentration of H2O2 induces a state resembling senescence (18–20).

Recently, it has been demonstrated that a replicative senescence state could be rapidly triggered by expression of an activated ras gene (V12ras) in primary human fibroblasts (21). Oncogenic Ras expression in these experiments also resulted in an increase in the expression of the cyclin inhibitor proteins p21 and p16, which are linked to the senescent phenotype (22, 23). Although Ras proteins activate a number of distinct downstream pathways, some evidence suggests that these proteins also play a role in the regulation of the redox state of the cell (24, 25). Based on the previous evidence linking oxidants to aging, we have explored in this study the possibility that V12Ras expression induces rapid replicative senescence by altering the intracellular levels of ROS.

MATERIALS AND METHODS

Retroviral Infection—A V12Ras-expressing retrovirus was constructed in a bicistronic vector that also encoded the IL-2 receptor α-chain as a cell surface tag. Primary human diploid fibroblasts were infected with a retrovirus encoding both V12Ras and the surface tag or, as a control, with a retrovirus encoding the surface tag alone. Five days after infection, infected cells were magnetically immunoaffinity sorted using an IL-2 receptor antibody coupled to magnetic beads (26). Using this system, growth arrest was evident from day 6 onward. By day 15 following infection, cells had developed an enlarged and flattened morphology and demonstrated endogenous β-galactosidase activity (pH 6.0), which was assessed as described previously (27). Low passage cells (<35 population doublings) or late passage (>35 population doublings) primary human fibroblasts were employed. Western blot analysis for protein expression was performed using 10 μg of protein lysate and detected by enhanced chemiluminescence (7).

Antibodies to Ras proteins and the cyclin-dependent kinase inhibitor p21 were obtained from Santa Cruz Biotechnology. Adenoviral Infection—Adenoviruses encoding either an activated Rac1 (Ad.V12Rac1 (28)), a dominant negative Rac1 (Ad. N17Rac1 (28)), Cu,Zn superoxide dismutase (Ad.SOD (29)), or the histological marker gene lacZ (βGAL (30)) have been previously described. An additional adenovirus, Ad.d1312, which is E1-negative but lacks a transgene, was used as a control (31). All viruses were amplified in 293 cells and purified by double cesium gradients (30). Adenoviral infections were on day 6 following retroviral infection and were performed at a multiplicity of infection of 200. Detection by Western blot analysis of the Myc epitope-tagged form of V12Rac or N17Rac was as described previously (28).

ROS Measurements—Levels of cytosolic ROS were assessed by loading cells with 5 μM/ml of 2′,7′ dichlorodihydrofluorescein diacetate (DCF)
(Molecular Probes) for 5 min and then imaging with a Leica laser scanning confocal microscope (7). Alternatively, cells were loaded with DCF and analyzed by FACS (Epics Elite-ESP cytometer, Coulter). Direct visualization of mitochondrial ROS was achieved using dihydrorhodamine 123 (DHR123) as described previously (32), and the resulting fluorescence was imaged using confocal microscopy. To confirm the mitochondrial localization of DHR123, cells were also incubated with Mitotracker red (Molecular Probes). Quantification of DCF of DHR123 fluorescence was achieved by measuring fluorescent intensity of approximately 60 random cells from six different fields (7).

**RESULTS**

We first sought to ascertain whether in our primary cultures of human diploid fibroblasts, levels of ROS differed between young and old cells. Cells from mid- or late passage were loaded with the peroxide-sensitive fluorophore DCF and assayed by FACS analysis. As demonstrated in Fig. 1A, older cells had markedly increased levels of DCF fluorescence, consistent with higher levels of intracellular hydrogen peroxide. The relationship between intracellular levels of \( \text{H}_2\text{O}_2 \) and senescence was further extended by analyzing mid-passage cells by FACS analysis and sorting cells based on the level of DCF fluorescence. The senescent phenotype was assessed by subsequently analyzing the percentage of cells exhibiting \( \beta \)-galactosidase activity at a neutral pH. Cells exhibiting the highest DCF fluorescence (top 10% fluorescence) had a significantly higher percentage of \( \beta \)-galactosidase-positive cells (Fig. 1B). Nonsorted cells at this passage exhibited approximately 7% \( \beta \)-galactosidase-positive cells.

**Fig. 1.** Levels of intracellular \( \text{H}_2\text{O}_2 \) correlate with senescence. A, mid- or late passage fibroblasts were loaded with DCF and analyzed by FACS. B, mid-passage cells were sorted based on DCF fluorescence intensity, replated, fixed, and stained for \( \beta \)-gal. Percentage of \( \beta \)-gal-positive cells in the bright (top 10% DCF fluorescence) and dim (bottom 10% DCF fluorescence) groups are shown. Nonsorted cells at this passage exhibited approximately 7% \( \beta \)-gal-positive cells.

**Fig. 2.** V12Ras expression increases levels of ROS. Cells were infected with either a control retrovirus (A and C) or a retrovirus encoding V12Ras (B, D, and E). Shown are levels of DCF fluorescence in control cells (A) and in V12Ras-expressing cells (B) 7 days after retroviral infection and levels of mitochondrial oxidant production as assessed by DHR123 fluorescence in control cells (C) and in V12Ras-expressing cells (D). E, the cells shown in D were simultaneously incubated with Mitotracker red to confirm the mitochondrial localization of DHR123. F, quantification (mean \pm S.D.) of fluorescence intensity from approximately 60 random cells. Levels of fluorescence were quantitated on a gray scale (0–256), and values were normalized for each fluorophore to the levels observed in control cells. *, \( p < 0.05 \) compared with control cells.
percentage of senescent cells when compared with cells of the identical passage number but containing the lowest decile of DCF fluorescence (Fig. 1B).

We next infected low passage human diploid fibroblasts with a bicistronic retrovirus encoding V12Ras and the subunit of the IL-2 receptor. Infected cells were purified by magnetic immunoaffinity sorting using an antibody to the IL-2 receptor. Compared with cells infected with a control retrovirus expressing only the IL-2a receptor, fibroblasts expressing V12Ras had elevated levels of DCF fluorescence (Fig. 2, A and B). This rise in ROS was evident by 7 days after retroviral infection and therefore coincided with the time when cells underwent growth arrest but preceded by at least 1 week the development of senescence markers, such as endogenous β-galactosidase activity and an enlarged and flattened morphological phenotype (27, 33). In an attempt to identify the source of the Ras-induced rise in ROS levels, DHR123 fluorescence, consistent with the notion that the mitochondria are a source of the Ras-induced rise in ROS levels. To confirm the mitochondrial localization of DHR123, cells demonstrated recombinant protein expression (Fig. 3A).

We next asked what the effect of N17Rac1 or V12Rac1 expression was in the setting of concomitant V12Ras expression. Consistent with its known ability in primary human fibroblast, we superinfected retroviral-infected cells with recombinant adenovirus encoding either an activated (Ad.V12Rac1) or dominant negative (Ad. N17Rac1) rac1 gene product. Similar infection with an adenovirus encoding a marker gene (lacZ) revealed evidence of successful gene transfer in over 90% of V12Ras-expressing cells (data not shown). Western blot analysis from lysates of cells infected with adenoviruses encoding the epitope-tagged form of Rac1 readily demonstrated recombinant protein expression (Fig. 3A).

We next sought to better understand the importance of the rise in ROS levels in the development of the Ras-induced se-
nescent phenotype. We therefore cultured Ras-expressing cells in varying oxygen environments, an established method to regulate the production of intracellular ROS (36). As demonstrated previously (21), as well as in Fig. 4, V12Ras-expressing cells maintained in the standard tissue culture environment of 20% oxygen undergo near complete growth arrest. In contrast, lowering ambient oxygen to 3 or 1% enabled cells expressing V12Ras to continue to proliferate.

Previous studies (21) have demonstrated that expression of V12Ras in primary fibroblast results in an increase in the levels of the cyclin inhibitor p21, which in turn appears to be essential for the development of the senescent phenotype (22). As demonstrated in Fig. 5, although reducing the ambient oxygen concentration did not alter the expression of Ras proteins, it did, however, inhibit the ability of Ras to signal an increase in the level of p21. Consistent with these results, when the senescence-associated β-galactosidase (β-gal) activity was assessed 15 days after infection, as previously shown, V12Ras expression resulted in an approximate 5–7-fold increase in β-gal-positive cells in 20% oxygen, whereas only a 1–1.5-fold increase was observed when cells were maintained in a 1% oxygen environment (Fig. 6).

FIG. 6. Low oxygen rescues Ras-induced senescence. Percentage of β-galactosidase-positive cells 15 days after retroviral infection. Results are expressed relative to the percentage of β-gal-positive cells seen in the control-infected population maintained in 20% oxygen (absolute percentage ≤ 5%).

The mitochondrial redox state is maintained primarily by manganese superoxide dismutase (Mn-SOD) and glutathione, which allows for the coordinated dismutation of O2− and the subsequent elimination of hydrogen peroxide. To attempt to understand the specific contribution of each of these reactive oxygen species to Ras-induced senescence, we treated cells with NAC, which replenishes intracellular glutathione, and MnTMPyP, a member of a class of SOD mimetics that have been demonstrated to be capable of rescuing mice made homozygous deficient in Mn-SOD (37).

Treatment of cells expressing V12Ras with the SOD mimic MnTMPyP did not restore growth, and in fact, these cells exhibited toxicity in a concentration-dependent fashion (Fig. 7A). At low concentrations of MnTMPyP (0.1 μM), approximately 50% of V12Ras-expressing cells died, and the remaining cells were viable but remained growth-arrested.

In contrast to the effects of an SOD mimic, addition of the peroxide scavenger NAC to the medium rescued V12Ras-expressing cells from growth arrest (Fig. 7A). Surprisingly, although MnTMPyP was incapable by itself of restoring proliferation, the combination of an SOD mimic and a peroxide scavenger (NAC) proved slightly more effective than NAC alone (Fig. 7A). Similarly, assessment of the effects of antioxidants on the subsequent development of the senescent phenotype demonstrated that V12Ras-expressing cells that survived treatment with MnTMPyP had a higher percentage of β-gal-positive cells than untreated cells. In contrast, treatment with NAC or the combination of NAC and MnTMPyP substantially reduced the rate of senescence (Fig. 7B).

To further confirm the inability of superoxide scavengers to rescue cells, we coexpressed V12Ras with Cu, Zn-SOD using a recombinant adenovirus encoding superoxide dismutase. We have previously shown that this construct allows for a 3–5-fold increase in SOD activity in cells (29). As seen in Fig. 7C, consistent with the effects of MnTMPyP, Ad.SOD infection failed to rescue V12Ras-induced senescence.

DISCUSSION

Our results suggest that V12Ras expression leads to an increase in intracellular H2O2, the source of which appears to be the mitochondria. The ability of Ras proteins to increase ROS levels is essential for the Ras phenotype because V12Ras-expressing cells grown in the presence of either a peroxide-scavenging antioxidant or low oxygen are rescued from senescence. Take together, these results provide strong support for the concept that a rise in ROS is an important intracellular trigger for replicative senescence.

Expression of an activated ras gene has been previously demonstrated to result in growth arrest in several types of nontransformed cells (21, 38, 39). Recently, it has been demonstrated in Swiss 3T3 cells that although when expressed alone V12Ras leads to G1 arrest, the simultaneous coexpression of an activated rhoA GTPases leads to S-phase progression (40). These results are similar to what we observed with coexpression of V12Rac in Ras-induced growth arrest and senescence in normal fibroblasts. Nonetheless, in our case, V12Rac was only able to partially rescue the V12Ras phenotype. The basis for this decreased efficacy is not entirely clear, although it may...
Expression of V12Ras has been previously demonstrated to increase ROS levels in cells (24, 25), and this property has been linked to the ability of ras to act as a transforming gene (25). Initially, it would appear contradictory that V12Ras expression leads to transformation of NIH 3T3 cells and senescence of primary fibroblasts. Nonetheless, both states are thought to be a result of the accumulation of somatic mutations. Given the known ability of ROS to induce DNA damage, it is conceivable that in the context of a normal cell, Ras-induced increases in ROS levels may result in oxidative modifications of DNA and, as previously demonstrated (21), the subsequent up-regulation of p53 and p21. Continued high level oxidative stress may eventually result in senescence. In contrast, in immortalized NIH 3T3 cells, oxidatively modified DNA may not be appropriately sensed or repaired, and the cell may respond to DNA damage not through growth arrest but by transformation. In this regard, it is interesting to note that Ras does not induce growth arrest in primary fibroblasts devoid of p53 (21). In addition, the overexpression of p53 can by itself increase ROS levels in cells (24, 25), and this property has been linked to the ability of Ras to act as a transforming gene (25).

Our results with the SOD mimetic MnTMPyP and the per-oxide scavenger NAC suggest that H₂O₂ is the most important mediator of Ras-induced growth arrest. Treatment with an SOD mimetic alone raised H₂O₂ levels and resulted in the death of some V12Ras cells and an increased rate of senescence in the remaining viable cells. This is consistent with previous data suggesting that cells treated with high levels of exogenous H₂O₂ undergo cell death, whereas moderate, nonlethal concentrations can induce a senescent phenotype (19). Nonetheless, because the combination of MnTMPyP and NAC produced the most efficient rescue of V12Ras-expressing cells, it is conceivable that hydroxyl radicals, the formation of which requires both H₂O₂ and O₂⁻, may also participate.

Although our results suggest that Ras proteins may regulate the level of mitochondrial ROS, based on the subcellular location of Ras, it is most likely that such regulation is indirect. One potential mediator is ceramide, a lipid linked to the senescent phenotype (42, 43), that recently has been demonstrated to affect directly the mitochondrial electron transport chain, leading to an increase in the release of reactive oxygen species (44). This suggests that mitochondria may be direct targets of signalling molecules and that leakage of mitochondrial ROS may be more intricately regulated than has been previously appreciated. As described in this study, the use of a simple, rapid, genetic model should provide the framework to further dissect the relevant signalling molecules in senescence and potentially provide important insights and new therapeutic strategies to combat human aging.

Acknowledgments—We are grateful to Martha Kirby for help in FACS analysis, S. Gutkind for the V12Ras cDNA, R. Crystal for Ad.SOD, and Ilsa Rovira for aiding in preparation of the manuscript.

2 A. C. Lee and T. Finkel, unpublished observations.

REFERENCES

1. Finkel, T. (1998) Curr. Opin. Cell Biol. 10, 248–253
2. Meier, B., Tadele, H. H., Selle, S., Younes, M., Sies, H., Resch, K., and Habermehl, G. G. (1989) Biochem. J. 263, 539–545
3. Krieger-Brauer, H. I., and Kather, H. (1992) Carcinogenesis 13, 1049–1054
4. Greig, D. J., and Collmer, A. (1993) J. Biol. Chem. 268, 4501–4506
5. Kato, K., Miura, C., Oda, M., and Kato, M. (1994) J. Biol. Chem. 269, 11505–11510
6. Strasser, A., and Korsmeyer, S. J. (1993) Annu. Rev. Immunol. 11, 743–764

7. Sohal, R., and Weindruch, R. (1996) Science 273, 49–53
8. Martin, G., Austad, S., and Johnson, T. (1996) Nat. Genet. 13, 25–34
9. Beckman, K., and Ames, B. N. (1998) Free Radical Biol. Med. 24, 495–516
10. Hagen, T. M., and Tai, Y. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 13742–13747
11. Serrano, M., Yu, Z. X., Ferrans, V. J., Lowenstein, R., and Finkel, T. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 11848–11852
12. Bladier, C., Wolvetang, E., Hutchinson, P., deHaan, J., and Kola, I. (1997) Cell Growth Diff. 8, 589–598
13. Chen, Q. M., and Shibanuma, M., Kuroki, T., and Nose, K. (1994) Nature 367, 541–544
14. Chock, P. B., and Chock, J. C. (1996) Adv. Enzymol. 68, 147–160

15. Bae, Y. S., Kang, S. W., Seo, M. S., Baines, I. C., Tekle, E., Chock, P. B., and Rhee, S. G. (1997) J. Biol. Chem. 272, 217–221
16. Johnson, T., Yu, Z., Ferrans, V., Lowenstein, R., and Finkel, T. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 5948–5952
17. Bladier, C., Wolvetang, E., Hutchinson, P., deHaan, J., and Kola, I. (1997) Cell Growth Diff. 8, 589–598
18. Chen, Q. M. (1998) Proc. Natl. Acad. Sci. U. S. A. 93, 13742–13747
19. Serrano, M., Yu, Z. X., Ferrans, V. J., Sulciner, D. J., Gutkind, J. S., Kinzler, K. W., Goldschmidt-Clermont, P. J., and Finkel, T. (1996) Biochem. J. 318, 379–382
20. Imai, K., Xia, Y., Zweier, J. L., Sollott, S. J., Der, C. J., Fournier, E. R., Sundaresan, M., Finkel, T., and Goldschmidt-Clermont, P. J. (1997) Science 275, 1649–1652
21. Ogryzko, V. V., Wang, P., and Howard, B. H. (1997) Mol. Cell. Biol. 17, 4877–4882
22. Dimri, G., Lee, X. H., Basile, G., Acott, M., Scott, C., Roskelley, C., Meidan, E. E., Linskens, M., Rubelj, I., Pereira-Smith, O., Peacocke, M., and Campisi, J. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 9363–9367
23. Pracyk, J. B., Tanaka, K., Hegland, D. D., Kim, K. S., Sethi, R., Rovira, I. I., Blazina, D. R., Lee, L., Bruder, J. T., Kovess, E., Goldschmidt-Clermont, P. J., Imai, K., and Finkel, T. (1998) J. Clin. Invest. 102, 929–937
24. Crawford, L. E., Milliken, E. E., Imani, K., Zweier, J. L., Becker, L. C., Johnson, T. M., Elissa, N. T., Crystal, R. G. Finkel, T and Goldschmidt-Clermont, P. S. (1996) J. Biol. Chem. 271, 26863–26867
25. Guzman, R. J., Hirschowitz, E. A., Brody, S. L., Crystal, R. G., Epstein, S. E., and Finkel, T. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 10732–10736
26. Stein, G., and Dulic, V. (1995) BioEssays 17, 537–543
27. Stein, G., and Dulic, V. (1995) BioEssays 17, 537–543
28. Stein, G., and Dulic, V. (1995) BioEssays 17, 537–543
29. Stein, G., and Dulic, V. (1995) BioEssays 17, 537–543
29. Stein, G., and Dulic, V. (1995) BioEssays 17, 537–543
30. Stein, G., and Dulic, V. (1995) BioEssays 17, 537–543
31. Stein, G., and Dulic, V. (1995) BioEssays 17, 537–543
32. Stein, G., and Dulic, V. (1995) BioEssays 17, 537–543
