Nuclear Accumulation of Globular Actin as a Cellular Senescence Marker

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ABSTRACT

We evaluated the nuclear actin accumulation as a new marker of cellular senescence, using human diploid fibroblast (HDF), chondrocyte primary cultures, Mv1Lu epithelial cells, and Huh7 cancer cells. Nuclear accumulation of globular actin (G-actin) and dephosphorylated coflin was highly significant in the senescent HDF cells, accompanied with inhibition of LIM kinase (LIMK) -1 activity. When nuclear export of the actin was induced by 12-O-tetradecanoylphorbol-13-acetate, DNA synthesis of the senescent cells increased significantly, accompanied with changes of morphologic and biochemical profiles, such as increased RB protein phosphorylation and decreased expressions of p21VAF1, cytoplasmic p- extracellular signal-regulated kinase 1/2, and caveolins 1 and 2. Significance of these findings was strengthened additionally by the fact that nuclear actin export of young HDF cells was inhibited by the treatment with leptomycin B and mutant coflin transfection, whose LIMK-1 phosphorylation site was lost, and the old cell phenotypes were duplicated with nuclear actin accumulation, suggesting that nuclear actin accumulation was accompanied with G1 arrest during cellular senescence. The aforementioned changes were observed not only in the replicative senescence but also in the senescence induced by treatment of HDF cells, Mv1Lu, primary culture of human chondrocytes, or Huh7 cells with HRas virus infection, hydroxyurea, deferoxamine, or H2O2. Nuclear actin accumulation was much more sensitive and an earlier event than the well-known senescence-associated β-galactosidase activity.

INTRODUCTION

Proliferation of normal diploid cells is arrested irreversibly after a certain number of divisions, called replicative senescence, which is a paragon of the general process of cellular senescence (1–3). Once senescent, cells continue to function metabolically but will not respond to mitogens. Decreased growth rate, limited cell division, flat and large cell shapes, and tight binding of the cells to a culture dish are well-known characteristics of cells entered into senescence (4, 5). Replicative senescence also is thought to be a tumor-suppressive mechanism and an underlying cause of aging. There is much evidence to indicate that escape from senescence is important for malignant transformation. Citing a few, cellular senescence is thought to be a role in the nucleus.

In vivo screening revealed that a serine-3 mutant (S3A) of coflin was able to inhibit serum response factor reporter activation by LIMK and to block LIMK-induced formation of actin aggregates in the cytosol, indicating that the role of coflin in signal transduction to serum response factor response elements is solely to increase G-actin levels and large cell shapes, and tight binding of the cells to a culture dish are well-known characteristics of cells entered into senescence (4, 5). Replicative senescence also is thought to be a tumor-suppressive mechanism and an underlying cause of aging. There is much evidence to indicate that escape from senescence is important for malignant transformation. Citing a few, cellular senescence is thought to be a role in the nucleus.

In vivo

We reported previously that during the replicative senescence and H-ras mutant-induced senescence of human diploid fibroblast (HDF) cells, a large fraction of actin molecules were accumulated in the nucleus (40) and that significantly high levels of reactive oxygen species generated in the senescent HDF cells inactivated protein phosphatases 1 and 2A and mitogen-activated protein kinase phosphatase 3, accompanied by sequestration of p-extracellular signal-regulated kinase (p-Erk) in cytoplasm (41). These observations have been supported strongly by two recent reports that actin polymerization was essential for nuclear translocation of p-Erk, which occurred in a caveolae compartment of rat cardiomyocytes through the stretch-induced RhoA and Rac1 activation (42), and that senescent phenotype was reversed by reduction of caveolin expression (43). In this study, we attempted to elucidate the biological significance of nuclear accumulation of actin molecules in the replicative senes-

Received 6/24/03; revised 11/4/03; accepted 11/10/03.

Grant Support: Grant R11-2002-097-0100-2 to Aging & Apoptosis Research Center, Grant R05-2002-000-00054-0 to I. K. L. from Korea Science & Engineering Foundation, and a grant from Health Fellowship Foundation.

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cence and induced senescence of various cell types. We also aimed to test the applicability of nuclear actin accumulation as a valuable and robust marker for cellular senescence and the causal link between senescence and nuclear G-actin accumulation in various normal cells and H$_2$O$_2$-treated cancer cells. We observed that G-actin accumulation in the nucleus and perinucleus of the senescent cells was accompanied by nuclear accumulation of dephosphorylated cofilin. Immunoprecipitation of LIMK-1 and its in vitro cofilin phosphorylation activity were decreased markedly in the senescent cells compared with that in the young cells. However, 12-O-tetradecanoylphorbol-13-acetate (TPA) treatment could induce nuclear export of actin, cofilin phosphorylation concomitant with increased LIMK activity, and induction of DNA synthesis only in the old cells. Moreover, the aforementioned phenotypes almost could be reproduced in the induced senescence of various cell types, especially H$_2$O$_2$-induced senescence. Because nuclear actin accumulation was a more sensitive and earlier event than the appearance of senescence-associated β-galactosidase (SA-β-gal) activity, we suggest strongly nuclear actin accumulation as a new marker of cellular senescence.

MATERIALS AND METHODS

Cell Cultures and Induction of Cellular Senescence. HDF primary culture was prepared in our laboratory from the foreskin of a 4-year-old boy and maintained in DMEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum at 37°C in 5% CO$_2$ incubator (40). To induce premature senescence, different methods were applied to various cells: H-ras mutant virus infection (V$_{12}$C$_{40}$, V$_{12}$G$_{37}$, and V$_{12}$S$_{35}$) to HDF cells, treatments of HDF cells with H$_2$O$_2$ (0.2 $\times$ 10$^{-3}$ M) every 24 h, or Mv1Lu mink lung epithelial cells with hydroxyurea (1 $\times$ 10$^{-3}$ M). The cells were maintained in DMEM (low glucose) with 10% fetal bovine serum. Young, mid-old, and old cells used in the present experiments were based on their doubling time of ~24 h, 7–10 days, and 12–20 days, respectively. For replicative senescence of human chondrocytes, female knee cartilage was obtained from the operation room at Ajou University Hospital with informed consent, and its primary culture was split 1:5 when the cells became 75% confluent. Number of the passage was counted from 0 to 6. Cancer cell senescence was induced by daily treatment of Huh7 human hepatoma cells with H$_2$O$_2$ (0.2 $\times$ 10$^{-3}$ M or once with 0.5 $\times$ 10$^{-3}$ M deferoxamine (44, 45).

Preparation of Nuclear Matrix and Immunocytochemistry. Nuclear matrix was prepared using the method described by Clubb and Locke (46). Cells were cultured on glass coverslips, and soluble proteins were then extracted by incubation for 1.5 min with cytoskeleton (CSK) buffer (0.01 M HEPES (pH 6.8), 0.1 M NaCl, 0.3 M sucrose, 3 $\times$ 10$^{-3}$ M MgCl$_2$, 1 $\times$ 10$^{-3}$ M EGTA, and 0.5 $\times$ 10$^{-3}$ M phenylmethylsulfonyl fluoride) containing 0.5% Triton X-100. To remove chromatin, the aforementioned Triton X-100-treated cell residues were treated additionally with 100 units/ml DNase I in CSK buffer for 30 min and rinsed with 0.25 M (NH$_4$)$_2$SO$_4$ for 5 min. Coverslip was immersed in fresh CSK buffer, and an equal volume of 4 M NaCl was added gradually to remove the residual protein. The preparation was fixed subsequently in 4% paraformaldehyde in PBS (pH 7.4) for 20 min at room temperature, permeabilized in 0.5% Triton X-100 in PBS for 5 min, and incubated with 3% BSA. The aforementioned preparation was incubated with primary antibodies against antiactin (Sigma Chemical Co., St. Louis, MO) and anti-

Fig. 1. Nuclear accumulation of globular actin (G-actin) and cofilin during human diploid fibroblast (HDF) senescence. A, young and old cells up to 50% confluent were incubated with antiactin and anticofilin antibodies (1:200) at 4°C in a chamber overnight and treated additionally with FITC or Texas red conjugated secondary antibody and Hoechst 33258 (0.1 µg/ml) for 1 h. Actin and cofilin were accumulated in the nucleus of old cells; confocal microscope (×400 Fluoview; Olympus, Tokyo, Japan). B, distribution of fibrous actin (F-actin) in HDF cells was examined by immunofluorescence microscope (×400 Axiophot; Zeiss, Esslingen, Germany) before chromatin removal. Much more F-actin expression was found in the old cells than in the young cells over the cytoplasm. C, no evidence of F-actin accumulation in the nucleus of HDF cells, except for G-actin in the nuclear matrix. Nuclear matrix was prepared after removal of soluble proteins by 1.5 min of Triton X-100 and DNase I (100 units/ml) treatment. The cells were permeabilized to stain F-actin and G-actin in the nuclear matrix. Rhodamine-phalloidin was applied for 1 h at room temperature, and actin was visualized with FITC-conjugated secondary antibody.
cofilin (Cytoskeleton, Denver, CO; 1:200) overnight at 4°C in a humidified chamber, and then with secondary antibody conjugated with FITC, Cy3 (1:200), or Texas red (Jackson ImmunoResearch Laboratories, West Grove, PA) and Hoechst 33258 (0.1 μg/ml for 1 h at 4°C. For assessing F-actin dynamics, cultured cells were fixed in 4% paraformaldehyde in PBS for 20 min at room temperature and then permeabilized in PBS containing 0.1% Triton X-100 and 3% BSA. F-actin was stained with 5 units of rhodamine phalloidin in methanol/PBS (Molecular Probes, Eugene, OR) for 1 h at room temperature and then observed after mounting the coverslip.

Cell Synchronization, Fluorescence-Activated Cell Sorter, and IEF Analyses. To harvest S-phase-specific cells, young HDF cells were treated with 2 × 10−3 m thymidine for 24 h, followed by the second treatment for 16 h after an 8-h interval. The cells were harvested at 4 h and 6 h after incubation in the thymidine-free medium. To harvest mitotic cells, the cells were first synchronized twice with thymidine and then treated with nocodazole (100 ng/ml) for 16 h after 4 h of thymidine release. Mitotic cells were harvested by shake-off at 0 h and then 3 h after removal of nocodazole. To harvest G1-phase cells, young cells were incubated with serum-free medium for 24 h and then stimulated with 10% serum-containing medium for 7, 16, and 20 h. Each cell cycle was confirmed with 2 × 105 cells by fluorescence-activated cell sorter analysis after 70% ethanol fixation. Cofilin phosphorylation was measured by isoelectric focusing (IEF) with 5 × 10−5 m cells. The young and old HDF cells were washed with ice-cold PBS; the nuclei were broken by sonication (Sonic Dismembrator 550; Fisher Scientific, Hampton, NH) in IEF lysis buffer ([0.02 M Tris-HCl (pH 7.5), 2 × 10−3 m MgCl2, 0.07 M KCl, 1 × 10−3 m Na3VO4, 0.3 × 10−6 m okadaic acid, 2 μg/ml leupeptin, 1 × 10−6 m phosphomethylsulfonyl fluoride, and 0.5% Triton X-100]; and the suspension was centrifuged at 4400 × g for 10 min. IEF of the supernatant was performed with IEF gel (pH 3–10) according to the manufacturer’s instruction. Immunoblot analyses were performed with anticofilin antibody. IEF criterion gel (pH 3–10) and cathode buffer were obtained from Bio-Rad ( Hercules, CA).

[^3H]-Thymidine Incorporation Assay. Old HDF cells were plated, and the monolayer was stabilized for 48 h until 70% confluent, refed with a fresh medium, and incubated for another 24 h. HDF young cells also were plated on a six-well plate and incubated for 24 h until 70% confluent. TPA (50 ng/ml), and DMSO as a vehicle was added individually to the culture medium, and the cells were harvested at 4, 8, 20, and 32 h of incubation. Before harvest, the cells were treated with 2 μCi/ml[^3H]thymidine for 4 h; the harvested cells were lysed with 200 μl of 0.04 m Tris-HCl buffer (pH 8.0) containing 0.12 m NaCl and 0.5% NP-40 by vortex mixing at 4°C for 20 min; and the lysate was rinsed thoroughly with PBS. Radioactivity of the lysate (100 μl) was determined by liquid scintillation counter (Microbeta Counter 1450; Perkin Elmer, Wellesley, MA) using 1 ml scintillation mixture solution. Data presented are mean ± SD of quadruplicate wells from more than three experiments. Multiple linear regression analyses were applied for the data analyses obtained from[^3H]-thymidine incorporation assay by calculating B value in the equation composed of the affecting factors and using P = 0.05 as cutoff for significance.

LMB Treatment and Mutant Cofilin Transfection to HDF Cells. To inhibit nuclear export of cofilin, HDF young cells (1.3 × 105 cell/6 well) were treated with leptomycin B (LMB; 10 nm or 20 nm; Sigma Chemical Co.) for 24 h after 18 h of cell seeding, and the cells were treated additionally with TPA (50 ng/ml) for 32 h and then subjected to cell growth analysis and[^3H]-thymidine incorporation. To prove nuclear accumulation of actin caused by dephosphorylated cofilin, S3A cofilin (pKEX2-A3 Flag) was transfected to HDF young cells (1 × 105 cells/100 mm) using 20 μl of lipofectamine (Invitrogen). The control vector, pKEX2-Flag, was prepared by ligating the XbaI-XhoI sites of the pKEX2. Sense and antisense flag oligonucleotides, which were synthesized in our laboratory, were denatured (100 pmol each) at 100°C and annealed slowly at room temperature for 30 min before ligating into pKEX2-XR plasmid. After transfection with 4 μg of pKEX2-A3 Flag or the control vector, hygromycin (100 μg/ml)-resistant HDF cells were selected and used for additional experiments.

Preparation of Recombinant Cofilin Protein. Wild-type cofilin clone (6-His tagged) was cultured in Luria-broth medium with ampicillin (100 μg/ml; Sigma Chemical Co.) at 37°C until absorbance at 600 nm reached 0.6 and incubated additionally with 0.4 × 10−3 m isotopropyl β-D-thiogalactoside (Sigma Chemical Co.) overnight at 28°C. Bacterial pellet was centrifuged at 1500 × g for 15 min at 4°C and washed with extraction/wash buffer ([50 × 10−3 m sodium phosphate and 300 × 10−3 m NaCl (pH 7.0)]. Additional procedures were carried out basically according to the manufacturer’s recommendation (BD Biosciences, Palo Alto, CA). Hexa-histidine tagged cofilin protein was purified with TALON resin (50% slurry; BD Biosciences), and cofilin was eluted three times from the resin with 300 μl of elution buffer ([50 × 10−3 m sodium phosphate, 300 × 10−3 m NaCl, and 150 × 10−3 m imidazole (pH 7.0)].

Immunoprecipitation of LIMK-1 and Its Activity Assay. Cells were washed twice with ice-cold PBS and broken by sonication in LIMK assay buffer ([50 × 10−3 m HEPES (pH 7.4), 150 × 10−3 m NaCl, 1% NP-40, 5% glycerol, 1 × 10−3 m DTT, 1 × 10−3 m MgCl2, 1 × 10−3 M MnCl2, 10 × 10−3 m NaF, 1 × 10−3 m NaVO3, 1 × 10−3 m phenylmethylsulfonyl fluoride, and 2 μg/ml leupeptin]. Whole cell lysates (50 μg) were incubated with anti-LIMK-1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) for 8 h at 4°C. Using protein G-agarose (Invitrogen), LIMK-1 immune complex was harvested at 4°C for 4 h and then washed vigorously three times with 500 μl of kinase buffer. LIMK-1 assay was performed with 5 μg cofilin as a substrate, 50 × 10−6 m ATP, and 5 μCi of [γ-32P]ATP in 30 μl of kinase buffer at 30°C for 45 min. The reaction was terminated by boiling for 5 min, and 32P-labeled cofilin was analyzed by autoradiography in 15% (w/v) SDS-PAGE.

Evaluation of Senescence Marker Expressions. To monitor degree of cellular senescence, expressions of phosphorylated RB, p21WAF1, p-Erk1/2, and cavin-1 and cavin-2 in the old cells were examined by immunoblot analyses before and after TPA treatment. For quantitation, the number of cells with nuclear actin accumulated was counted under a confocal microscope; however, the cells in mitosis were excluded from the total counting, which included >1000 cells. Assay of SA-β-gal activity followed basically the published method (47). Cells were fixed with 3% formaldehyde for 5 min after washing with PBS and then were incubated overnight in freshly prepared staining solution [0.04 m citric acid/sodium phosphate (pH 6.0), 1 mg/ml of X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), 5 × 10−3 m potassium ferrocyanide, 5 × 10−3 m potassium ferricyanide, 0.15

![Fig. 2](http://example.com/fig2.png)
m NaCl and 2 × 10⁻³ m MgCl₂]. Stain was visible after 12 h of incubation at 37°C. By counting the number of the cells with blue color and the total cells per field (0.5 × 0.5 cm) under an inverted microscope, the percentage of the SA-β-gal-positive blue-stained cells was calculated. More than 1000 cells were counted from five fields and presented as mean ± SD.

RESULTS

Nuclear Accumulation of G-Actin and Cofilin during HDF Senescence. To investigate a mechanism of actin accumulation in the nucleus, subcellular localization of actin and cofilin was examined by immunocytochemistry, and markedly increased actin and cofilin expressions were found in the nucleus of the old, but not the young, cells (Fig. 1A). When treated with rhodamine-conjugated phalloidin for 1 h, F-actin was found spread over the cytoplasm; however, the F-actin was not concentrated in nuclei of young and old cells (Fig. 1B). To eliminate the possibilities that the phalloidin-binding sites were not accessible to the potential binding site in the nucleus of fixed cell and that long actin filaments in the nucleus might have been covered with a protein that obstructs the phalloidin-binding pocket, HDF cells were treated briefly with Triton X-100 and DNase I. This treatment revealed no actin accumulation in the nucleus (Fig. 1C), suggesting that the accumulated actin in the nucleus (Fig. 1A) would be G-actin.

When the nuclear matrix was extracted, nuclear actin was seen as a matrix scaffold, which is located inside nuclear membrane. Furthermore, IEF revealed dephosphorylated cofilin exclusively in the old HDF (Fig. 2A), whereas young HDF and NIH3T3 cells contained phosphorylated and dephosphorylated forms of cofilin. To elucidate possible dependency of changes of cofilin activity on the cell division cycle, IEF analysis of cofilin also was performed with HDF cells synchronized (Fig. 2B) by double thymidine block, nocodazole treatment, or serum deprivation and refeeding. When harvested by shake-off after nocodazole treatment, fluorescence-activated cell sorter analysis showed 100% mitotic cells (N0). On analyses of the synchronized cells by fluorescence-activated cell sorter and IEF, completely phosphorylated cofilin was found exclusively in the mitotic cells (Fig. 2C). These data could explain why the dephosphorylated cofilin was present in the old HDF cells, which were arrested in late G1 phase, potentially dissociating G-actin from F-actin.

Nuclear Export of G-Actin Significantly Increased DNA Synthesis of Senescent Cells. To investigate the consequence of nuclear export of G-actin, young and old HDF cells were treated with either EGF or TPA. As shown in Fig. 3, TPA induced export of actin from the nucleus to cytoplasm 8 h after the treatment, followed by morphologic changes of large and flat old cells to small and cylindrical young cell-like ones in 20 h. However, EGF failed to stimulate actin export from nuclei and to change the old cell morphology. To elucidate the effect of TPA-induced nuclear export of actin on G1 arrest of senescent cells, [³H]-thymidine incorporation assay was performed, and statistically significant induction of DNA synthesis was found only in the old cells in 20 h (Fig. 4B) as compared with no response.
cofilin is responsible for nuclear actin accumulation in the senescent young HDF cells.

To investigate whether the dephosphorylated form was accompanied by reversed expression of several other senescence markers, including RB phosphorylation (Fig. 4D), inhibition of p21WAF1 and p-Erk1/2 expressions (Fig. 4C), and reduced caveolin-1 and caveolin-2 expressions in 20 h of TPA treatment (Fig. 4D). RB protein in the senescent HDF was mostly dephosphorylated as compared with that in the young cells (Fig. 4A). These results are in accordance with previous findings that phosphorylated and underphosphorylated forms of RB proteins are found in young cells, whereas only under-phosphorylated RB protein is found in senescent cells (48) and in the long-term confluent cultures (49). These findings indicate strongly that TPA induced export of nuclear actin in old cells and changed not only the old morphologic phenotypes but also biochemical markers.

Biochemical Evidence of Reversal of Cellular Senescence. In addition to [3H]-thymidine incorporation, TPA-induced actin export also was accompanied by reversed expression of several other senescence markers, including RB phosphorylation (Fig. 6B), inhibition of p21WAF1 and p-Erk1/2 expressions (Fig. 6C), and reduced caveolin-1 and caveolin-2 expressions in 20 h of TPA treatment (Fig. 6D). RB protein in the senescent HDF was mostly dephosphorylated as compared with that in the young cells (Fig. 6A). These results are in accordance with previous findings that phosphorylated and underphosphorylated forms of RB proteins are found in young cells, whereas only under-phosphorylated RB protein is found in senescent cells (48) and in the long-term confluent cultures (49). These findings indicate strongly that TPA induced export of nuclear actin in old cells and changed not only the old morphologic phenotypes but also biochemical markers.

Regulation of LIMK-1 Activity in the Old and H2O2-treated Young HDF Cells. To investigate whether the dephosphorylated cofilin is responsible for nuclear actin accumulation in the senescent cells, S3A cofilin-transfected HDF cells were used for cofilin and actin immunocytochemistry. S3A cofilin transfection revealed nuclear accumulation of cofilin (Fig. 7A) and actin (Fig. 7B) compared with that in the vector-transfected cells. The regulation of the accompanying LIMK-1 activity during cellular senescence then was investigated by in vitro LIMK-1 assay with recombinant cofilin and LIMK-1 immunoprecipitate. As shown in Fig. 8A, the activity was lower

![Fig. 4. 12-O-tetradecanoylphorbol-13-acetate (TPA) induced DNA synthesis only in the old, but not in the young, human diploid fibroblast cells. A, young cells were treated with epidermal growth factor (EGF), TPA, or DMSO when the cell density reached 70% and were incubated with [3H]-thymidine (2 μCi/ml) for 4 h before harvest at the indicated times. B, mid-old (doubling time, 10 days) cells with 70% cell density were stabilized for 48 h before being subjected to [3H]-thymidine incorporation assay. Note the significantly increased DNA synthesis after 20 h of TPA treatment (*P = 0.024 versus TPA 8 h; *P < 0.01 versus all other samples). C, to evaluate whether cofilin phosphorylation occurred after TPA treatment, the mid-old cells were subjected to isoelectric focusing, and cofilin phosphorylation induced by TPA treatment was determined. D, to evaluate the role of protein kinase C (PKC), Go6976, inhibited significantly the growth rate (*P < 0.001 versus all other samples; **P < 0.005 versus all other samples).](cancers.aacrjournals.org)
Actin Accumulation in the Nucleus of Senescent Cancer Cells. To investigate whether the nuclear accumulation of actin molecule is the universal phenomenon applicable even to the cancer cells, HuH7 human hepatoma cells were treated with either deferoxamine (0.5 × 10^{-3} M) once or H_{2}O_{2} (0.2 × 10^{-3} M) for 3 days. Induction of senescence phenotype was monitored by SA-β-gal assay (Fig. 10A) and the actin immunocytochemistry with nuclear stain with Hoechst 33258 (Fig. 10B). Deferoxamine and H_{2}O_{2} treatments induced senescence phenotypes of HuH7 cells in morphology and expression of senescence markers such as SA-β-gal and actin accumulation. This finding suggests strongly that nuclear actin accumulation may be used as a sensitive and universal marker of senescence in normal and cancer cells.

Nuclear Actin Accumulation Is Highly Sensitive and an Earlier Event during Cellular Senescence. To evaluate the possibility of nuclear actin accumulation as a new marker of cellular senescence, various senescence models were examined, including premature senescence of HDF primary culture by H-ras double mutant virus infection (Fig. 9A), induced senescence of HDF cells by treatment with H_{2}O_{2} (0.2 × 10^{-3} M), induced senescence of Mv1Lu epithelial cells by treatment with hydroxyurea (1 × 10^{-3} M), and replicative senescence of human chondrocyte primary cultures (Fig. 9B) in addition to replicative senescence of HDF cells. The results obtained indicate strongly that nuclear actin accumulation is a universal phenomenon in cellular senescence. When compared with SA-β-gal activity, nuclear actin accumulation was much more sensitive and an earlier event. Nuclear actin accumulation was observed in ~40% of the cells in 2 days and in >50% in 4 days after H_{2}O_{2} treatment; however, there were no SA-β-gal-positive cells until 4 days under the same condition (Table 1), although morphology of the cells changed to large and flat at this time point.

Fig. 7. Nuclear accumulation of actin occurred in the serine-3 mutant (S3A) cofilin-transfected cells but not in the vector-transfected cells. Young human diploid fibroblast cells were (1 × 10^{5} cells/100 mm) transfected with 4 μg of S3A cofilin or vector plasmids and selected with hygromycin (100 μg/ml) treatment. Anticofilin (A) and antiaactin (B) antibodies were used for immunocytochemistry, and Hoechst 33258 was applied for nuclear staining. C, immunoblot analyses reveal pRb and p21^{WAF1} changes in the young (Y) HDF cells and H_{2}O_{2}-induced premature senescent cells (Y+H_{2}O_{2}). Note the decrease of hyperphosphorylated Rb and the increase of p21^{WAF1} proteins in the H_{2}O_{2}-treated cells.
DISCUSSION

It has been reported that actin contains two putative leucine-rich nuclear export signals (50); therefore, it is expected to be localized in cytoplasm as an important cytoskeleton protein. Thus, a question of why G-actin is accumulated in the nucleus during cellular senescence arises. In our study, because actin accumulated in the nucleus concomitantly with colocalization of dephosphorylated cofilin in the senescent cells (Fig. 1A), there is a strong possibility of generating actin monomers or short oligomers by the active cofilin. IEF analysis revealed that the old cells contained only dephosphorylated (active) cofilin as opposed to the mixture of phosphorylated and dephosphorylated forms in young HDF and NIH3T3 cells (Fig. 2A). Furthermore, maximum level of p-cofilin was found at G2/M phase (N0; Fig. 2C). However, once the cells entered into G1 phase, p-cofilin was dephosphorylated within 3 h after the release from the mitotic arrest (N3; Fig. 2C). This supports the previous report that LIMK phosphorylates cofilin (31) and that LIMK is most active during mitosis (51). To investigate whether the LIMK activity can be regulated in the replicative and induced senescent cells, LIMK-1 activity was measured in vitro in the young and old cells treated with either H2O2 or DFO.

Table 1 Comparison of nuclear actin accumulation and SA-β-galactosidase activity in the H2O2-induced senescent HDF cells

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<td>Nuclear actin accumulation</td>
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Human diploid fibroblast (HDF) cells were treated with H2O2 (0.2 × 10^-5 M) every 24 h and subjected to immunocytochemistry with anti-actin antibody and SA-β-galactosidase (SA-β-gal) assay. Both changes were compared at the indicate times with >1000 cells. Note the much earlier event of actin accumulation in the nucleus as compared with that in the SA-β-gal activity.

Actin accumulation in the nucleus of the Huh7 cancer cells treated with either deferoxamine (DFO) or H2O2. A, inverted microscopic findings show senescence-associated β-galactosidase (SA-β-gal) expression in the Huh7 hepatocellular carcinoma cells by treatment with either DFO once or H2O2 once a day for 3 days (∼100). SA-β-gal was positive in 3 days after DFO and H2O2 treatment. We have reported previously that DFO induced premature senescence of Huh7 cells in the culture system (44, 45). B, actin immunocytochemistry of Huh7 cells treated with either DFO once or H2O2 every day. Note the cells became large and flat with actin accumulation in the nucleus by DFO and H2O2 treatments as compared with the control. Lower, site of the nucleus in each cell.

Fig. 9. Nuclear accumulation of actin is a universal event in the replicative senescence and the variously induced senescent cells. A, premature senescence of human diploid fibroblast (HDF) cells was induced by H-ras double mutant (V12C40, V12G37, or V12S35) virus infection to HDF cells, and the virus-infected HDF cells revealed its doubling time over 3 days (V12C40) and 6 days (V12G37 and V12S35). Actin immunocytochemistry was performed with FITC-conjugated secondary antibody with Hoechst 33258 stain (×400). B, induced senescence was also reproduced by treating HDF primary culture with 0.2 × 10^-5 M H2O2 for 4 days (confocal ×900) and by treating Mv1Lu epithelial cells with hydroxyurea (1 × 10^-5 M) for 4 days (confocal ×1200). Chondrocyte primary cultures obtained from female knee were maintained by culture from passage 2 (control cell) to passage 6 (treated cell) and evaluated by rhodamine-conjugated actin immunocytochemistry (confocal ×1200). All of the flat cells revealed nuclear actin accumulation, regardless of the origin of cells and the methods of senescence induction used.

Fig. 10. Actin accumulation in the nucleus of the Huh7 cancer cells treated with either deferoxamine (DFO) or H2O2. A, inverted microscopic findings show senescence-associated β-galactosidase (SA-β-gal) expression in the Huh7 hepatocellular carcinoma cells by treatment with either DFO once or H2O2 once a day for 3 days (∼100). SA-β-gal was positive in 3 days after DFO and H2O2 treatment. We have reported previously that DFO induced premature senescence of Huh7 cells in the culture system (44, 45). B, actin immunocytochemistry of Huh7 cells treated with either DFO once or H2O2 every day. Note the cells became large and flat with actin accumulation in the nucleus by DFO and H2O2 treatments as compared with the control. Lower, site of the nucleus in each cell.
TPA (Fig. 8), and the activity was found to be regulated by the treatment in the replicative and induced senescence, supporting the potential role of dephosphorylated cofilin for actin accumulation in nuclei of the senescent cells. Furthermore, the assay of S3A mutant cofilin transfected into young HDF cells (Fig. 7) supported the cooperative function of cofilin phosphorylation by LIMK-1 and G-actin accumulation during cellular senescence. Because senescent cells are arrested at G1 phase, it is evident that the dephosphorylated cofilin may have severed G-actin from F-actin. Cofilin and gelsolin, one of the actin-solubilizing factors, were accumulated in the nucleus of old HDF (52). Contemporaneous accumulation of active cofilin and gelsolin in the nucleus also could explain continuous severing of actin and accumulation of G-actin during cellular senescence.

A few questions then arise as to whether nuclear export of actin could reverse the senescence phenomenon and what signal could induce actin export and stimulate the cell division cycle of the senescent cells. It has been shown recently that, when activated by TPA, PKC in vascular smooth muscle cell line A7r5 disassembles actin stress fiber to membranous ruffles through Src- and Rho-dependent pathways (53), suggesting strongly that the PKC isozymes are involved in the nuclear actin accumulation during the senescence process. In addition, phospholipase D and diacylglycerol pathways are defective in senescent WI38 human fetal lung fibroblast (54), indicating significantly reduced PKC activity in the senescent cells. Furthermore, stimulation of PKC-α translocation by serum is possible only in the young cells but not in the old cells; however, exogenous PKC activator, TPA, strongly induces PKC-α translocation in the young and old cells. Conversely, depletion of G-actin pool could stimulate serum response factor activity in neuronal cell lines (55). Therefore, our observations that G-actin accumulated in the nucleus of senescent cells and that TPA induced G-actin export in the old cells (Fig. 3) accompanied by the induction of DNA synthesis (Fig. 4B) imply strongly that the G1 arrest during cellular senescence may be caused by defect of the PKC pathway. Involvement of PKC was confirmed additionally by the use of Go6976, a PKC inhibitor, which inhibited TPA-induced DNA synthesis in the old cells (Fig. 4D), indicating a significant role of PKC in nuclear actin export and the reversal of senescence phenotypes. The failure of PKC activation by EGF may be explained by recent reports that up-regulation of cavelolin and down-regulation of amphiphysin-I attenuate EGF signaling in the senescent HDF cells through the receptor-mediated endocytosis (56–58). These findings are strengthened additionally by the recent observation that reduction of cavelolin expression reverses the senescent phenotype in HDF cells (43). However, we are unable to explain the failure of PKC activation by EGF even in the young cells. Detailed signal transduction mechanism of actin nuclear export through a specific PKC isozyme remains to be explored further.

The earlier report that RB protein phosphorylation occurred 10–20 h after serum stimulation, accompanied by induction of DNA synthesis, is in good accord with our present observation that TPA started to reverse senescent cell morphology to young cell-like phenotypes in 8 h and that the reversal became evident in 20 h (Fig. 3; Ref. 48). TPA treatment also reversed the well-known biochemical markers of cellular senescence in 20 h, including RB protein phosphorylation, changing cellular morphology, and accumulation of G-actin during cellular senescence. Because senescent cells are arrested at G1 phase, it is evident that the dephosphorylated cofilin may have severed G-actin from F-actin. Cofilin and gelsolin, one of the actin-solubilizing factors, were accumulated in the nucleus of old HDF (52). Contemporaneous accumulation of active cofilin and gelsolin in the nucleus also could explain continuous severing of actin and accumulation of G-actin during cellular senescence.

Finally, we examined the feasibility of nuclear accumulation of actin as a senescence marker, not only in the replicative but also in the induced senescence, by using H2O2-treated HDF cells, hydroxyurea-induced senescence of Mv1Lu, H-ras virus infection of HDF, culture passage-induced senescence of human chondrocyte primary cultures (Fig. 9), and Huh7 cancer cell senescence by treatment with either deferoxamine or H2O2 (Fig. 10). To our delight, actin accumulation in the nucleus was found to be a much earlier and universal event for the various cellular senescence than for SA-β-gal activity (Table 1). Nuclear actin accumulation was highly sensitive to H2O2 exposure; ~40% of the cells exposed to H2O2 for 2 days revealed the typical nuclear actin accumulation, whereas it took 10 days for SA-β-gal expression. Therefore, we suggest strongly the nuclear actin accumulation as a sensitive and new marker for cellular senescence.

ACKNOWLEDGMENTS

We thank Professors Woon Ki Paik at Hanyang University and Ronald A. DePinho at Harvard Medical School for their encouragement and careful discussion. pKEX2-A3 Flag was donated by Prof. Dr. Yvonne Samstag at Ruprecht-Karls-University, Heidelberg, Germany. 6-His tagged wild-type mouse cofilin clone was donated by Dr. Kensaku Mizuno at Tohoku University, Japan, and V5_S35, V5G34, and V5C40 in pWZL constructs were from Dr. Scott W. Lowe at Cold Spring Harbor Laboratory.

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