Up-regulation of Caveolin Attenuates Epidermal Growth Factor Signaling in Senescent Cells*

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Senescent human diploid fibroblasts do not respond to growth factors like epidermal growth factor (EGF), although they have a normal level of receptors and downstream signaling molecules. To examine the mechanism of signaling attenuation, we investigated Erk activation after EGF stimulation in senescent cells. Senescent cells did not phosphorylate Erk-1/2 after EGF stimulation, whereas young cells did. In those senescent cells, we found an increased level of caveolin proteins and strong interactions between caveolin-1 and EGF receptor. Electron microscopic analysis demonstrated an increased number of caveola structures in senescent cells. More interestingly, brain, spleen, and lung from 26-month-old rats showed substantial increases of caveolin proteins. However, in the case of p53-induced senescence, caveolin-1 was not induced, and EGF stimulation phosphorylated Erk-1/2 as much as young control cells. Finally, we overexpressed caveolin-1 in young human diploid fibroblasts in which the activation of Erk-1/2 upon EGF stimulation was significantly suppressed. These results suggest that the unresponsiveness of senescent fibroblasts to EGF stimulation may be due to the overexpression of caveolins, which seems to be independent of growth arrest and other aging phenotypes.

Normal human diploid cells show cellular senescence in vitro after a finite number of population doublings (1). Although senescent cells can maintain their metabolic activity, the loss of proliferation capacity may be due to a diminished response to growth factors (2–4). In aged human diploid fibroblasts, the decreased response to growth factors is suggested to be associated with a repression of c-fos expression, a reduced AP-1 DNA binding, and diminished DNA synthesis. Senescent cells in culture or tissues have been reported to have normal numbers of EGF receptors (EGFRs) (5, 6), and the binding capacity of EGF to EGFR is normal (7). However, there is no mitogen-activated protein kinase activation upon EGF stimulation in senescent cells, and the mechanism for the age-related modulation of EGF stimulation in response to the EGF stimulation is not yet fully resolved.

Caveolae are vesicular invaginations of the plasma membrane with a diameter of 50–100 nm (8, 9) and regulate signal transduction, potocytosis, and transcytosis (10, 11). Caveolin, a 21–24-kDa integral membrane protein, is a principal structural component of caveolae membranes in vivo. The stable expression of the caveolin-1 or -3 gene to the mammalian cells without caveolin induced the formation of caveolae structures (12). Caveolin functions as a scaffolding protein within the caveole membrane and interacts with signaling proteins, namely EGFR, G-proteins, Src-like kinases, Ha-Ras, protein kinase C, endothelial nitric-oxide synthase, integrin and so on (13–18). A short cytosolic N-terminal region of caveolin is involved in the formation of oligomers and mediates the interaction with these signal molecules, which results in the inactivation of signaling (11). The suppression of these signaling molecules can be circumvented by a tumor, wherein caveolins are generally downregulated. Targeted down-regulation of caveolin-1 is sufficient to drive cell transformation and hyperactivates the Erk kinase cascade (19). Co-expression with caveolin-1 dramatically inhibits signaling from EGFR, Raf, MEK-1, and Erk-2 to the nucleus in vivo (10).

In order to find an explanation for the attenuated responsiveness to EGF stimulation in senescent cells, we examined the signaling pathways of Erk activation after EGF stimulation. Interestingly, these differences in signaling behavior were consistent with the expression of caveolins in aged cells. Moreover, p53-mediated senescent cells respond differently to EGF stimulation, although they showed the same senescence-associated β-galactosidase activity and delayed doubling time. EJ-p53 cells did not express caveolin-1 proteins and did not show the attenuated EGF response despite the phenotypic cellular senescent changes. To know the direct effect of caveolin in HDFs, we overexpressed caveolin-1 in young HDFs. The phosphorylation of Erk-1/2 upon EGF stimulation was significantly blocked by caveolin-1 expression in young HDFs. Our results suggest that the expression of caveolin and the resulting formation of caveolae might be responsible for the attenuation of EGF signaling in senescent cells.

EXPERIMENTAL PROCEDURES

Materials—Human diploid fibroblasts were isolated from newborn foreskin (20), and IMR-90 was purchased from ATCC (CCL-186). EJ-p53 cells were obtained from Dr. D. Y. Shin (21), and Harlan Sprague-Dawley rat embryo fibroblasts (HDFs) were obtained from Dr. W.-Y. Park (22). The stable expression of p53 cells were obtained from Dr. D. Y. Shin (21), and Harlan Sprague-Dawley rat embryo fibroblasts (HDFs) were obtained from Dr. W.-Y. Park (22).

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1 The abbreviations used are: EGF, epidermal growth factor; EGFR, EGF receptor; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; PDL, population doubling level; HDF, human diploid fibroblast; Erk, extracellular signal-regulated kinase.
Dawley rats were purchased and maintained from the Animal Laboratory of Seoul National University College of Medicine. Monoclonal anti-caveolin-1 antibody (C43420), monoclonal anti-caveolin-2 antibody (C57820), and monoclonal anti-caveolin-3 antibody (C38420) were purchased from Transduction Laboratories. Polyclonal anti-EGFR antibody (sc-09), polyclonal anti-phospho-Erk antibody (sc-7383), polyclonal anti-phospho-Erk-1/2 antibody (sc-84), anti-p53 antibody (sc-126), and anti-p21 antibody (sc-6246) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Secondary horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibodies were purchased from Jackson Immunothericals. Other biochemical reagents were purchased from Sigma and Life Technologies, Inc.

Cell Culture and Aged Rates—Human diploid fibroblasts and IMR-90 were kept in 10-cm plates in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and antibiotics and subcultured at a 1:4 ratio. Young cells are defined as resulting from less than 25 population doublings, whereas old cells were from more than 60 population doublings. The senescence of all old cells was confirmed by their delayed population doubling times and by senescence-associated β-galactosidase activity assay as described by Dimri et al. (22). EJ-p53 cells were maintained as described by Sugrue et al. (21).

Harlan Sprague-Dawley rats were maintained on a normal diet for more than 24 months; tumor-free female rats were used for this experiment. 8–10-week-old female rats were used as young controls.

Senescence-associated β-Galactosidase Analysis—After being grown in a semiconfluent state, senescence-associated β-galactosidase (pH 6.0) activity was examined (22). Briefly, cells were washed with phosphate-buffered saline and fixed with 2% paraformaldehyde, 0.2% glutaraldehyde in phosphate-buffered saline for 5 min at room temperature. After washing with phosphate-buffered saline, cells were incubated with β-galactosidase reagent (1 mg/ml X-gal, 40 mM citric acid/sodium phosphate buffer, pH 6.0, 5 mM potassium ferrocyanide/potassium ferricyanide, 150 mM NaCl, 2 mM MgCl₂) at 37 °C. Western Blot and Analysis of Erk Activation—Total cell or tissue lysates were extracted using extraction buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 150 mM NaCl, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 2 mM NaF, 0.2 mM Na₃VO₄) and sonicated briefly. Lysates were analyzed by Western blot using polyclonal anti-EGFR antibody and polyclonal anti-Erk-1/2 antibody. Immune complexes were precipitated with protein A-Sepharose for 4 h, washed with phosphate-buffered saline, incubated at 4 °C for 30 min, and spin down at 9,000 rpm for 5 min, and supernatants were incubated with anti-EGFR antibody, polyclonal anti-caveolin-1 antibody, polyclonal anti-caveolin-2 antibody, monoclonal anti-caveolin-3 antibody, anti-EGFR antibody, and monoclonal anti-Erk-1/2 antibody (sc-09). Secondary horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibody was added, and absorption was visualized using enhanced chemiluminescence detection kit (ECL kit; Amersham Pharmacia Biotech).

Immunoprecipitation—Cells were lysed in IP lysis buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 150 mM NaCl, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 2 mM NaF, 0.2 mM Na₃VO₄) and sonicated briefly. Lysates were separated using SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose filters (Protran; Schleicher & Schuell) using standard techniques. After immunoblotting the membrane with a primary antibody, the membrane was incubated with a peroxidase-conjugated anti-mouse or anti-rabbit secondary antibody and finally visualized using an enhanced chemiluminescence detection kit (ECL; Amersham Pharmacia Biotech).

Western Blot Analysis of Erk Activation—Total cell or tissue lysates were extracted using extraction buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 150 mM NaCl, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 2 mM NaF, 0.2 mM Na₃VO₄) and sonicated briefly. Lysates were separated using SDS-polyacrylamide gel electrophoresis and transferred onto nitrocellulose filters (Protran; Schleicher & Schuell) using standard techniques. After immunoblotting the membrane with a primary antibody, the membrane was incubated with a peroxidase-conjugated anti-mouse or anti-rabbit secondary antibody and finally visualized using an enhanced chemiluminescence detection kit (ECL; Amersham Pharmacia Biotech).

RESULTS

Reduced Phosphorylation of Erk-1/2 by EGF Stimulation—Senescent cells like old rat hepatocytes or old human diploid fibroblasts have been demonstrated to show an age-dependent decline in DNA synthesis and/or cell division upon growth factor stimulation. To explore the mechanism of the reduced response to EGF stimulation with aging, we first examined the phosphorylation and activation of Erk kinases in old cells. Two kinds of primary human fibroblasts were used: primary HDFs isolated from newborn foreskin (20) and IMR-90, human lung fibroblast ATCC. These cells were kept as described previously (23) and classified as young (PDL less than 30), middle-aged (PDL 35–45), and old (PDL more than 60) cells.

Erk activation by EGF stimulation was attenuated in old human diploid fibroblasts. HDFs were starved of fetal bovine serum for more than 24 h and treated with EGF at 100 ng/ml for the indicated times. A, using young cells at PDL 20–30, middle-aged cells at PDL 35–45, and old cells at PDL 55–65, total cell lysates were analyzed by Western blot using polyclonal anti-phospho-Erk antibody to detect the activation of Erk-1/2 kinases. Using polyclonal anti-Erk-1/2 antibody, the amounts of each sample were normalized. B, total cell lysates from each group of HDFs were analyzed for the expression of EGFR and Erk-1/2 using polyclonal anti-EGFR antibody and polyclonal anti-Erk-1/2. Y, young; M, middle-aged; O, old.
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Previously (17), lysates from young and old HDF were precipitated with normal mouse sera, anti-caveolin-1 antibody, or anti-EGFR antibody. Then each immune complex was separated by SDS-polyacrylamide gel electrophoresis and analyzed by Western blot using anti-caveolin-1 antibody or anti-EGFR antibody. The immune complex of EGFR from old HDF contained caveolin-1 proteins, while young fibroblasts did not show a comparable amount of caveolin-1 and subsequent interactions with EGFR (Fig. 2B). Although we did not check the kinase activity of EGFR in old HDF, molecular interaction between caveolin and EGFR may mediate the attenuation of EGFR signaling in old HDF.

Caveolin-2, a homolog of caveolin-1 (25), is expressed ubiquitously in most cell types, supposedly forming a hetero-oligomer in basolaterally localized caveolae (26). Although the overexpression of caveolin-2 was not sufficient to form functional caveolae structures (27), it would be important to know whether the up-regulation of caveolin-1 is accompanied by an overexpression of caveolin-2. Fig. 2A shows that the expression of caveolin-2 in both HDF and IMR-90 was increased with aging, like caveolin-1. The role of caveolin-2 is not well defined, but we suggest that caveolin-2 might potentiate caveolae formation in aged cells. Although the expression of caveolin-3 is restricted to striated muscle cells (28), heterologous expression of caveolin-3 in old human fibroblasts was detected in both HDF and IMR-90 (Fig. 2A).

Caveolin-1, a key constituent of caveolae structures, forms caveolae structures in old HDF. Electron microscopic experiments of plasmalemmal profiles from old (PDL 55–65) HDF revealed the presence of numerous non-clathrin-coated vesicular structures, 50–100 nm in diameter, that are juxtaposed to the plasma membrane and are morphologically indistinguishable from caveolae (Fig. 3A). In contrast, profiles of young (PDL 20–25) cells contained significantly less caveolae-like structure. Quantitative analysis of multiple plasmalemmal profile revealed that old fibroblasts contained over 10 times more caveolae-like structure than young cells (0.5 μm versus 5.2/μm, respectively) (Fig. 3B).

**In Vivo Analysis of Caveolin in the Aged Rats—Extrapolation of in vitro changes of old cells to in vivo old rat tissues will confer a biological significance in aging research. Every old tissue consists of heterogeneous cell types, especially in the case of proliferation potential. However, the overall function and responsiveness of each organ was significantly down-regulated with aging. To compare the expression of caveolin proteins in each organ, young 2-month-old and old 27-month-old Harlan Sprague-Dawley rats were sacrificed, and the solid organs were dissected out for Western blot analysis. In Fig. 4, all of the major organs showed markedly increased level of caveolin-1 proteins. Aged brain, kidney, lung, and spleen in particular expressed significant quantities of caveolin-1 proteins as well as caveolin-2 and caveolin-3. Moreover, some tissues such as brain, thymus, and spleen showed that the expression of caveolins was highly increased in the old tissues, which is in contrast to their undetectable levels of expression of the young tissues. From these results, the up-regulation of caveolin proteins was consistently found in vivo in old tissues, implicating the role of caveolins in various aging-related phenomena.**

**Erk Activation in p53-induced Senescence—Senescence-like growth arrest by expression of p53 in EJ human bladder carcinoma cells induced cellular senescence (21). To find the causal relationship between caveolin and signaling in old cells, we used EJ-p53 cells as an induced aging model. EJ-p53 cells were induced for senescence by removal of tetracycline from the culture media for more than 7 days. p53 and p21 were induced in p53-induced EJ human bladder carcinoma cells (Fig. 5A). Senescence-associated β-galactosidase activity was also increased in p53-induced EJ cells (data not shown). However, the responses to EGF stimulation in p53-induced senescent cells were intact despite their phenotypic changes (Fig. 5B) and different from those responses in naturally senescent cells. Namely, after treatment of EGF to EJ-p53 cells, Erk-1/2 kinases were well phosphorylated as much as control proliferating cells. The ready responsiveness of EGF signaling in EJ-p53 cells regardless of the senescent phenotypic changes suggests that some different biochemical changes other than overexpression of p53 might be involved for the regulation of EGF signaling, for which we assumed that caveolin would play an important role. Therefore, we examined the expression of caveolin proteins in EJ-p53 cells, but we could not detect the overexpression of caveolin-1 proteins, which was quite different from the behavior of naturally aged cells (Fig. 5A).

Moreover, an age-related increase in free cholesterol is associated with a decrease in the phospholipid content (29, 30), which might contribute to the increased caveolin expression (31). To know whether the aging of HDF correlates with the increasing level of cholesterol, we examined the total cholesterol in old HDF. As shown in Fig. 5C, total cholesterol level of old HDF was 1.8 times higher than young HDF. However, in the case of EJ-p53 cells, the level of cholesterol was not increased despite the induction of phenotypic senescence. The increased level of cholesterol was well matched with the up-regulation of caveolin-1 protein, both of which are the key constituents of caveolae structures.

**Transient Expression of Caveolin-1 in Young HDFs—**If caveolin acts dominantly in age-related attenuation of EGF signaling, the overexpression of caveolin in young fibroblast might inhibit the activation of Erk-1/2 upon EGF stimulation. To this end, we overexpressed human caveolin-1 cDNA in young HDFs and checked the activation of Erk-1/2 upon EGF stimulation. First, we examined the expression of caveolin-1 in mock- and caveolin-1-transfected cells (Fig. 6A). After EGF stimulation of those cells, we checked the activation of Erk-1/2...
by Western blot using anti-phospho-Erk antibody. As shown in Fig. 6B, the phosphorylation of Erk-1/2 was inhibited by the overexpression of caveolin-1 in young HDFs. The expression level of Erk-1/2 was not affected by transfection. These data suggest that the overexpression of caveolin-1 is sufficient to inhibit the EGF signaling in human diploid fibroblasts.

**DISCUSSION**

Senescent cells are generally resistant not only to extrinsic noxious stimuli (32, 33) but also to growth factor stimulation (2–4). Limited responsiveness to extrinsic and intrinsic stresses might characterize the old cells. Several hypotheses have been suggested for the mechanism involved, such as the repression of genes, modulation of signal cascades, changes in membrane properties, etc. (5–7). However, the general mechanism or the common denominator for the unresponsiveness of the old cells has not yet been established. In this work, we have demonstrated that mechanistically caveolins or caveolae would play an important role in the regulation of signaling in the old cells. We have found that late passage old cells, either HDF or IMR-90, are enriched with all subtypes of caveolin-1, -2, and -3 and the numerous non-clathrin-coated vesicular structures. Immunoprecipitation analysis showed that caveolins were strongly bound to EGFR in the old cells, while binding was negligible in young cells, implicating the functional role in the modulation of EGF signaling in the old cells. Moreover, we showed that the overexpression of caveolin-1 in young HDF suppressed the signaling from EGF to Erk-1/2. These data strongly indicate that senescent cells are enriched with caveolae structures and caveolin proteins, which might be involved in the regulation of cellular signal transduction pathways through the direct interactions with signaling molecules.

It is well known that transformed cells, including cancer cells, show a reduced level of caveolins and caveolae organelles (10, 19, 34, 35). Targeted down-regulation of caveolin-1 is sufficient to drive cell transformation and to hyperactivate the mitogen-activated protein kinase cascade, which suggests that caveolins would suppress the transformation (19). The expression of caveolin-1 in CHO cells inhibits signaling from EGFR, Raf, MEK-1, and Erk-2 to the nucleus (10). The anchorage-independent growth of transformed fibroblasts also disappeared by the overexpression of caveolin-1 (36). Cellular senescence was presumed to be the opposite phenomenon of the transformation. In contrast with the transformed cells, senescent cells showed reduced responsiveness, which could have been due to the elevated level of caveolins. We could successfully prove that the overexpression of caveolin-1 in young HDF directly down-regulated the phosphorylation of Erk-1/2 in response to EGF.

We have compared the expression levels of caveolins in vivo to confirm our in vitro data. As summarized in Fig. 4, the organs from “young” 2-month-old (Y) and “old” 27-month-old (O) Harlan Sprague-Dawley rats were isolated and analyzed for the expression of caveolin proteins by Western blot, using anti-caveolin-1 antibody, anti-caveolin-2 antibody, and anti-caveolin-3 antibody. Cx, cerebral cortex; Mb, midbrain; Hp, hippocampus; Cl, cerebellum; Ht, heart; Lu, lung; Li, liver; Sp, spleen.
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protein both in vivo and in vitro, augmented expression of caveolins may alter amyloid precursor protein processing and lead to an overproduction of toxic amyloid metabolites (37, 38). In addition, the up-regulation of caveolin with aging is suggested to relate to insulin resistance, resulting in susceptibility to diabetes mellitus (39). Therefore, it may be worthwhile to study the age-related degenerative disorders in association with the expression of caveolins.

Recently, there have been several reports that cellular senescence can be induced by a variety of agents such as H2O2, sodium butyrate, UV irradiation, and induction of p53 or several cyclin-dependent kinase inhibitors (21, 23), evaluated by irreversible growth arrest, cytomorphometry, and senescence-associated β-galactosidase activity. Although the general mechanism for induced senescence is debatable, in the genetic aspect, the forced expression of p53, as one of major senescence-associated genes, has been emphasized. Using EJ-p53 cells (21), we could readily induce the cellular senescence-like changes by the control of tetracycline, resembling the morphological and biochemical characteristics of naturally senescent cells. However, when the senescence was induced in EJ-p53 cells, the expressions of caveolins were not significant, while those of p53 and p21 were markedly increased (Fig. 5A). Moreover, the downstream signal cascade of EGFR was not inhibited in senescence-induced EJ-p53 cells (Fig. 5B). This finding proves that the down-regulation of EGFR signaling in the aged cells can be directly ascribed to the level of caveolins in one aspect and that the overexpression of p53 is not sufficient to convert the cells into the functionally senescent state in other aspect. Although caveolin-2 was induced in EJ-p53 cells, the sole expression of caveolin-2 cannot form the structurally and functionally compatible caveola structure in vivo (27). From these results, we could reach a tentative conclusion that the expression of caveolins is essential to maintain the senescent property.

It was suggested that a high level of cholesterol is required to form caveola structure and that cholesterol plays an important role in the activation of caveolin (40). In addition, it has been proposed that the general unresponsiveness of old cells either to growth signals or to apoptotic stimuli could be ascribed to the alteration of the cell membrane properties. Membrane lipids constantly undergo peroxidation by repeated toxic insults, and the lipid composition changes; levels of cholesterol are increased, and, inversely, phospholipid is decreased (29, 30, 32). When we checked cholesterol levels, it was found that the old HDF cells showed higher levels of cholesterol than young HDF cells (Fig. 5C), but the senescence-induced EJ-p53 cells did not show these high levels of cholesterol. This result may provide a clue to the low level of caveolins in EJ-p53 cells. Actually, in a study of caveolin-1 gene promoter, it was reported that caveolin-1 gene promoter contains sterol regulatory element-like sequences, which are comparable with those identified in several genes for the regulation of influx and biosynthesis of cholesterol (41). This may explain that induction of senescence by overexpression of p53 in EJ-p53 cells is not accompanied by the high expression of caveolins, because the increase in cholesterol level does not occur. The importance of cholesterol in the regulation of caveolin expression or activation might imply the significance of nutritional intervention in the aging process.

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FIG. 5. Caveolin-1 was not increased in the p53-induced aging of EJ-p53 cells. A, EJ-p53 cells were incubated with tetracyclin (Tet (+)) or without tetracyclin (Tet (-)) for 7 days. After confirming their senescence using a β-galactosidase assay, the lysates from each point were analyzed for their expression of p53, p21, and caveolin proteins, in tetracyclin-positive control and tetracyclin-negative senescent EJ human bladder carcinoma cells. B, the activation of Erk-1/2 kinases by EGF stimulation was monitored using Western blot with anti-phospho-Erk antibody and anti-Erk antibody. C, the level of total cholesterol was monitored using cholesterol oxidase and plotted at each PDL number. Each column represents the mean of two independent experiments using three different wells of cells. S.E. for each value are indicated as error bars.

FIG. 6. The Erk-1/2 activation in caveolin-1-overexpressing young HDFs. A, at 48 h after the transient expression of caveolin-1 in HDF, total cell lysates were analyzed for their expression of caveolin-1 by Western blot using anti-caveolin-1 antibody. B, transfected cells were stimulated with EGF at 100 μg/ml for the indicated periods. Total cell lysates were analyzed for the phosphorylation and the expression level of Erk-1/2 by Western blot using anti-phospho-Erk-1/2 or anti-Erk-1/2 antibody, respectively.
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