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Abstract: The proliferative responses of cells to mitogens decrease during aging, and this may result from age-related defects in signal transduction in response to mitogens. In this study, we have investigated the age-related alteration of responses to epidermal growth factor (EGF) in cultured human keratinocytes that were senesced *in vitro* by repeated passage. The stimulation with EGF increased the DNA-binding activity of activator protein 1 (AP-1), an important transcription factor for cell proliferation, in young keratinocytes, whereas the binding activity showed little or slight change in the senescent cells. The induced DNA-binding activity of AP-1 in young cells was inhibited by PD 98059, an inhibitor of MEK, and partially inhibited by GF 109203X, an inhibitor of protein kinase C. Western blot analysis demonstrated that EGF induced dramatic increase in the phosphorylation of EGF receptor (EGFR) and extracellular signal-regulated kinases (ERK) in young cells, while this phosphorylation was much less profound in senescent cells. Finally, the application of EGF to young cells resulted in increased phosphorylation of Fra-2, a Fos protein component of the Jun/Fos heterodimer AP-1 complex. This EGF-induced Fra-2 phosphorylation was attenuated in senescent cells. Taken together, our study suggests that the signal transduction mediated by EGF/ERK pathway is altered in senescent human keratinocytes, and this change may be attributed, in part, to the decreased AP-1 transcription activity observed in senescent keratinocytes.

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Key words: AP-1-cellular senescence – human epidermal keratinocytes – EGF – ERK

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Introduction

Although all organs of the human body change with age, nowhere are the changes as readily apparent as in the skin. Chronologically, aged skin becomes atrophic with slowed metabolic activity, resulting in a lower rate of epidermal turnover, thinning and decreased elasticity, poorer clearance of chemical substances, and delayed re-epithelialization after wound healing (1,2). The age-related loss of wound healing capacity leads to a high risk of surgical wound dehiscence and infection, constituting major medical problems for aging adults (3–5).

Intrinsic tissue aging is believed to be mirrored by *in vitro* behavior of that tissue's cells in culture. Normal diploid cells have a finite culture lifetime, sometimes called the Hayflick limit (6). After a limited number of population doublings in culture, cells no longer respond to mitogens, thus lose their replicative and proliferative capacity. This process of cellular aging in culture is termed replicative senescence. Recent studies have demonstrated that replicative senescence not only occurs in tissues as organisms including man age, but also has significant consequences for aging of organisms (7–9). In human skin, Campisi (10) has reported that senescent keratinocytes and fibroblasts appear to accumulate with age. With increasing age, the culture life span and proliferative rate of keratinocytes and fibroblasts progressively decline (2), correlating with the clinical observation of compromised tissue repair and wound healing in aged adults (11–13). Cultured cells thus may provide important insight into the mechanisms of organismal aging and mortality and can be used as a cellular model for aging (7,14).

Cellular proliferation is the result of growth factor receptor-mediated signaling and subsequent regulation of gene expression (15). One hallmark of cellular aging is the decreased proliferative response resulting from age-related defects in signal transduction for mitogens (12,16,17). When this signaling process is impaired with age, cells will consequently arrest growth in G1 phase and will not enter S phase in response to physiologic mitogens. Conceptually, young cells are more sensitive to extracellular signals and react more appropriately through various signal transduction pathways after stimulation as compared to their aged counterparts.

Keratinocytes make up more than 95% of the epidermis, and their proliferation and differentiation are controlled by a number of mitogens and growth factors (13). Among those that are most robust are the factors that activate the epidermal growth factor receptor (EGFR) (18). Specific age-dependent defects in mitogen-activated protein (MAP) kinases pathway, which play an important role in transmission of EGF-mediated mitogenesis to the nucleus, have been observed in senescent human fibroblasts (12,19). In an *in vivo* human skin study, Chung et al. (20) reported that the extracellular signal-regulated MAP kinase (ERK) activity is reduced, whereas the stress-activated MAP kinase (c-Jun-N-terminal kinase) activity is increased in old, compared with young, human skin. A major nuclear target of EGF/MAP kinase pathway is activator protein 1 (AP-1) (21,22). AP-1 is a family of transcription factors that bind specific DNA sequences (5′-TGAC/GTCA-3′) in the promoter region of target genes. Activation of AP-1 represents the early nuclear response to mitogenic stimuli, and it is involved in modulation of the expression of a variety of genes that are implicated in cell proliferation (23–25) and differentiation (26). Moreover, the cumulative effects of oxidative damage are widely recognized to contribute to the aging process (13,27). AP-1 can be activated by many antioxidants, thus is considered to be a potent antioxidant transcription factor (24). To determine whether keratinocytes exhibit an age-dependent alteration in signal transduction for mitogenic stimuli, we studied the DNA-binding activity of AP-1 in young and senescent human keratinocytes in response to EGF stimulation.

Our studies were carried out using neonatal foreskin-derived human epidermal keratinocytes that were senesced *in vitro*. Under current culture conditions, human epidermal keratinocytes possess a relatively short replicative life span (18,28), and the senescence phenotype has been found to increase with passage number (29). Dimri et al. (30) reported that 23% of cultured human keratinocytes become senescent after about 10 population doublings. Saunders et al. (17) reported that cultured human keratinocytes entered the senescent state after 34 population doublings. This limited doubling potential makes normal keratinocytes in culture a convenient system for studying the mechanisms underlying the aging process. In our experiments, cultured keratinocytes were senesced by serial passage until they were no longer capable of division in their normal growth medium.

Methods

Chemicals and materials

The kit of Gel Shift Assay System including oligonucleotide of AP-1 and T4 polynucleotide kinase was purchased from Promega (Madison, WI, USA). Anti-p44/42 (ERK1/2), anti-phospho-p44/42 (ERK1/2), anti-EGFR, and anti-phospho-EGFR (Tyr1068) antibodies were purchased from Cell Signaling Technology (formerly New England Biolabs, Inc) (Beverly, MA, USA). Anti-pan Jun, anti-pan Fos, anti-c-Fos, anti-Fra-1, anti-Fra-2, and other antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). [γ-^{32}P]ATP was purchased from ICN (St. Louis, MO, USA). Inhibitors of protein kinases PD 98059, PD 158780, and Compound 32 were purchased from Calbiochem (San Diego, CA, USA).

Cell culture and senescence

Normal human keratinocytes were derived from neonatal foreskin. The cultured keratinocytes were routinely grown in Medium 154 with growth supplements (Cascade Biologics, Inc, Portland, OR, USA) as described (31). *In vitro* senescence of keratinocytes was done by continued passage (2.5–3 population doublings per passage) until cells lost their proliferative capacity. In the experiments, keratinocytes were maintained in medium containing growth supplements until they reached 80–90% of confluence, and then maintained in a quiescent state in the growth supplements-free medium for 24–48 h before treatment with growth factors.

SA-β-galactosidase assay

Senescence-associated β-galactosidase (SA-β-gal) activity assay of keratinocytes was assessed using a SA-β-gal staining kit from Cell Signaling Technology following the manufacturer's instruction. Cells were fixed in 2% formaldehyde and 0.2% glutaraldehyde for 15 min at room temperature, and then incubated in a staining solution (pH 6.0) containing 1 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranodase (X-gal) at 37°C overnight. Senescent cells were identified as blue-stained cells by light microscopy. Cells in control were incubated in the staining solution containing N-N-dimethylformamide (DMF) that was used as the solvent for X-gal stock solution.

Preparation of nuclear extracts and cell lysates

Nuclear extracts for electrophoretic mobility shift assay (EMSA) were prepared essentially as described (32,33). Cells were rinsed with phosphate buffer saline (PBS), then frozen by placing the plates on a dry ice/methanol freezing bath. The cells were thawed and scraped in 250 μl hypotonic buffer (10 mM HEPES, 10 mM KCl, 0.2 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 3 mM Na$_3$VO$_4$, and protease inhibitors, pH 7.9), rocked for 20 min on ice, then passed through a 27.5 gauge needle. After addition of an additional 250 μl hypotonic buffer containing 2% Nonidet P-40, the suspension was centrifuged at 10 000 × g for

2 min. The yielded supernatant was the cytoplasmic extract. The obtained pellets were resuspended in a hypertonic buffer (20 mM HEPES, 400 mM NaCl, 25% glycerol, 1 mM EDTA, 1 mM EGTA, 0.5 mM PMSF, 3 mM Na_3VO_4, and protease inhibitors, pH 7.9), homogenized, rocked at 4°C for 30 min, and pelleted by centrifuge at $10\,000 \times g$ for 15–20 min to collect the nuclear extract supernatant. For Western blot analysis, cells were lysed in a lysis buffer containing 10 mM Tris, 10 mM NaCl, 2 mM EDTA, 2 mM PMSF, 2 mM Na_3VO_4, protease inhibitors, and 0.25% Triton X-100. After being rocked for 30 min, cell lysates were centrifuged at $14\,000 \times g$ for 20 min. Protein concentrations of both nuclear extracts and cell lysates were determined using the Bradford protein assay (Bio-Rad Laboratories, Richmond, CA, USA). Samples were stored at -80°C.

EMSA

Electrophoretic Mobility Shift Assay (EMSA) was done according to the protocol included in Gel Shift Assay System (Promega). AP-1 oligonucleotide was labeled with [γ-^{32}P]ATP in a buffer containing T4 polynucleotide kinase for 10 min at 37°C. The reaction was terminated by adding 0.25 M EDTA/10 mM Tris-HCl solution. The labeling solution was centrifuged through a Sephadex G-50 column to remove unincorporated [γ-^{32}P]ATP. For DNA-protein binding, the nuclear extracts containing 5–10 μg of protein were mixed with ^{32}P-labeled AP-1 oligonucleotide and poly(dI:dC)poly(dI:dC) in 15 μl binding buffer. The reaction was typically performed for 15 min at 4°C. The generated complexes were electrophoresed on a denaturing 6% acrylamide gel in Tris/boric acid/EDTA buffer, and the gel was dried and subjected to autoradiography. For the gel mobility shift interference assay (antibody supershift EMSA), the nuclear extracts were preincubated with a specific antibody in the binding solution for 2 h on ice. Then ^{32}P-labeled AP-1 was added to initiate the DNA-protein-binding reaction.

Western blot analysis

Samples with equal amounts of protein were loaded into a 10 or 7.5% acrylamide gel, and SDS-PAGE was run at 150 or 200 mV. The proteins on the gel were transferred onto a PVDF membrane at 100 mV for 60 or 75 min. The membranes were blocked with 5% non-fat dry milk in PBS, then probed with appropriate primary antibodies. The immunoreactive proteins were detected using horseradish peroxidase-linked anti-IgG and then stained with ECL Western blotting detection reagents (Amersham Life Science, Inc, St. Louis, MO, USA).

Images of EMSA or Western blotting were obtained by scanning the films on a UMAX S-6E scanner.

Results

Human keratinocytes displayed a finite replicative life span *in vitro*. After approximately 15 passages (2.5–3 population doublings per passage), cells largely lost their proliferative capacity. Cells at late passages (14–16) lost the characteristic 'cobblestone' morphology and acquired an enlarged and flattened appearance with vacuolated cytoplasm. The senescent phenotype of cultured keratinocytes was confirmed using senescence-associated-β-galactosidase activity assay (30,34). As expected, SA-β-gal activity in keratinocytes increased with passage number. Essentially none of cells of passage 4 displayed SA-β-gal activity, whereas the majority of cells of passage 14 were positive for SA-β-gal

activity (Fig. 1). In this study, cultured cells of the same strain at early (3–5) passage, defined as young cells, and at late (14–16) passage, defined as senescent (or old) cells, were used for experiments.

EGF-induced DNA-binding activity of AP-1 decreases in senescent keratinocytes

The DNA-binding activity of AP-1 was detected using EMSA. The activity was lower in both young and senescent keratinocytes maintained in growth supplements-free Medium 154 for 24–48 h, as compared to cells maintained in the medium containing growth supplements (data not shown). Within 1 h after addition of EGF (100 ng/ml) to growth factor-starved young keratinocyte cultured in the growth supplements-free medium, an increase in the AP-1–DNA binding was observed. The peak value observed at 2 h was 1.3-fold higher than the baseline level, and this increase persisted for 12 h. In senescent keratinocytes, however, there was little increase in AP-1–DNA-binding activity in response to EGF addition (Fig. 2a). A robust dose-dependent increase in AP-1–DNA-binding activity was observed in young cells with maximal binding activity at 10–100 ng/ml EGF (Fig. 2b). TGF-α, another ligand of EGFR, induced a similar increase in the DNA-binding activity of AP-1 in young but not senescent keratinocytes (data not shown).

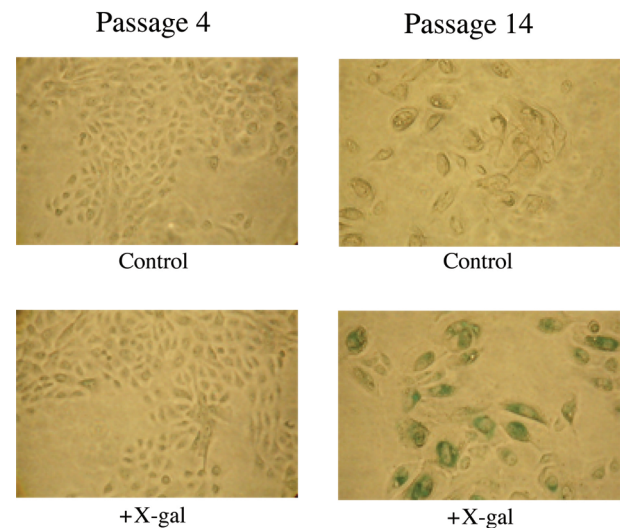The signaling pathway(s) involved in EGF-induced AP-1–DNA-binding activity was studied

Passage 4   Passage 14

Figure 1. Cultured human keratinocytes were senesced using serial passage. Keratinocytes were subjected to repeated passage in Medium 154 with growth supplements. Senescent phenotype of cells at passage 4 or passage 14 was detected using senescence-associated β-gal activity assay described in *Materials and methods*. Cells were examined by a light microscope (×200), and senescent cells could be identified by development of blue color. Cells in control were incubated in the staining solution containing dimethylformamide that was used as the solvent for X-gal stock solution.
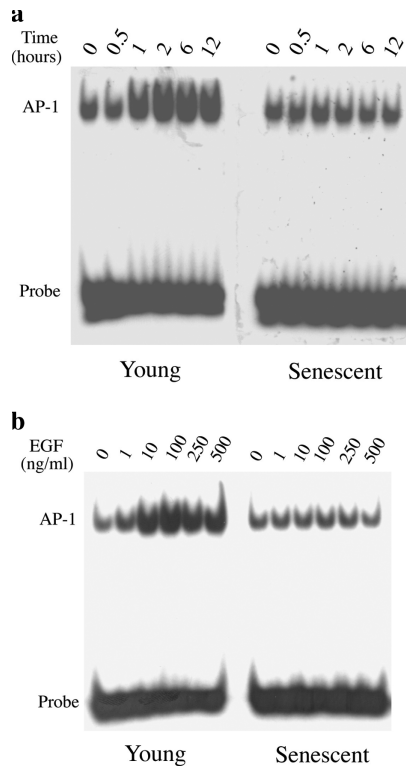
Figure 2. Epidermal growth factor (EGF) induces an increase in DNA-binding activity of AP-1 in young but not in senescent keratinocytes. (a) Cells were treated with 100 ng/ml EGF for varying times. (b) Cells were treated with EGF of varying concentrations for 2 h. Electrophoretic mobility shift assay was performed as described in *Materials and methods*. The data shown are representative of two independent experiments with similar results.

Figure 3. The mitogen-activated protein kinase inhibitor PD 98059 and the protein kinase C inhibitor GF 109230 reduce epidermal growth factor (EGF)-induced activator protein (AP)-1 DNA-binding activity. (a) Cells were incubated with 50 μM PD 98059 for 30 min prior to treatment with 50 ng/ml EGF for 1 h. The data shown are representative of two independent experiments with similar results. (b) Cells were treated with EGF (100 ng/ml) or 12-O-tetradecanolphorbol-13-acetate (TPA) (100 nM) for 1 h. GF 109203X (0.5 μM) was added 1 h before EGF treatment.

by pharmacologically inhibiting members of the activation pathway. Prior to EGF treatment, cells were incubated with PD 98059 (50 nM, 30 min), an inhibitor of MEK1/2, the kinase that activates extracellular signal-regulated kinases (ERK). The EGF-induced DNA-binding activity of AP-1 in young keratinocytes was reduced to nearly basal (control) level (Fig. 3a). In addition, after cells had been pretreated with the protein kinase C (PKC) inhibitor GF 109203X (0.5 μM, 30 min), the EGF-induced DNA-binding activity of AP-1 in young cells was partially inhibited (Fig. 3b). The phorbol ester 12-O-tetradecanolphorbol-13-acet-ate (TPA) is a well-known stimulator of AP-1, and it stimulates AP-1 by the induction of AP-1 components Jun and Fos (35). When TPA (100 nM, 1 h) was applied to cells, AP-1-binding activity was increased not only in young cells but also in senescent cells. This fact suggests that AP-1 is activatable in senescent keratinocytes. Thus, the observed decrease in AP-1-binding activity in senescent cells treated with EGF was not because of an intrinsic impairment of the transcription factor itself, rather than an upstream signaling defect.
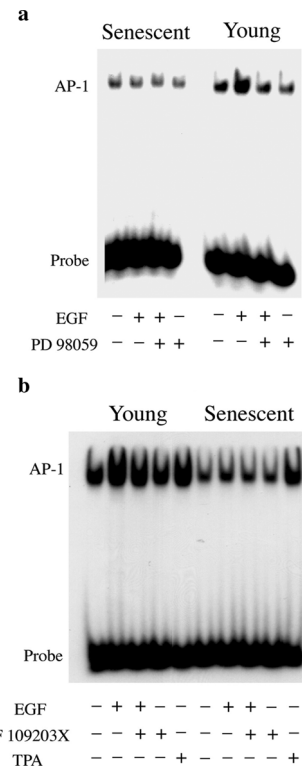
AP-1 can exist as either a Jun/Jun homodimer or a Jun/Fos heterodimer, and the heterodimer generally displays more stable binding to the promoter (36). The composition of AP-1 in keratinocytes was determined by gel mobility shift interference assay (antibody supershift EMSA). Preincubation of nuclear extracts with anti-pan Jun antibody before the protein–DNA-binding reaction took place had a minor effect on the DNA-binding activity of AP-1 in either young or senescent samples, revealed by the appearance of the slower shifting smear ladders. On the other hand, preincubation of nuclear extracts with anti-pan Fos antibody completely abolished the AP-1-binding activity, resulting in a supershifted band of an antibody/AP-1/DNA ternary complex (Fig. 4). These findings indicate that the binding activity of a heterodimeric AP-1 in keratinocytes is more dependent on the Fos subunit. Nevertheless, after treatments with EGF, the supershift patterns of AP-1-DNA binding in both young and senescent cells remained unchanged. Therefore, AP-1 in
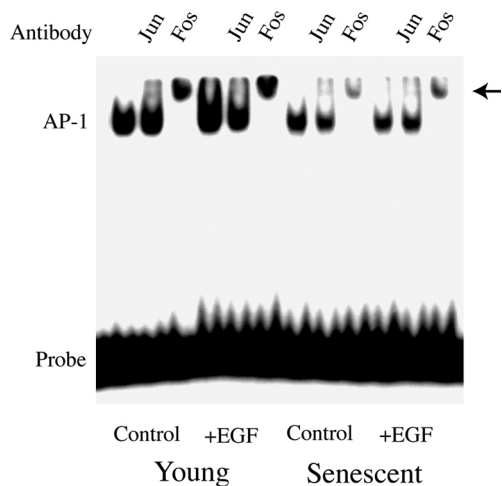
Figure 4. Young and senescent keratinocytes have a similar Jun/Fos heterodimer architecture of AP-1 complex. Gel mobility shift interference assay was performed by preincubation of nuclear extracts with anti-pan-Jun or antipan-Fos anti-body for 2 h prior to addition of ^{32}P-ATP-labeled AP-1 oligonucleotide. Supershift of bands is indicated by an arrow. The data are representative of two independent experiments with similar results.

young and senescent keratinocytes appears to share a similar Jun/Fos heterodimer architecture.

EGF-induced phosphorylation of ERK and EGFR decreases in senescent keratinocytes

Because AP-1 activity is mediated by EGF primarily through the EGF receptor (EGFR)/ERK cascade, a question was raised: Whether the observed attenuation of AP-1 activity resulted from a senescence-related alteration in EGFR/ERK pathway? We investigated this possibility.

We compared the expression and activation of the EGFR in young and senescent human keratinocytes. We found that the basal levels of EGFR expression were comparable in keratinocytes at early and at late passages maintained in growth supplements-free Medium 154 for 24 h. The EGFR abundance in young and senescent keratinocytes responded to EGF (50 ng/ml) stimulation with a similar pattern: It remained unchanged within 30 min after addition of EGF and declined at 1 h (Fig. 5a) probably because of degradation after internalization (37,38). To detect the EGF-induced activation of EGFR tyrosine kinase in keratinocytes, we measured the phosphorylation of EGFR at Tyr1068 that is reportedly crucial to the EGF-induced Grb2/Ras/ERK signaling cascade (39). As shown in Fig. 5b, the basal phosphorylation of EGFR at Tyr1068 was almost not detectable in either young or senescent keratinocytes. Following EGF stimulation, young cells displayed a marked phosphorylation of EGFR at Tyr1068, which
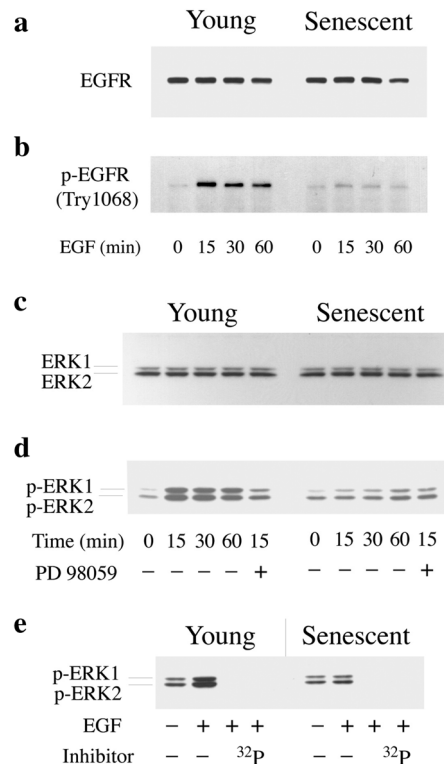

Figure 5. Effects of epidermal growth factor (EGF) on EGF receptor (EGFR) phosphorylation and extracellular signal-regulated kinases (ERK) phosphorylation in young and senescent keratinocytes. (a and b) EGF induces EGFR phosphorylation in young but not senescent keratinocytes. Cells were treated with EGF (50 ng/ml) for 15, 30, or 60 min. Cell lysates were resolved by SDS-PAGE. Proteins on the gel were transferred onto a membrane and detected by immunostaining with (a) an anti-EGFR antibody or (b) an anti-phospho-ERKR (Tyr1068) antibody. The data are representative of two independent experiments with similar results. (c–e) EGF induces a greater increase in ERK phosphorylation in young keratinocytes than in senescent keratinocytes. In panels c and d, cells were treated with EGF (50 ng/ml) for 15, 30, or 60 min. The MAP kinase inhibitor PD 98059 (40 μM) was added 30 min prior to EGF treatment. In panel (e), cells were treated with 50 ng/ml EGF for 30 min. The EGFR inhibitor compound 32 (32) (0.1 μM) or PD 158780 (P) (5 μM) was added 30 min prior to EGF treatment. Cell lysates were resolved by SDS-PAGE and detected by immunostaining with an (c) anti-ERK1/2 antibody or (d and e) anti-phospho-ERK1/2 antibody. The data are representative of two independent experiments with similar results.

signals MAPK/ERK cascade through Grb2/Ras mechanism, whereas senescent cells maintained a very low level of the phosphorylation of Tyr1068.

We then detected how senescence would affect the ERK response in keratinocytes. In the absence of EGF, ERK1 and ERK2 levels in the cytoplasm were similar in keratinocytes at early and late passages. The application of 50 ng/ml EGF only marginally increased the levels of ERK in both young and senescent cells (Fig. 5c). Although EGF failed to induce ERK synthesis in keratinocytes in the period of time tested, it induced a rapid

and dramatic increase in ERK phosphorylation in young keratinocytes (Fig. 5d). Within 15 min following EGF treatment, the phosphorylation of ERK1 and ERK2 in young cells increased by 9.9-fold (ERK1) and 4.0-fold (ERK2), respectively. The increased level of ERK phosphorylation was sustained up to 1 h. On the other hand, EGF-mediated phosphorylation of ERK in senesced cells was much less robust with increases of only 2.9-fold (ERK1) and 1.4-fold (ERK2), respectively. When cells were preincubated with PD 98059, the EGF-induced ERK1 phosphorylation in young keratinocytes was markedly reduced, from 9.9-fold without PD 98059 to 3.9-fold with PD 98059. Moreover, when cells were pretreated with compound 32 or PD 158780, inhibitors of EGFR tyrosine kinase activity, the phosphorylation of ERK1/2 was completely abolished (Fig. 5e).

EGF-induced phosphorylation of Fra-2 decreases in senescent keratinocytes

One component of the AP-1 heterodimer, proteins of Fos family, is known to be downstream target of ERK, and our investigation next focused on how the Fos component was affected by attenuation of EGFR/ERK signaling, leading to change in AP-1 activity. There are four members of Fos protein family: c-Fos, Fos B, Fra-1, and Fra-2, we first detected which members are expressed in normal human keratinocytes. Western analysis data demonstrated the presence of c-Fos, Fra-1, and Fra-2, while Fos-B was not detectable (Fig. 6a). A similar expression pattern of c-Fos, Fra-1, and Fra-2 proteins in human keratinocytes was recently reported by Schmuth et al. (40). Our data are also consistent with the finding in mouse keratinocytes, where Fra-2 is reported to be a major Fos component in AP-1 (41). Fra-2 is reportedly involved in the control of cell proliferation and growth arrest-specific gene expression (42), and change in Fra-2 has been noted during aging (27). We, therefore, investigated how it would respond to EGF stimulation in the young and senescent cells. Addition of EGF induced the phosphorylation of Fra-2, demonstrated by slower migrating bands of Fra-2 on the gel, in young keratinocytes within 15 min, which then declined gradually (Fig. 6b). The induced phosphorylation was inhibited by preincubation of cells with the inhibitor PD 98059 (10 μM, 30 min), which inhibited ERK phosphorylation through the inhibition of MEK1/2. In senescent keratinocytes, EGF-mediated phosphorylation of Fra-2 was notably diminished. Fra-2 was, however, phosphorylatable in senescent cells when TPA was applied as a stimulator (Fig. 6c).
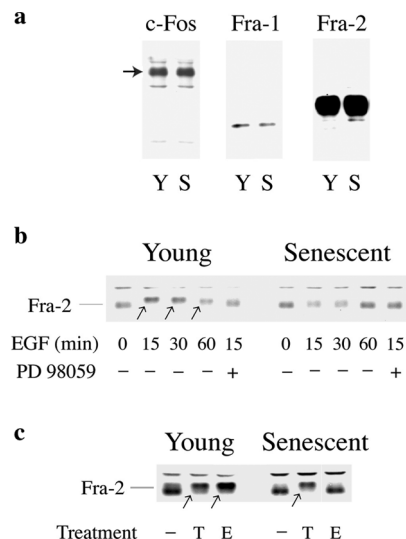

Figure 6. Effects of epidermal growth factor (EGF) on Fra-2 phosphorylation in young and senescent keratinocytes. (a) Constituents of Fos protein family in human keratinocytes. Samples of young (Y) or senescent (S) keratinocytes were resolved by SDS-PAGE, and detected by immunostaining with anti-c-Fos, anti-Fra-1, or anti-Fra-2 antibody, respectively. The data are representative of two independent experiments with similar results. (b and c) EGF increases Fra-2 phosphorylation in young but not senescent keratinocytes, whereas 12-O-tetradecanolphorbol-13-acetate (TPA) increases Fra-2 phosphorylation in both young and senescent keratinocytes. In panel b, cells were treated with EGF (50 ng/ml) for 15, 30, or 60 min. PD 98059 (40 μM) was added 30 min prior to EGF treatment. In panel c, cells were treated with 100 nM TPA (T) or 50 ng/ml EGF (E) for 15 min. Samples were subjected to SDS-PAGE and detected by immunostaining with a Fra-2 antibody. Slower migrating bands are indicated by arrows. The data are representative of two independent experiments with similar results.

Discussion

Cellular proliferation is the result of growth factor-receptor binding and activation of cellular signaling pathways that regulate specific mitogenic gene transcription. Senescent cells are generally resistant to growth factor stimulation. Replicative senescence largely results from failure to express or/and activate growth regulatory transcriptional modulators, and this is very likely sufficient to irreversibly arrest cell proliferation (43). Cellular aging is a multifactorial process that presumably involves different biochemical pathways (44,45). One current theory of cell aging maintains that exposure to oxidative stress is primarily responsible for aged phenotype in multiple cellular and tissue levels (45). The accumulated oxidative damages induce profound effects on cell signaling, thus modulating the expression of gene products that are associated with aging through functional alteration of transcription factors (24).

In this study, we demonstrated that EGF-induced DNA-binding activity of the transcription

factor AP-1 is attenuated with senescence of keratinocytes. The impairment of DNA-binding activity of AP-1 during cellular senescence has been documented in several cell types (36,46–48). In a case of a genetic syndrome of geroderma, which exhibits premature skin aging, Gherzi et al. (49) demonstrated that AP-1 DNA-binding activity, in response to proliferative stimuli such as serum, was attenuated in skin fibroblasts from a patient of this disease. However, the proposed linkage between cell senescence and dwindled AP-1 activity is not without controversy (50). Grassilli et al. (51) reported that fibroblasts from centenarians exhibited the similar DNA-binding activity of AP-1 in response to serum stimulation, compared to fibroblasts from young donors.

EGF activates the AP-1 transcription factor primarily through EGFR/MAP kinase pathway (21,22). In this cascade, activation of EGFR results in phosphorylation of ERK through a kinase cascade(s). The activated ERK then activates the Fos component of the AP-1 complex through the ternary complex of TCF, Elk-1, and Sap-1a (22). Attenuation of EGFR/ERK signaling pathway has been linked to age-related decline in the proliferative response to mitogenic stimuli (52–54). For instance, senescent human fibroblasts do not phosphorylate ERK after EGF stimulation, whereas young cells do (19). The downregulation in EGFR/ERK/AP-1 mitogenic signaling in aged cells could be because of changed EGFR expression/activity (12,55), weaker association between EGFR and its downstream adopter proteins (15,53), diminished expression/activation of MAP kinase or its upstream kinase elements such as Raf or MEK (20,56), or altered AP-1 composition/AP-1 component activity (23,44). To find an explanation for the attenuation of EGF-reduced AP-1 activity in senescent keratinocytes, we examined the major upstream and downstream events along this signaling cascade.

Human fibroblasts have been a model widely used in the study of cell senescence. At the receptor level, Reenstra et al. (55) noted that aged fibroblasts lacked a population of high affinity EGFRs that young fibroblasts possessed. Later, Shiraha et al. (12) found that, as fibroblasts approached senescence, they presented reduced EGF responsiveness because of preferential loss of EFG receptor. Recently, Tran et al. (38) observed, besides reduction of EGFR numbers on cell surface, attenuation of EGFR phosphorylation in senescent human fibroblasts because of increased protein tyrosine phosphatase activity. In our study, Western analysis data indicated that there was virtually no difference in EGFR abundance between young and senescent keratinocytes. However, the phosphorylation of EGFR at Tyr1068 was markedly reduced in senescent cells, although, we do not know what mechanism was implicated. The impairment of the EGFR/ERK signaling in keratinocytes during senescence was further substantiated by the investigation on ERK phosphorylation. Following EGF stimulation, despite young and senescent keratinocytes presented similar levels of ERK, senescent cells did not respond with the same robust ERK phosphorylation as did young cells. Interestingly, similar results were obtained with human skin *in vivo*. Chung et al. (20) observed that total ERK protein levels did not differ between young (mean age 23.8 years) and old (mean age 83.4 years) skin, whereas phosphorylation ERK protein was reduced 60% in old skin. This resulted in 45% lower ERK activity in old skin relative to young skin.

In some types of cells, the altered DNA-binding activity of AP-1 with age is associated with the differential regulation of the steady state levels of individual AP-1 components, resulting in changes in the AP-1 composition (27,44). For instance, the abundance of Fos proteins was reportedly reduced dramatically in old, as opposed to young fibroblasts, despite equivalent amount of Jun proteins being expressed (46). Consequently, old fibroblasts produce predominantly the Jun/Jun homodimer replacing the Jun/Fos heterodimer found in young cells (23). However, here we demonstrated that the architecture of AP-1 appears unchanged with senescence in keratinocytes. It retains a Jun/Fos heterodimer in either young or senescent cells, and this architecture was not affected by application of EGF.

The observed decrease in ERK phosphorylation with senescence in keratinocytes may lead to altered expression or activity of Fos proteins, a downstream target for ERK. Members of Fos proteins are known to be growth regulatory transcriptional modulators, and their expression and/or phosphorylation are found less inducible by mitogenic stimuli in aged cells (23,36,57). In lymphocytes, while there is no difference of c-jun expression in cells derived from young (3 months) and old (>18 months) mice, c-fos expression was diminished in cells from old mice, resulting in loss of AP-1–DNA-binding activity (47). In cultured fibroblasts, induction of fos-B showed a significant decrease in serum-stimulated senescent cells (50). In this study, we demonstrated that the binding activity of the Jun/Fos heterodimer of AP-1 in human keratinocytes seems largely dependent on the Fos component, and Fra-2 appears to be a significant member of Fos protein in this heterodimer. Fra-2 could be phosphorylated on its C-terminal *in vivo* through ERK activation, and the

extent of Fra-2 phosphorylation will determine the transcriptional activity of AP-1 (58). Therefore, any attenuation of Fra-2 phosphorylation in aged cells would be expected to decrease the DNA-binding activity of AP-1. Our data support this hypothesis by demonstrating that EGF-mediated Fra-2 phosphorylation was indeed decreased in senescent keratinocytes. Meanwhile, Fra-2 itself in senescent keratinocytes appeared to be phosphorylatable when alternate activators like TPA were applied, thus, the observed reduction of EGF-mediated Fra-2 phosphorylation is probably because of an age-related upstream defect in the EGF-signaling pathway.

In summary, cultured human keratinocytes at early passages (young) and late passages (senescent) display different responses to EGF stimulation. While EGF induces phosphorylation of ERK, resulting in the activation of the AP-1 component Fra-2, and consequently an increase in the DNA-binding activity of the AP-1 in young keratinocytes, these responses to EGF are apparently attenuated with *in vitro* senescence. These alterations may likewise be related to the loss of proliferative capacity in senescent keratinocytes.

Acknowledgements

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