mRNA level of alpha-2-macroglobulin as an aging biomarker of human fibroblasts in culture

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Abstract

Cellular senescence is a well-established model system for studying the molecular basis of aging. To identify a reliable biomarker for cellular age and further study the gene expression of aging, we profiled the gene expression difference between aged and young cultured human embryonic lung fibroblasts by high-density complementary deoxyribonucleic acid (cDNA) arrays. Among the differentially expressed genes, alpha-2-macroglobulin (α2M) was selected for further study. Its gene expression level as a function of population doubling level (PDL) in cultured fibroblasts was determined by RT-PCR and northern hybridization. mRNA level of α2M showed a positive linear-correlation with cumulative PDL. Additional assays revealed that the levels of α2M increased in irreversible growth arrest induced by sublethal H2O2, but not in quiescent state of cultured fibroblasts induced by serum-deprivation, and remained stable in Hela cells. These results suggest that mRNA level of α2M can be used as a biomarker of aging in cultured fibroblasts. mRNA level of α2M showed significant difference between newborn and old human leucocytes, which suggest that the mRNA level of α2M may be used as a biomarker of aging in vivo.

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1. Introduction

Normal human fibroblasts do not divide indefinitely. This property, termed the finite replicative lifespan of cells, leads to a permanent and irreversible arrest of cell division by a process termed replicative or cellular senescence (Hayflick, 1965). To understand the mechanisms that regulate cellular senescence, many genetic factors and environmental factors that affect the processes of cell senescence have been studied. Besides accumulation of cell generations, cellular senescence occurs as a consequence of exposure to subcytotoxic conditions, stimulation with cytokines, over-expression of proto-oncogenes and tumor suppressor genes, and deficiency of JunD (Toussaint et al., 2002). However, a reliable biomarker for cellular age and further study of the gene expression profile of aging are needed to elucidate the mechanism of cellular senescence.

Although no consensus exists as to what constitutes an acceptable biomarker of cellular aging, the suggested criteria include that it (1) is highly reproducible and reflects biological age; (2) shows significant age-related changes within a relatively brief period of time; (3) is easily measured and the sample for measurement is small; (4) is stable in immortal cells (Ma et al., 2002). Markers characterizing the transition from young replicating to old growth-arrested cultured cells in different experimental systems were reported to be an increase in neutral β-galactosidase activity (Dimri et al., 1995), telomere shortening (Allsopp et al., 1992), over-expression of collagenase (Sottile et al., 1989), two-chain cathepsin B (DiPaolo et al., 1992) and Alzheimer disease β-amyloid precursor protein (APP) (Alder et al., 1991), increasing resistance of DNA to damage by H2O2 (Caldini et al., 1998), reduced inducibility of c-fos in response to serum stimulation (Seshadri and Campisi, 1990), as well as reduced expression of heat shock protein 70 in response to stress (Choi et al., 1990, 1995) and

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EPC-1 in response to serum deprivation (Pignolo et al., 1993; Tresini et al., 1999). The most widely used biomarkers of cellular senescence are neutral β-galactosidase (SA-β-gal) activity and telomere shortening. Recent reports state that SA-β-gal activity is variable in different culture conditions have made it unreliable as a biomarker of cellular senescence (Severino et al., 2000; Dumont et al., 2000). Telomere shortening as an aging biomarker is also challenged by the existence of telomere-independent senescence (Chen et al., 2001; Litaker et al., 1998).

α2M is a 718-kDa homotetrameric glycoprotein. It is one of the most abundant proteins in human plasma with a concentration of 2–4 mg/ml (Scottrup-Jensen et al., 1989). It is well characterized as an extra cellular panproteinase inhibitor and as a carrier of specific growth factors, including transforming growth factor-β (TGF-β) and nerve growth factor (NGF-β) (Crookston et al., 1994; Goniais et al., 1994). The cleavage of a peptide bond at the bait region of the α2M molecule by a protease leads to a conformational change in α2M, which is referred to as the activated α2M (α2M*). TGF-β1 preferentially binds to α2M*, whereas TGF-β2 binds both α2M and α2M* with an affinity higher than that of TGF-β1 (Liu et al., 2001).

In this study, we used cDNA arrays to profile the gene expression difference between the senescent and young cultured human embryonic lung fibroblasts. As quantification of high-abundance mRNA is less interfered by ‘noise’, α2M, which was expressed most highly, both in aged and young fibroblasts, among the five most differentially expressed genes according to cDNA array result, was selected as a biomarker candidate of cellular aging. We further determined mRNA level of α2M as a function of PDL in human fetal lung fibroblasts by RT-PCR and northern hybridization, and we also studied the possibility of mRNA level of α2M as a biomarker of aging in vivo.

2. Materials and methods

2.1. Cell culture

Human embryonic lung diploid fibroblasts (2BS cells) were provided by the National Institute of Biological Products, Beijing, China. 2BS cells were previously isolated from female fetal fibroblast tissue and have been fully characterized (Tang et al., 1994). 2BS cells were cultured in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.), which contained 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cultures were defined at the end of their proliferative life span or Hayflick limit, when they were unable to complete one population doubling during a 4-week period that included three consecutive weeks of re-feeding with fresh medium containing 10% FBS. Their current expected Hayflick limit is 62–65 population doublings (PD). For concise expression, in accordance with the definition by Pignolo RJ and Cristofalo VI (Pignolo et al., 1993), 2BS cells were described as young cells at PD 31 or below, whereas senescent cells were at 62 PD or above over.

2.2. Peripheral blood leukocyte isolation

The donors of peripheral blood were divided into two groups. In the newborn group a total of 5 cm³ of peripheral blood was drawn from umbilical cord of 10 newborn babies with a heparinized syringe and needle at the Department of Gynaecology and Obstetrics, the Third Affiliated Hospital of Peking University, China. In the old group, a total of 5 cm³ of peripheral blood was drawn from forearm vein of 10 old people (65–70 years) with a heparinized syringe and needle, at the Geriatric Care Center, The Hospital of Peking University Health Science Center, China. None of the donors had been diagnosed with any significant disease. Leucocytes were isolated from peripheral blood according to QIAamp RNA Blood Mini Handbook.

2.3. cDNA array hybridizations and data mining

The total RNA of 2BS cells at different population doubling levels (PDL) was harvested using the RNeasy mini kit (QIAGEN company, Germany). Cells were pelleted and processed according to the animal cell protocol. A260 measurements were used to quantify the preparation, which were then run on native agarose gels for integrity assessment. mRNA extraction was performed using the Oligotex mRNA Midi kit (QIAGEN company, Germany).

The cDNA probes were synthesized and purified as described previously (Ma et al., 2002). cy3-dUTP was used to label cDNA from 2BS cells (PD 28), and cy5-dUTP was used to label cDNA from 2BS cells(PD 64).

After denaturing at 95 °C for 5 min, labeled probes were hybridized to HEGC-40S cDNA arrays (Biostar Genechip Inc., Shanghai, China) in low stringent condition for 15–17 h at 60 °C. The array was washed three times with 2 × SSC + 0.2% SDS, 0.1 × SSC + 0.2% SDS, 0.1% SSC, for 10 min each, and dried at room temperature.

The assay was scanned with GenePix 4000B. Background-subtracted element signals were used to calculate cy5/cy3. 40 housekeeping genes were used as internal controls to balance and modify the original signal. Data were analyzed with GenePix 3.0.

2.4. Northern blot

A total of 30 μg RNA was run on a 1% denaturing Agarose gel in 1.8 mM formaldehyde and transferred onto hybond-N Nylon membrane. The DNA fragment representing unique sequence of α2M was labeled with [α-32P] dATP according to the manufacturer’s protocol of prime-α-Gene labeling system (Promega). Hybridization was carried overnight in a 5 × SSC, 0.1%SDS, and 50% deionized formamide. GAPDH mRNA level was used as reference.
Membranes were washed with 0.1%  

\( \times \) SSC, 0.1% SDS for 2–4 h in 37 °C, exposed to Kodak film at −80 °C for 1–5 days. Data were analyzed by IBAS analyzer 2000.

2.5. RT-PCR

Two micrograms of RNA were subjected to reverse transcription using the Access RT-PCR system (Promega). The sequences of the primers for \( \alpha_2 \)M were \( 5'- \) TTGTCAGTGACGTTTGCCTC-3' (upstream) and \( 5'- \) CAAAACCTCGTCTCGTAG-3' (downstream); for \( p16^{INK4a} \) were \( 5'- \) GAATAGTTACGGTCGGAG-3' (upstream) and \( 5'- \) CGGTGACTGATGATCTAAG-3' (downstream) and for \( \beta \)-actin were \( 5'- \) GTGGGCATGGGTCAGAAG-3' (upstream) and \( 5'- \) GAGGCGTACAGGGA-TAGCAC-3' (downstream). All of the primers were checked by sequencing. For \( \beta \)-actin control, the linear range was at cycle 30. 1% reverse transcription reaction was amplified for 20 s at 95 °C, 1 min at 58 °C, and 30 s at 72 °C for 30 cycles. The products were examined by 1% agarose gel electrophoresis and quantified by computerized gel documentation (Bio-Rad).

3. Results

3.1. Screening the candidate genes by cDNA array

The mRNA obtained from senescent cells and young cells were converted to cy3-labeled and cy5-labeled probes and hybridized to HGEC-40 s arrays. The hybridization results are presented on a scattered plot graph (Fig. 1). Each point on the plot represented a gene hybridization signal. Signals were corrected for intensity via internal controls. The red points represent the ratios of cy5 to cy3 signal in the range 0.5–2.0, and belong to the undifferenital group. The yellow points represent the ratios greater than 2.0 or smaller than 0.5, whose gene expression was most possibly altered. \( \alpha_2 \)M is marked by the black arrow.

3.2. Confirmation by Northern hybridization

Expression of \( \alpha_2 \)M was tested by northern hybridization using RNA preparations from different experiments, and the predicted changes were confirmed. The observed signal difference in northern hybridization was lower than the value of balanced differential expression determined from cDNA array. To determine the progressive change of gene expression of \( \alpha_2 \)M, RNA preparations from two middle-aged fibroblast cultures were also processed for northern hybridization (Fig. 2A). The northern hybridization result showed that the gene expression of \( \alpha_2 \)M increased gradually during the aging of cultured fibroblasts.

3.3. RT-PCR analysis of gene expression of \( \alpha_2 \)M

Comparing with northern hybridization, RT-PCR is easier to perform and more sensitive, and is a more suitable method for detecting an aging biomarker. To determine mRNA level of \( \alpha_2 \)M as a function of PDL in 2BS cells,
the gene expression of \( \alpha_2M \) was measured by RT-PCR by using RNA preparations from 2BS cells of PD27, 35, 42, 48, 54, 60, and 64 (Fig. 2B). Mean mRNA level of \( \alpha_2M \) was plotted versus cumulative PDL, and regression analysis showed a linear increase in mRNA level of \( \alpha_2M \) with cumulative PDL as modeled by linear equation (Fig. 3A). The difference between means of all measured generations showed statistical significance (Student’s \( t \)-test, \( p < 0.01 \)).

The same RNA preparation and statistical methods were also used to determine the gene expression of p16\(^{INK4a} \), a well-known senescence-associated gene. Mean mRNA level of p16\(^{INK4a} \) increased in 2BS cells with cumulative PDL, and the increase can be modeled as an exponential equation (Figs. 2B and 3B). There is no statistical significance between neighboring groups below 54.

The reversible growth-arrested condition referred to as the G0 or quiescent state is achieved through serum deprivation; the irreversible G1 arrest induced by sublethal H\(_2\)O\(_2\) is the most acceptable model of stress-induced premature senescence (Toussaint et al., 2000; Ma et al., 2003), while Hela cells are a typical kind of active immortal cells. The result of RT-PCR using RNA preparations from serum-deprived 2BS cells, H\(_2\)O\(_2\)-induced premature senescent 2BS cells, and Hela cells revealed that the mRNA levels of \( \alpha_2M \) in serum-free young 2BS cells did not increase while that in the premature senescence induced by sublethal H\(_2\)O\(_2\) was up-regulated, and that in the immortal Hela cells is much lower and stable (Fig. 2C) (additional data not shown).

### 3.4. Gene expression level of \( \alpha_2M \) in human peripheral blood leucocytes

Human peripheral blood leucocytes are easily obtained and are suitable substrates for testing a biomarker of aging in vivo. The gene expression of \( \alpha_2M \) can not be detected by RT-PCR using the same amount of cDNA template and the same PCR cycle numbers as \( \beta \)-actin gene in all the human leukocyte samples. We therefore used five times the amount of template, namely 5% of the reverse transcriptional cDNA, for RT-PCR of \( \alpha_2M \). The comparison of \( \alpha_2M \) mRNA levels showed a significant difference between new-born
and old human leucocytes according to the Student’s test (Fig. 4, p < 0.01).

4. Discussion

Replicative senescence of human diploid fibroblasts has been used as a well-established model system for studying aging (Martin et al., 1970; Salk, 1982; Rohme, 1986; Goldstein, 1990). To find a new biomarker candidate of aging, we have surveyed the gene expression alterations in senescent fibroblasts by cDNA arrays. We found that the gene expression level of α2M was up-regulated in senescent fibroblasts, and confirmed this finding by northern hybridization and RT-PCR. α2M protein fragment was recently reported to be accumulated in senescent fibroblasts, and down-regulated in immortal cells, but the protein fragment was considered to be taken up from the culture medium and fragmented by the cells (Kondo et al., 2001). The relatively high amount of α2M protein in bovine serum makes it complicated to detect the protein expression of α2M in cultured cells. The gene expression level of α2M can easily be measured by semi-quantitative RT-PCR with a small sample and it is a more suitable biomarker candidate of aging than well-known senescence-associated gene such as p16INK4a. Furthermore, the study showed that mRNA level of α2M has a positive linear-correlation with cumulative PDL. Additional assays revealed that the mRNA level of α2M was up-regulated in premature senescence induced by sublethal H2O2 but unchanged in quiescent cultured fibroblasts. These results suggested that up-regulated gene expression of α2M can be used to mark the progress of cellular senescence in fibroblasts like telomere shortening, rather than act as a biomarker of growth arrest like SAβ-gal. α2M protein level in plasma of healthy subjects has been reported to correlate with age (Wager et al., 1982). The comparison of α2M mRNA levels between newborn and old human leucocytes showed significant difference, while the gene expression level of α2M in immortal Hela cells was very low and stable. These results indicate that up-regulated expression of α2M could be a universal age-related phenotype. Further experiments are necessary to identify if α2M protein or mRNA level can be a biomarker of aging in other types of cells in vitro or in vivo.

It has been previously reported by our research group that over-expression of p16INK4a in human fibroblasts induced permanent and irreversible growth arrest at a PDL below the Hayflick limit, and fibroblasts harboring an antisense construct to p16INK4a mRNA had a prolonged life span (Duan et al., 2001). Our results in this study show that gene expression of p16INK4a at passage was exponentially correlated with cumulative PDL, in keeping with previous reports on p16INK4a, and provides further evidence that up-regulation of p16INK4a mRNA level is one of the causes of cellular senescence. The linear relationship between gene expression level of α2M and cumulative PDL suggests that up-regulated expression of α2M may play a different role from that of p16INK4a. α2M is the major binding protein for TGF-β1 and TGF-β2. α2M and TGF-β1 to be induced in both replicative senescence and H2O2-induced senescence of human fibroblasts were reported (Ma et al., 2003; Frippiat et al., 2001). Previous studies (Dumont et al., 2000; Frippiat et al., 2001) have shown that TGF-β1 is responsible for the induction of biomarkers of replicative senescence. The up-regulation of α2M may be caused by the progression of cellular aging to bind and neutralize the activity of TGF-β1 or other proteases up-regulated in senescent cells. α2M has been demonstrated to increase DNA synthesis and cell division in macrophages (Misra and Pizzo, 2002).

A possible contribution of α2M to the etiology of Alzheimer’s disease has been suggested by many lines of biological evidence. First, α2M has been identified as a genetic risk factor for Alzheimer’s disease by genetic linkage analyses (Blacker et al., 1998). Second, α2M has been demonstrated to mediate the clearance and degeneration of Alzheimer β-protein by making a complex with serine protease or enhancing internalization into the cells through low-density lipoprotein-related protein (Hughes et al., 1998; Qiu et al., 1999). Third, the previously demonstrated ability of α2M bind to and neutralize the activity of TGF-β may promote Alzheimer’s disease progression because TGF-β stimulates Alzheimer β-protein clearance by microglial cells and reduces Alzheimer β-protein accumulation in brain parenchyma of mice that over-express human APP (Mettenburg et al., 2002). Further studies on the gene regulation of α2M will contribute greatly to understand both the mechanism of cellular senescence and its role in the etiology of Alzheimer’s disease.
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References

Pignolo, R.J., Cristifalo, V.J., Rotenberg, M.O., 1993. Senescent WI-38 cells fail to express EPC-1, a gene induced in young cells upon entry into the G0 state. J. Biol. Chem. 268, 8949–8957.
Pignolo, R.J., Cristifalo, V.J., Rotenberg, M.O., 1993. Senescent WI-38 cells fail to express EPC-1, a gene induced in young cells upon entry into the G0 state. J. Biol. Chem. 268, 8949–8957.


