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Analysis of expression of growth factor receptors in replicatively and oxidatively senescent human fibroblasts

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Abstract Replicatively and oxidatively senescent human fibroblasts demonstrate an impaired response to mitogens. To investigate whether this is due to downregulation of growth factor receptors we examined their expression in these two types of senescence. mRNA and protein levels of the insulin receptor and platelet-derived growth factor (PDGF) α-receptor decreased in replicatively senescent cells. The PDGF β-receptor and insulin-like growth factor 1 receptor at the protein level also decreased but remained readily detectable. However, these major growth factor receptors remained unchanged in oxidatively premature senescent cells. This suggests that mechanisms underlying diminished responsiveness to mitogens might be different in replicative senescence and oxidatively premature senescence.

Keywords: Senescence; Oxidative stress; Insulin receptor; Growth factor receptor; Mitogen

1. Introduction

Cellular senescence is an irreversible growth arrest state in which cells have lost proliferative capacity but stay viable for extended periods of time. Replicative senescence results from serial passage in culture and is generally believed to be triggered by critically shortened telomeres [1–3]. Cellular senescence can be induced prematurely in early passage cells by agents that cause DNA damage [4–7] or disrupt heterochromatin [8], or by strong mitogenic signals [9–11]. Premature senescence can also be induced by exposure of the telomere 3′ overhang sequence [12]. The phenotype of premature senescence often shares many features of replicative senescence, including distinct morphology, senescence associated β-galactosidase (SA-β-gal) activity and upregulation of p53 pathway. Telomere shortening, however, is not associated with such acutely induced premature senescent cells [13]. This is possibly because cell replication is rapidly halted in the premature senescent cells and cell replication is believed to be required for the telomere shortening [14]. Nevertheless, premature senescence can also be achieved in a telomere-dependent manner by exposure to mild oxidative stress [14,15]. This is because oxidative stress causes single strand breaks in telomeric regions which consequently cause accelerated telomere shortening [16,17].

One of the prominent hallmarks of both replicative senescence and premature senescence is their diminished response to serum or growth factors. The mechanisms underlying the hyporesponsiveness to mitogenic stimuli are not fully elucidated. Cellular responses to external signals are mediated via activation of complex signalling cascades that ultimately lead to changes in gene expression. The first event in response to mitogenic signals is the activation of respective cognate receptors, followed by formation of multi-protein complexes, phospholipid turnover, calcium mobilization and activation of protein kinases, phosphatases and transcription factors. This leads to coordinated regulation of proteins that ensure successful cell proliferation. The impaired response of senescent fibroblasts to mitogens suggests inappropriate transmission of signals. Decrease in expression of cell surface receptors in senescent cells may be partly accountable for the diminished response to mitogens. This has been supported by evidence obtained from replicative senescence [18,19]. For example, age-related downregulation of the epidermal growth factor (EGF) receptor has been reported in human microvascular endothelial cells [20], human fibroblasts [21–23] and rat articular chondrocytes [24]. Similarly, age-related reductions in the platelet-derived growth factor (PDGF) binding sites or PDGF receptor have also been demonstrated in several cell systems including human smooth muscle cells [25] and human fibroblasts [26]. Decreased expression of the PDGF β-receptor has been suggested to be a causative factor for the decreased mitogenic response of fibroblasts in Werner's syndrome, a premature ageing condition [27]. However, little is known as to whether premature senescent cells also share the same alteration in terms of receptor expression and downstream pathways.

The aim of this study was to systematically compare expression of the insulin receptor and other growth factor receptors at mRNA and protein levels in replicatively and oxidatively premature senescent human fibroblasts. This may provide insight into the potential different mechanisms by which replicative and oxidative damage induced senescence occurs.

2. Materials and methods

2.1. Cell culture and H_2O_2 treatment

IMR-90 cells at population doubling (PD) 24.5 were obtained from the America Type Culture Collection (ATCC). The cells were grown in ATCC modified Eagle's minimal essential medium supplemented with

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Abbreviations: PDGF, platelet-derived growth factor; FGF, fibroblast growth factor; IGF, insulin-like growth factor; EGF, epidermal growth factor; SA-β-gal, senescence associated β-galactosidase; Rb, retinoblastoma; BrdU, 5-bromo-2′-deoxyuridine; PBS, phosphate-buffered saline

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10% fetal bovine serum (FBS, ATCC), 100 U/ml penicillin and 0.1 mg/ml streptomycin (penicillin-streptomycin solution from Invitrogen). Cells for H_2O_2 treatment were prepared from the exponential phase around PD 30. H_2O_2 treatment was carried out 24 h after seeding by incubating 2×10^6 cells in 100-mm dishes in 13 ml of the culture medium containing 600 μM H_2O_2 for 2 h. For a second H_2O_2 treatment the cells were 1:2 split after being cultured for 4 days following the first treatment and treated again for 2 h with 600 μM H_2O_2 24 h after seeding. Trypan blue exclusion assay was carried out to demonstrate that the treatment did not cause any cell death. IMR-90 cells reached replicative senescence at PD 59.

2.2. BrdU labeling and SA-β-gal assay

Cells were seeded and treated as described above in dishes containing autoclaved coverslips. For 5-bromo-2′-deoxyuridine (BrdU) labeling, coverslips were transferred into a 6-well plate and incubated in 2 ml of the culture medium containing 10 μM BrdU for 48 h. Cells on coverslips were then washed with phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde in PBS, washed twice in PBS, permeabilized in 0.2% Triton X-100 and washed in PBS. DNA was denatured by incubation in 2 M HCl for 1 h followed by three washes with PBS. Coverslips were incubated for 1 h with fluorescein-conjugated mouse anti-BrdU monoclonal antibody (1:20 dilution; Alexis Biochemicals) and with DNA stain TOTO-3′-iodide (0.5 μM; Molecular Probes), washed three times with PBS, and mounted in Antifade mounting solution (Molecular Probes). For SA-β-gal assay, cells on coverslips were washed twice in PBS, fixed for 5 min in 4% paraformaldehyde in PBS, washed three times in PBS and incubated at 37 °C overnight in fresh SA-β-gal staining solution (1 mg of 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside [stock = 100 mg/ml of dimethylformamide]/ml of 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide, 150 mM NaCl, 2 mM MgCl in 40 mM citric acid/sodium phosphate, pH 6.0) [26]. At least 500 cells from each time point were scored under the microscope for BrdU- or SA-β-gal assay.

2.3. Semi-quantitative RT-PCR

Total RNA was extracted from cell pellets using TRI reagent (Sigma) and quantified spectrophotometrically in capillary cuvettes on GeneQuant (Amersham Pharmacia Biotech). First strand cDNA was reverse transcribed from 0.8 μg of total RNA using a 1st Strand cDNA Synthesis Kit from Roche with random primers $p(dN)_6$ supplied with the kit. PCRs were carried out as follows: 1 cycle of 94 °C for 4 min and receptor specifically defined number (see Table 1) of cycles of 94 °C for 30 s, 56 °C for 45 s, 72 °C for 1 min. Primers used for receptor PCR amplifications and sizes of PCR products are shown in Table 1. PCR products were analyzed by electrophoresis on a 1% agarose gel followed by densitometry quantitation using an Alpha Imager (Alpha Innotech Corporation, CA, USA).

2.4. Western blotting

Whole cell lysate was prepared by scraping cells in Laemmli buffer [0.12 M Tris, pH 6.8, 4% (w/v) SDS, 20% (v/v) glycerol with protease inhibitors (cocktail from Sigma)]. Protein concentration was determined by the bicinchoninic acid (BCA) method (Sigma) with BSA as a standard. 15 μg of proteins were separated by SDS–PAGE and electrophoretically transferred to nitrocellulose membrane. Blots were probed with the following antibodies: anti-p53 antibody (ab7757, abcam), anti-p21 antibody (H-164, Santa Cruz), anti-p16 antibody (H-156, Santa Cruz), anti-retinoblastoma (Rb) antibody (G3-245, BD PharMingen), anti-insulin receptor β-subunit antibody (sc-711, Santa Cruz), anti-PDGF α-receptor antibody (sc-338, Santa Cruz), anti-PDGF β-receptor antibody (06-498, Upstate), anti-fibroblast growth factor (FGF) receptor 1 antibody (05-149, Upstate), anti-insulin-like growth factor (IGF)-1 receptor antibody (sc-713, Santa Cruz), anti-EGF receptor antibody (05-104, Upstate) and anti-β actin antibody (ab6276, abcam). The bound primary antibodies were detected by horseradish peroxidase-conjugated secondary antibodies (Amersham), followed by enhanced chemiluminesence (Amersham). The densities of the bands were quantified using an Alpha Imager (Alpha Innotech Corporation).

3. Results

3.1. Characterization of replicatively and oxidatively senescent human fibroblasts

Replicative senescent cells were obtained when IMR-90 cells showed no more population doubling for 10 days after a serial passage. As shown in Fig. 1(b) replicatively senescent cells displayed typical enlarged morphology that was strikingly different to that of young cells as shown in Fig. 1(a). The senescence biomarker, SA-β-gal was also fully detectable in 98% of the cells at this stage for the senescent cells (Fig. 1(d)) whereas only 14% of young control cells showed SA-β-gal activity (Fig. 1(c)). Furthermore, p53, p21 and p16 were found to be upregulated with Rb being hypophosphorylated in the replicatively senescing cells (Fig. 1(i), lanes 2 and 3). It is interesting to note that in the deep senescent cells (4 weeks into senescence) while p53 and p21 decreased as compared to the newly senescent cells p16 showed a further increase (compare lane 4 to lane 3 in Fig. 1(i)). This is in keeping with cells undergoing replicative senescence in that p53 senses stresses (e.g., telomere shortening), which then activates expression of p21 resulting in cell cycle arrest, whereas p16 is responsible for the maintenance of the senescent state [28,29].

Premature senescence was induced by treating IMR-90 cells with H_2O_2 as reported previously [7]. As determined by 48 h BrdU labeling, permanent cell cycle arrest increased from 16% in young control cells to over 98% in the treated cells (Fig. 1(e) and (f)). SA-β-gal activity was detectable in 96% treated cells (Fig. 1(h)). In addition, hypophosphorylation of Rb and upregulation of p53, p21 and p16 were also detected in the H_2O_2-induced premature senescent cells (Fig. 1(i), lane 5).

3.2. Analysis by semi-quantitative RT-PCR

In order to investigate whether permanent cell cycle arrest in replicatively senescence and oxidative stress-induced premature senescence is due to alterations in expression of growth factor receptors we carried out semi-quantitative RT-PCR to determine relative mRNA levels in young and senescing IMR-90 cells. As shown in Fig. 2, the insulin

Table 1
Primers used for RT-PCR

	Forward primers (5′–3′)	Reverse primers (5′–3′)	PCR product size (bp)	Cycle
IR	GCGGAAGACAGTGAGCTGTT	GATGCGATAGCCCGTGAAGT	(11^+) 474/(11^-) 438	34
PDGFR-α	CCCAGATGTAGCCTTTGTAC	GACCGTCAAAGTGTACACCA	463	28
PDGFR-β	ACACTGCACGAGAAGAAAGG	CGGTTGTCTTTGAACCACAG	590	29
IGF-1R	CATTGAGGAGGTCACAGAG	CAAAGACGAAGTTGGAGGC	431	29
FGFR1	CTCTATGCTTGCGTAACCAG	TTGCTCCCATTCACCTCGAT	599	32
EGFR	ACGCAGTTGGGCACTTTTGA	TGGTCAGTTTCTGGCAGTTC	520	29
Actin	CAACTGGGACGACATGGAGA	ATACCCCTCGTAGATGGGCA	277	28