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Clusterin/apolipoprotein J is a novel biomarker of cellular senescence that does not affect the proliferative capacity of human diploid fibroblasts

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Abstract Normal human fibroblasts have a limited replicative potential in culture and eventually reach a state of irreversible growth arrest, termed senescence. In a previous study aiming to identify genes that are differentially regulated during cellular senescence we have cloned clusterin/apolipoprotein J (Apo J), a 80 kDa secreted glycoprotein. In the current report we pursue our studies and show that senescence of human diploid fibroblasts is accompanied by up-regulation of both Apo J mRNA and protein levels, but with no altered biogenesis, binding partner profile or intracellular distribution of the two Apo J forms detected. To analyze the causal relationship between senescence and Apo J protein accumulation, we stably overexpressed the Apo J gene in primary as well as in SV40 T antigen-immortalized human fibroblasts and we showed no alteration of the proliferative capacity of the transduced cells. Despite previous reports on tumor-derived cell lines, overexpression of Apo J in human fibroblasts did not provide protection against apoptosis or growth arrest induced by hydrogen peroxide. Overall, our results suggest that Apo J overexpression does not induce senescence but it is rather a secondary consequence of the senescence phenotype. To our knowledge this is the first report that provides a functional analysis of human Apo J during replicative senescence.

Key words: Clusterin/apolipoprotein J; Aging; Replicative senescence; Human fibroblast; Apoptosis; Oxidative stress

1. Introduction

Normal human fibroblasts have a limited replicative potential in culture and eventually reach a state of irreversible growth arrest, termed senescence [1]. During serial passaging primary cultures show a progressive decline in their ability to synthesize DNA that is accompanied by significant changes at the morphological, biochemical and molecular levels (reviewed in [2,3]). In addition, several studies have revealed that cellular senescence relies on a genetic background. For instance, cell fusion and micro-injection studies showed that the senescent phenotype is dominant over immortality (reviewed in [4]). Following these initial studies, many genes have been cloned and characterized as senescence-related genes on the basis of their differential expression upon senescence induction [5–8]. Interestingly, by using oligonucleotide-based arrays in different aging models it was found that about 1% of the genes examined were differentially regulated suggesting that aging and senescence are accompanied by significant alterations in gene expression patterns [9,10]. However, only a small fraction of these genes have been analyzed so far for their ability to affect normal life-span when introduced into primary proliferating and/or immortal cells (see Section 4). This kind of experimentation will provide insights into the senescence phenotype and will determine which of these genes are differentially expressed in order to induce senescence or alternatively, whether their overexpression is a consequence of the induction of the senescence phenotype.

Recently, we took advantage of conditionally immortalized SV40 T antigen (Ag) rat embryo fibroblast cell lines which undergo senescence upon T Ag inactivation [11] and, by applying differential screening methods, we have cloned several genes that associate with mammalian replicative senescence [8]. One of the cloned genes encodes clusterin/apolipoprotein J (Apo J). Initial analysis has shown that senescent cultures of human or rat embryonic fibroblasts as well as of human osteoblasts express high mRNA levels of Apo J [8,12]. Interestingly, stress-inducing agents such as H_2O_2, ethanol, t-BHP or heat shock also result in a significant up-regulation of Apo J mRNA levels in these cells [8,12].

Apo J (also called SGP-2, clusterin, SP40, 40, complement lysis inhibitor, gp80, glycoprotein III and T64) is a glycoprotein encoded by a single-copy gene. The protein is translated from a single mRNA as a preprotein that matures by limited proteolysis to form a disulfide-linked (α- and β-chains) heterodimeric glycoprotein of 70–80 kDa that is secreted by a number of cell types (reviewed in [13,14]). Apo J has been implicated in a variety of physiological processes, including sperm maturation, lipid transport, membrane remodelling and inhibition of the complement cascade (reviewed in [14]). In addition, the Apo J gene is overexpressed in many severe physiological disturbances such as castration-induced apoptosis in rat ventral prostate, neuronal apoptosis and various diseases including scrapie, Alzheimer's disease or AIDS (reviewed in [15]). Although Apo J induction is well established in such pathological states, its precise involvement in these disorders

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remains puzzling. It has also been shown that the gene is up-regulated in response to a variety of stress- and/or apoptosis-inducing agents including tumor necrosis factor α (TNFα) and transforming growth factor β treatment [16,17], heat shock, UVA exposure and oxidative stress [8,12,18,19] as well as treatment with chemotherapeutic drugs ([20], Trougakos et al., submitted). Although it was initially thought that Apo J was related to cell death it was then found that in fact, the protein is overexpressed in the surviving neighboring cells [16,21–23]. Moreover, it was shown that stable Apo J overexpression in cancer cells could confer protection against various cytotoxic agents such as UV radiation, heat shock, oxidative stress, TNFα and chemotherapeutic drugs ([16,17,19,20], Trougakos et al., submitted), thus raising the hypothesis that Apo J may be a survival gene exerting a protective function on surviving cells. In addition, Apo J response to some cytotoxic agents is accompanied by an altered biogenesis that results in the appearance of stress-specific cytoplasmic or nuclear forms [17,24,25]. To add to the complexity regarding Apo J function, recent reports propose that a truncated Apo J nuclear form may induce death signals through interaction with the DNA helicases Ku70 and Ku80 [26,27]. Therefore, no genuine function has been attributed to the Apo J protein so far.

All mentioned studies have focused on Apo J functions in tumor-derived cell lines. However, hardly anything is known regarding Apo J function in normal cells. In the current report we pursue our previous studies by examining Apo J function in proliferating and senescent human diploid fibroblasts (HDFs). We have verified, by a combination of molecular, biochemical and cellular techniques, the senescence-related induction of two Apo J protein forms and we provide evidence for no altered biogenesis, partner binding, or intracellular distribution between cellular proliferation and senescence. Furthermore, we have demonstrated that stable human Apo J overexpression in primary or immortal human fibroblasts does not affect their proliferative capacity. Finally, we have shown that Apo J does not protect these cells from H_2O_2-mediated apoptosis or growth arrest, contrary to recent reports in tumor-derived cell lines [28]. According to these results a putative role of Apo J in human fibroblasts is proposed.

2. Materials and methods

2.1. Cells and cell culture

Human diploid WI-38 fibroblasts and the SV40 T Ag-immortalized WI-38 VA 13 cell line (WI-38/SV40 T Ag) were obtained from the European Collection of Cell Cultures and were maintained in Dulbecco's modified Eagle's medium (Gibco Life Technologies), supplemented with 10% (v/v) fetal calf serum, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin and 1% non-essential amino acids (complete medium). WI-38 fibroblasts were subcultured at a split ratio 1:2 when cells were confluent until they reached senescence at about 45 cell population doublings (CPD). Primary rat embryonic fibroblast (REF) cultures were prepared from 12-day-old Sprague–Dawley rat embryos after passaging 10^4 cells/cm^2 every 72 h and they were maintained in the above-mentioned medium. In all experimental procedures described below, cells were fed approximately 16 h prior to the assay.

2.2. RNA analysis

Total RNA (20 μg) from early passage proliferating (20 CPD) and late passage senescent (43 CPD) WI-38 cells were extracted, electrophoresed, transferred to Hybond N membrane (Amersham) and hybridized to human Apo J or 28S rRNA probes by standard methods [29].

2.3. Immunoblot analysis

Cell monolayers were lysed in non-reducing or reducing Laemmli buffer [30]. Analysis of Apo J levels in cell culture supernatants was done in 100 μl of culture medium diluted directly 1:1 in 2×Laemmli buffer. Protein samples (25 μg) were separated by 10% SDS–PAGE, followed by immunoblotting analysis according to standard procedures [30]. Antibodies used were: goat polyclonal antibody against human Apo J (sc-6419; Santa Cruz Biotechnology), actin antibody (sc-1616; Santa Cruz Biotechnology) and $p21^{Waf1/Cip1/Sdi1}$ mouse monoclonal antibody (sc-817; Santa Cruz Biotechnology). All antibodies were diluted 1:1000 in 5% non-fat dry milk in Tris-buffered saline containing 0.1% Tween 20 (blocking solution). Secondary antibodies used (horseradish peroxidase-conjugated anti-goat or -mouse IgG; Santa Cruz Biotechnology) were diluted 1:2000 in blocking solution. Immunoreactive bands were visualized by ECL enhanced chemiluminescence (Amersham). Quantification of immunoreactive protein bands was carried out by a densitometric analysis directly from the X-ray films using a Molecular Dynamics (Sunnyvale) scanner and the ImageQuant software.

2.4. Apo J immunoprecipitation

Early and late passage (CPD 20 and 43 respectively) WI-38 fibroblasts were starved in methionine-deficient medium for 1 h, followed by pulse labeling for 1 h with 100 μCi/ml of [^{35}S]methionine in methionine-deficient medium. Cell monolayers were lysed in 50 mM Tris–HCl (pH 8.0) containing 150 mM NaCl and either 1% or 0.5% NP-40. Cell extracts were cleared by adding normal goat or mouse serum and protein G agarose beads (Santa Cruz Biotechnology) for 1 h at 4°C. Apo J was specifically immunoprecipitated by adding in the pre-cleared extracts either 2 μg of the goat anti-human Apo J antibody (sc-6419; Santa Cruz Biotechnology) or 1 μg of anti-human Apo J α-chain monoclonal antibody (Upstate Biotechnology) plus 1 μg of anti-Apo J monoclonal antibody (Quidel Company) for 1 h on ice. Following addition of protein G agarose beads the binding reactions were performed overnight at 4°C. Immunoprecipitated protein complexes were eluted from the agarose beads by boiling for 5 min in non-reducing Laemmli buffer, were separated on 10% SDS–PAGE gels, gels were fixed, amplified, dried and directly exposed to X-ray films.

2.5. Immunofluorescence microscopy

Immunofluorescence labeling of Apo J in early and late passage WI-38 cells was done according to standard procedures [30]. Briefly, viable cells grown on coverslips were fixed with 3% freshly prepared paraformaldehyde, followed by cell permeabilization with 0.2% Triton X-100. Apo J was immunolocalized using the goat polyclonal antibody against human Apo J (sc-6419; Santa Cruz Biotechnology) diluted 1:50 in phosphate-buffered saline containing 0.1% Tween 20 and 2% bovine serum albumin (blocking buffer) and then an anti-goat IgG/fluorescein isothiocyanate-conjugated antibody (Santa Cruz Biotechnology) diluted 1:250 in blocking buffer. Images of the mounted coverslips were taken by using a Nikon 2000 confocal laser scanning microscope (CLSM). Routine procedures applied as controls to demonstrate the specificity of the antibody used were: (a) the usage of normal goat serum instead of the reactive antibody and (b) omission of the first antibody. In all control cases no immunofluorescent background was observed.

2.6. Construction of Apo J expression vectors and generation of Apo J-expressing WI-38/SV40 T Ag, WI-38 and REF cells

The full-length human Apo J cDNA (−35 to +1507 bp) [31] was isolated from pGAD10 vector (a generous gift of Dr. Howe) and was directly subcloned either into the pcDNA3.1 (Invitrogen) mammalian expression vector (pcDNA3.1^{ApoJ}) or into the pBabe-puro retroviral vector (pBabe-puro^{ApoJ}). The WI-38/SV40 T Ag and REF cells were transfected with either pcDNA3.1^{ApoJ} or pcDNA3.1 empty vector using the electroporation method. Briefly, 10^7 cells mixed with 50 μg DNA were electroporated at 260 V, 960 μF (Gene Pulser™, Bio-Rad). Transfected cells were split 48 h later and were maintained in complete medium containing 400 μg/ml G418. Colonies of stable transfectants were isolated from the selection medium 3–4 weeks later and propagated accordingly. The production of amphotropic recombinant retroviruses and infection of WI-38 fibroblasts was per-

formed essentially as described by Kolettas and Rosenberger [32]. Briefly, amphotropic replication-defective recombinant retroviruses were produced by transfecting ecotropic ΨCRE cells with the retroviral constructs pBabe-puro (empty vector) or pBabe-puro^{ApoJ} and collecting the released retroviral particles, following selection in puromycin, to infect the amphotropic PA317 packaging cell line. WI-38 fibroblasts at CPD 23 were infected with the recombinant retroviruses Babe-puro or Babe-puro^{ApoJ} collected from overnight cultures of puro-resistant PA317, selected in 1.5 μg/ml puromycin for 2 weeks and propagated as mass cultures. In all cases, transfectants were tested for Apo J protein expression levels by immunoblotting, as described above.

2.7. TUNEL analysis, survival and clonogenic assays of WI-38/SV40 T Ag fibroblasts treated with H_2O_2

In order to detect DNA fragmentation in intact cells during H_2O_2-induced apoptosis, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) was performed using the in situ Cell Death Detection Kit (fluorescein, Roche). Briefly, cells were seeded in 6-well plates in duplicate at a density of 5×10^4 cells/well, allowed to attach overnight and exposed to various H_2O_2 concentrations for 2 h. TUNEL reaction was applied as recommended by the manufacturer and positive apoptotic nuclei were documented by fluorescence microscopy. For the survival and the clonogenic assays, cells were seeded in 6-well plates in duplicate at a density of 5×10^4 cells/well (survival assay) or at the clonal density of 5×10^3 cells/well (clonogenic assay) and allowed to attach overnight. Cells were then exposed for 2 h to either 400 and 800 μM (survival assay) or 100 and 400 μM H_2O_2 (clonogenic assay) diluted in complete medium and returned thereafter to normal complete medium. H_2O_2-treated surviving cells were identified by the trypan blue exclusion staining method [29] 24 h after H_2O_2 removal. Growth ability (i.e. colony formation) of the 100 and 400 μM H_2O_2-treated cells was assessed 1 week following H_2O_2 treatment by directly counting the methylene blue-stained colonies. Percentage of cell viability or colony formation refers to the cells surviving or to the colonies formed following H_2O_2 treatment as compared to the respective values obtained by the corresponding non-treated control cells. All experiments were done in duplicate and the presented results refer to the mean values and standard deviation of two independent experiments.

3. Results

3.1. Cellular senescence is accompanied by up-regulation of Apo J, but with no altered biogenesis or differentiation of its intracellular distribution

We have shown in previous studies that there is a transcriptional up-regulation of the Apo J gene during replicative senescence of various cell types [8,12]. Therefore we determined whether this mRNA up-regulation is also reflected at the protein level and whether there is an induction of any novel senescence-induced Apo J form(s) or alteration of the intracellular distribution of the protein, as has been reported in other cellular processes [17,24,25]. As shown in Fig. 1A RNA analysis revealed a significant overexpression of the Apo J mRNA levels in late passage senescent HDFs as compared to early passage proliferating cells, in agreement with previous data. Since Apo J is constitutively secreted by mammalian cells [14], we then analyzed the Apo J protein levels in cell culture supernatants and in whole cell lysates of proliferating and senescent cells. Immunoblotting analysis shown in Fig. 1B demonstrated an approximate 2.5-fold induction of all the immunoreactive Apo J bands in senescent HDFs. In both cell lysates two Apo J forms were detected with molecular weights, under non-reducing conditions, of about 80 and 68 kDa (Fig. 1B, right panel). In agreement with previous studies focusing on the various Apo J forms and their processing ([14] and references therein), the 80 kDa form that corresponds to the secreted Apo J was also found in cell culture supernatants (Fig. 1B, left panel) and dissociates into two subunits (α and β) under reducing conditions by cleavage at the Asp^{227}-Ser^{228} residues. Accordingly, the 68 kDa band that corresponds to the cytoplasmic Apo J, as expected, was not found in cell culture supernatants and it did not dissociate into α and β subunits under reducing conditions (Fig. 1B).

To verify further the enhanced secretion rate of the 80 kDa Apo J form in senescent HDFs, the secreted Apo J amount in the culture supernatants of about 70% confluent flasks of proliferating and senescent WI-38 cells was analyzed at different time intervals following the addition of fresh complete medium. As demonstrated in Fig. 1C at all eight distinct time points examined (from 15 min to 24 h), the secretion rate and thus the extracellular levels of the 80 kDa Apo J form are higher in late passage HDFs as compared to the early passage cultures. Moreover, the observation that the protein amount in the medium (in both cell states) is continuously increasing provides evidence that in senescent cells (as in the proliferating ones) the protein is continuously secreted via the constitutive secretory pathway. Thus the enhanced Apo J levels in senescent cells are not due to their inability to secrete the protein.

Finally, to examine whether the quantitative differences seen in the Apo J protein expression levels between proliferating and senescent HDFs corresponds to a differentiated intracellular distribution pattern, we immunolocalized Apo J in these cells. As is apparent in the CLSM images shown in Fig. 1D, in both cellular states a dispersed punctuated cytoplasmic distribution, indicative of vesicular packaging, was observed. In accordance with the previously shown results the immunofluorescence signal observed in senescent cells appeared to be significantly enhanced as compared to their young counterparts. In summary these data demonstrate that there is no Apo J altered biogenesis or intracellular distribution during replicative senescence. Moreover, both pathways (through which the nascent Apo J polypeptide is post-translationally processed) that lead to the accumulation of the extracellular (80 kDa) and the intracellular (68 kDa) Apo J forms are regulated in a similar manner in both proliferating and senescent HDFs.

3.2. Comparative Apo J immunoprecipitation in proliferating and senescent WI-38 fibroblasts

Next we analyzed whether the progression of replicative senescence is accompanied by an altered profile of Apo J co-immunoprecipitated intracellular proteins. For these experiments Apo J was comparatively immunoprecipitated from metabolically labeled early and late passage WI-38 cells. Cells were lysed under stringent (1% NP-40) or less stringent (0.5% NP-40) conditions and Apo J was immunoprecipitated either with the polyclonal antibody used for the immunoblotting analysis or with a combination of two monoclonal antibodies that recognize both the 80 and 68 kDa Apo J forms. A representative analysis is shown in Fig. 2. In all conditions used several polypeptides were found to co-immunoprecipitate with Apo J but none of them appeared to interact with Apo J in a selective manner in early or late passage fibroblasts. (As most of the reported Apo J binding ligands are extracellular molecules [14,24,27], we are currently characterizing by matrix-assisted laser desorption ionization mass spectrometry the major Apo J binding intracellular ligands.) Although these experiments do not exclude the possibility that Apo J may

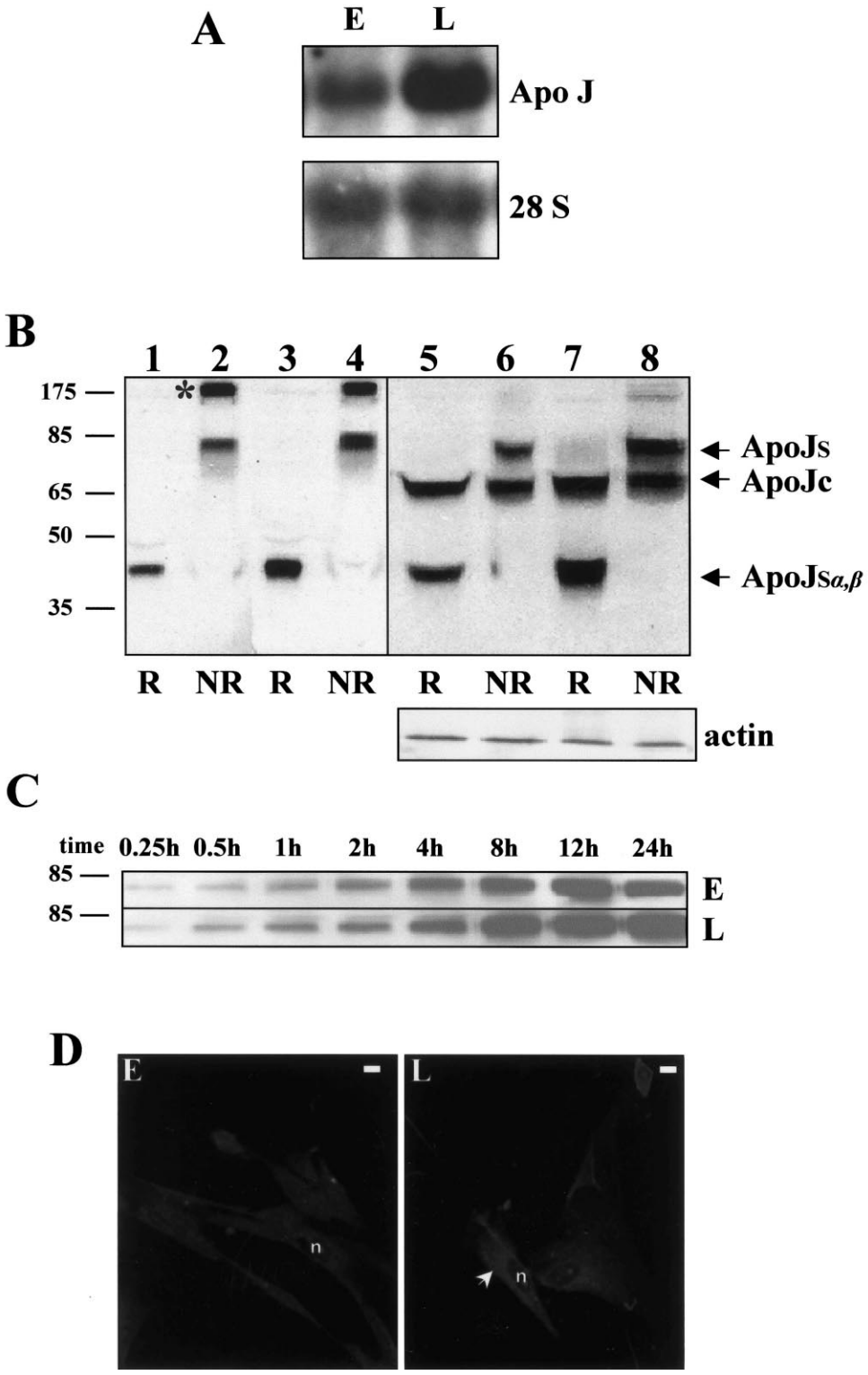


Fig. 1. Apo J gene expression, protein levels and cell distribution in early passage (E) proliferating (CPD 20) and late passage (L) senescent (CPD 43) WI-38 fibroblasts. A: Northern blot analysis of RNA transcript hybridized to the Apo J cDNA probe (upper panel). Stripping of the blot and reprobing with a 28S rRNA probe was done to ensure equal RNA loading (lower panel). B: Immunoblotting analysis of the Apo J amount present in the cell culture supernatants (lanes 1–4) and whole cell lysates (lanes 5–8) in early (lanes 1, 2, 5 and 6) and late passage (lanes 3, 4, 7 and 8) WI-38 fibroblasts in reducing (R) or non-reducing (NR) conditions (star in lane 2 indicates an antibody cross-reacting band of unknown origin). Equal protein loading was further verified by stripping the membrane and reprobing with an actin antibody (lower right panel). C: Immunoblotting analysis of Apo J accumulation in the cell culture supernatants at different time points (time 0 corresponds to culture medium renewal) in early and late passage WI-38 fibroblasts. D: CLSM images following Apo J immunofluorescence localization in early and late passage WI-38 fibroblasts. The protein is distributed in the cytoplasm, while the signal is significantly increased (arrow) in the senescent cells. The nucleus (n) in both cellular states appears devoid of any immunofluorescence. ApoJs: secreted Apo J form (~80 kDa); ApoJc: cytoplasmic Apo J form (~68 kDa); ApoJsα,β: α- and β-chains of the secreted Apo J form following reduction of the protein (~40 kDa); bars in D: 10 μm. Molecular weight markers (in kDa) are indicated on the left of each blot.

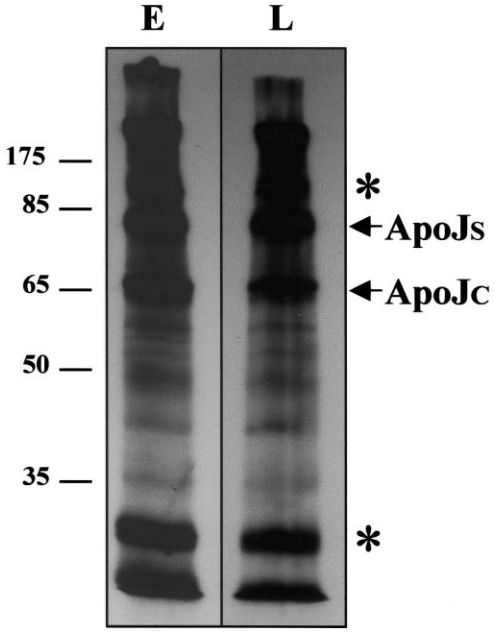


Fig. 2. Representative Apo J immunoprecipitation from whole cell extracts of early (E) and late passage (L) WI-38 fibroblasts. Equal numbers of early and late passage cells were lysed with 1% NP-40 lysis buffer and Apo J was immunoprecipitated using a goat anti-human Apo J polyclonal antibody as described in Section 2. The secreted (ApoJs) and cytoplasmic (ApoJc) Apo J forms are indicated. Stars indicate enriched polypeptides which we are currently identifying by mass spectrometry. Molecular weight markers (in kDa) are shown on the left of the autoradiographs.

weakly associate with different proteins in proliferating or senescent fibroblasts under conditions other than those used here, on the basis of the demonstrated recognition pattern of the antibodies used, we suggest that Apo J overexpression in senescent HDFs is not coupled with a differentiated pattern in terms of protein–protein interactions.

3.3. *Stable overexpression of the Apo J gene does not affect the proliferative capacity of WI-38 or WI-38/SV40 T Ag fibroblasts*

The appearance of the replicative senescence phenotype in primary cells is accompanied by the differential expression (negative or positive growth effectors) of a significant number of genes (see Section 1). However, despite the high number of senescence-related cloned genes only a limited number of them was shown to affect normal life-span when overexpressed in either immortal or primary mammalian cells (see Section 4). A major question, therefore, when analyzing the function of a senescence biomarker (such as Apo J), is whether the observed differentiated gene expression is directly related to the induction of the senescence phenotype or represents a secondary event of replicative senescence. Thus we have overexpressed the human Apo J gene in both primary and SV40 T Ag-immortalized WI-38 fibroblasts and determined the gene's ability to alter the proliferative capacity of the transduced cells.

The full-length human Apo J cDNA was cloned into the expression vector pcDNA3.1 and introduced by electroporation into the WI-38/SV40 T Ag fibroblasts, and after appropriate antibiotic selection several colonies were isolated and expanded into cell lines. Selected clones were then tested for Apo J protein expression levels by immunoblotting analysis. As demonstrated in Fig. 3A,B for two representative clones (WI-38/SV40 T $Ag^{ApoJ2.1}$ and WI-38/SV40 T $Ag^{ApoJ2.5}$) the introduced construct results in significant overexpression of both the 68 kDa and 80 kDa Apo J forms (Fig. 3A, lanes 7–10). Moreover, the engineered Apo J protein is processed along the secretory pathway and secreted in the cell culture supernatants (Fig. 3B, lanes 7–10) exactly as their endogenous counterparts in the parental WI-38/SV40 T Ag (Fig. 3A,B, lanes 3, 4) and the WI-38 (Fig. 3A,B, lanes 1, 2) cells. No Apo J overexpression was seen when cells were transfected with the empty pcDNA3.1 vector (WI-38/SV40 T $Ag^{pcDNA3.1}$; Fig. 3A,B, lanes 5, 6). It is worth mentioning that the difference noticed in the Apo J expression levels between the WI-38/SV40 T Ag and the WI-38 primary fibroblasts is consistent with previous data showing that senescence-induced genes are suppressed in immortalized and/or transformed cells [8,33]. All the obtained Apo J-overexpressing WI-38/SV40 T Ag^{ApoJ} cell lines did not show any difference in cell morphology when compared with the non-transfected WI-38/SV40 T Ag or with the WI-38/SV40 T $Ag^{pcDNA3.1}$ cells. Moreover, growth rates between the parental cell line and the WI-38/SV40 T Ag^{ApoJ} clones were similar even after several months of subcultivation (data not shown). It is therefore apparent that Apo J overexpression in the WI-38/SV40 T Ag immortal cell line does not induce any of the replicative senescence-related morphological alterations and does not affect the cellular growth rate.

Since SV40 T Ag binds several proteins involved in the regulation of irreversible growth arrest and senescence (for example p53) we pursued our studies by examining whether Apo J overexpression in primary WI-38 cells can affect the growth rate and the proliferative life-span of these cells. The full-length human Apo J cDNA was cloned into the pBabe-puro vector and introduced by retrovirus-mediated gene transfer in early passage proliferating WI-38 fibroblasts. Following initial antibiotic selection, cells infected with the Apo J-encoded retrovirus (WI-38^{ApoJ}; we have avoided the clonal selection step that could exhaust the limited proliferative capacity of primary cells) were examined by immunoblotting for Apo J expression levels (Fig. 3C, upper panel, lane 3), in parallel with cells infected with the empty retroviral vector (WI-$38^{pBabe-puro}$) and parental cells (WI-38; Fig. 3C, upper panel, lanes 2 and 1 respectively). Apo J-infected cells were found to express almost 10 times higher Apo J levels as compared to control vector-infected or uninfected human fibroblasts. All cell types (WI-38, WI-$38^{pBabe-puro}$ and WI-38^{ApoJ}) were propagated until they reached replicative senescence as concluded by the development of several markers related to the senescence phenotype (i.e. senescence-associated β-galactosidase activity, morphological features and DNA synthesis levels). WI-38 cells reached senescence at mean CPD 45, WI-$38^{pBabe-puro}$ cells at CPD 44 and WI-38^{ApoJ} cells at CPD 47 (average values of two independent experiments; Fig. 1D). Furthermore, the cells were kept for over 3 months in culture and no clones with extended life-span or with immortal phenotype emerged. In addition no differences were observed in cell morphology, growth rates or expression level of the cyclin-dependent kinase inhibitor $p21^{Waf1/Cip1/Sdi1}$ (an established biomarker of senescence; Fig. 3C, middle panel) among the Apo J-infected and control cells during the selection process and subsequent subcultivation. Therefore these data suggest that, as was found in WI-38/SV40 T Ag Apo J trans-

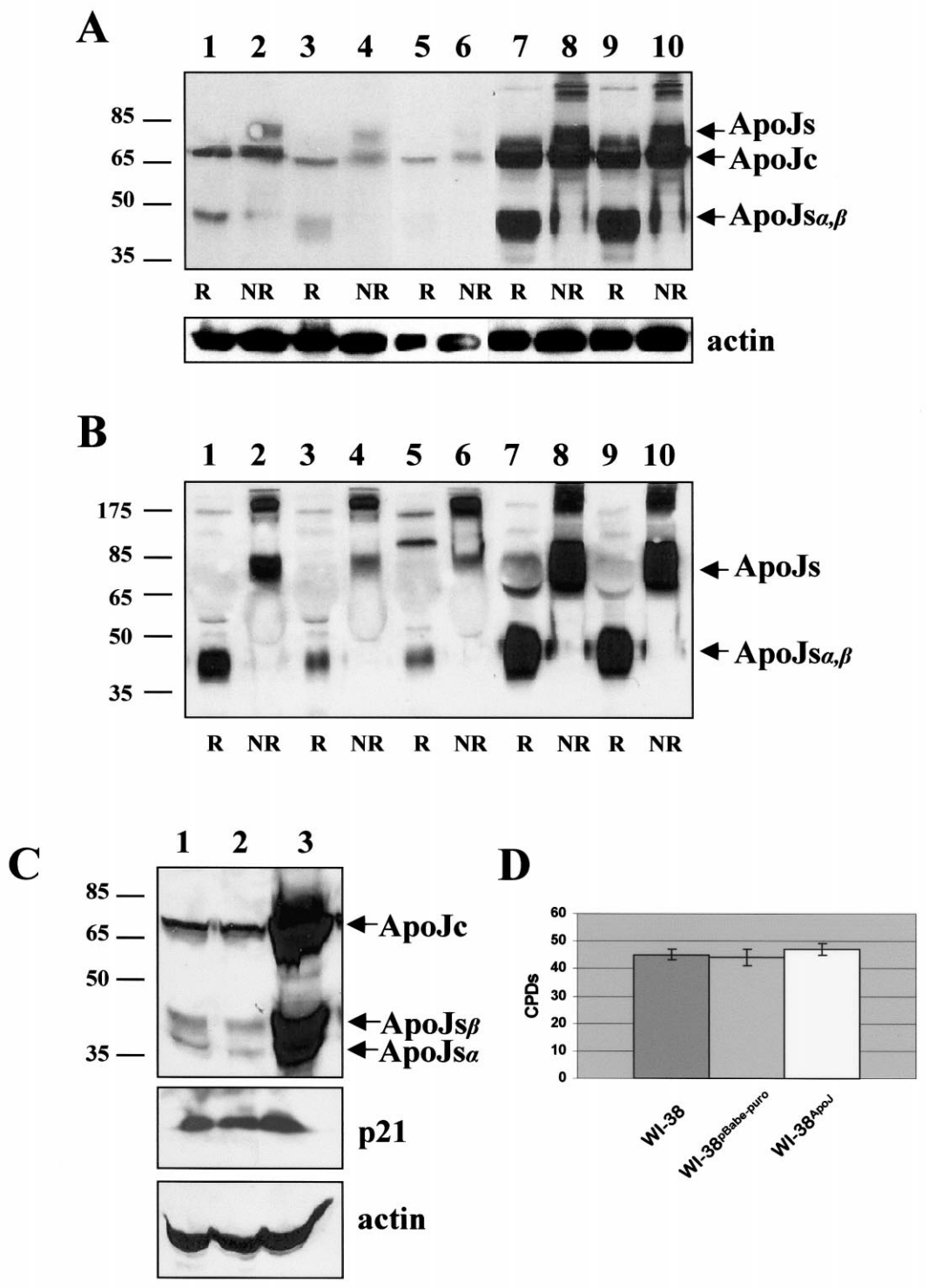


Fig. 3. Apo J protein levels following stable transfection of pcDNA3.1^{ApoJ} and retroviral infection of pBabe-puro^{ApoJ} constructs into the WI-38/SV40 T Ag (A,B) and WI-38 fibroblasts (C) respectively. A,B: Immunoblotting analysis of the Apo J protein levels, under reducing (R) and non-reducing (NR) conditions, in whole cell lysates (A) and cell culture supernatants (B). Lanes 1, 2: WI-38 cells; lanes 3, 4: WI-38/SV40 T Ag cells; lanes 5, 6: WI-38/SV40 T Ag$^{pcDNA3.1}$ cells; lanes 7, 8 and 9, 10: WI-38/SV40 T Ag$^{ApoJ2.1}$ and WI-38/SV40 T Ag$^{ApoJ2.5}$ selected clones respectively. C: Apo J expression levels in whole cell lysates of WI-38 (lane 1), WI-38$^{pBabe-puro}$ (lane 2) and WI-38^{ApoJ} fibroblasts (lane 3; upper panel); p21$^{Waf1/Cip1/Sdi1}$ (middle panel) and actin (lower panel) levels in the same cells at CPD 28. D: Maximum CPDs reached in WI-38, WI-38$^{pBabe-puro}$ and WI-38^{ApoJ} cells. Values represent the mean of two independent experiments. ApoJs: secreted Apo J form (~80 kDa); ApoJc: cytoplasmic Apo J form (~68 kDa); ApoJsβ, ApoJsα: β- (~40 kDa) and α-chain (~35 kDa) respectively, of the secreted Apo J form following reduction of the protein. Molecular weight markers (in kDa) are indicated on the left of each blot.

fectants, Apo J overexpression does not affect normal life-span in WI-38 fibroblasts. Similar results were also obtained following overexpression of human Apo J cDNA in REFs isolated from 12-day-old rat embryos. As in HDFs, Apo J stable overexpression did not affect the replicative life-span of transfected cells as compared with their control counterparts (data not shown). In agreement with these data, retroviral infection of a canine Apo J construct in WI-38 fibroblasts also did not affect normal life-span (Dumont et al., submitted). In conclusion, these data demonstrate that stable Apo J overexpression does not affect the proliferative capacity of human fibroblasts.

3.4. WI-38 and WI-38/SV40 T Ag fibroblasts overexpressing Apo J exhibit survival and growth ability to H_2O_2-induced cytotoxicity similar to their parental cell lines

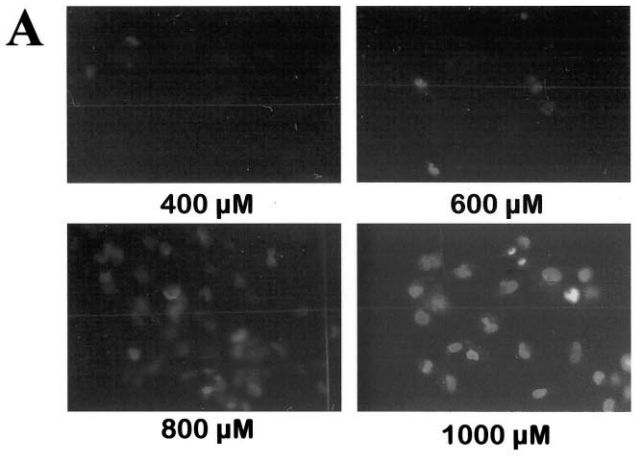
Recent studies focused on the Apo J function have revealed that it may act as a survival gene against cytotoxic stresses induced by different kinds of agents (see Section 1). These studies, however, are related exclusively to tumor-derived cell lines. Exposure of proliferating mammalian fibroblasts to various H_2O_2 concentrations can induce temporary growth arrest (100–120 μM), irreversible growth arrest (150–450 μM), or apoptosis (500–1000 μM) (reviewed in [34]). We [12] and others [35] have shown that treatment of young WI-38 fibroblasts with H_2O_2 concentrations ranging from 150 to 450 μM (single treatments) results in the eventual induction of a senescence-like phenotype, which is accompanied by a significant overexpression of Apo J mRNA levels. Moreover, initial analysis has shown that WI-38 and WI-38/S40 T Ag fibroblasts treated with various H_2O_2 concentrations exhibit an approximate two-fold increase in Apo J protein synthesis 24 h after treatment (data not shown), which is in agreement with previous reports in tumor-derived cell lines [19]. Therefore we have addressed two complementary and interrelated questions: (a) whether Apo J acts as a survival factor in primary or immortalized cells as is the case in tumor-derived cell lines and (b) whether WI-38 and WI-38/SV40 T Ag Apo J-overexpressing cells exhibit different growth rates after H_2O_2 treatment, as compared to parental cell lines.

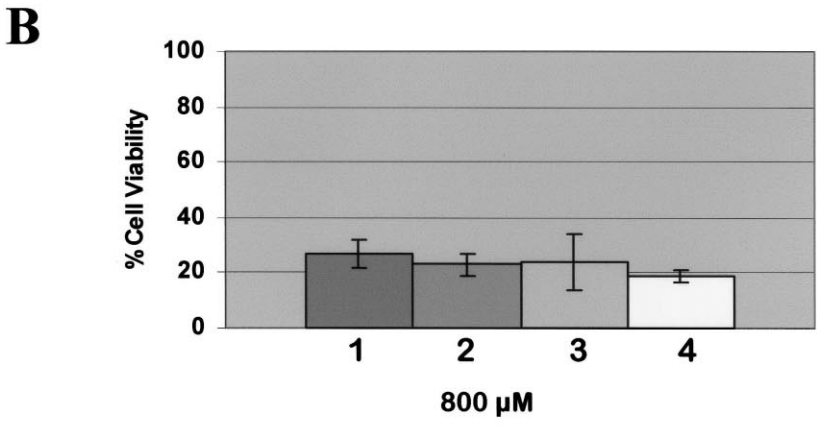
We have established first the H_2O_2 concentrations at which there is induction of apoptosis in WI-38/SV40 T Ag fibroblasts. Cells were treated with a range of H_2O_2 concentrations for 2 h and assayed for initiation of apoptosis by TUNEL. As shown in Fig. 4A and in accordance with previous reported data [34], only ∼10% of cells treated with 400 μM H_2O_2 were found to undergo apoptosis (the remaining cells eventually entered a state of irreversible growth arrest). However and as expected, apoptosis was massive at much higher H_2O_2 concentrations (800 or 1000 μM) as ∼75% and ∼90% of treated cells respectively were found to be apoptosis-positive (Fig. 4A). Treatment of WI-38/SV40 T Ag, WI-38/SV40 T $Ag^{pcDNA3.1}$ and WI-38/SV40 T $Ag^{ApoJ2.1\ and\ 2.5}$ cell lines with 800 μM H_2O_2 for 2 h did not result in any significant difference in their survival rates, as indicated by the trypan blue method (Fig. 4B). Similar results were also obtained at 400 μM H_2O_2, where apoptosis is only present at basal levels (Fig. 4C); these results, regarding the ability of the various clones to survive H_2O_2 treatment, were also verified by comparative TUNEL analysis (data not shown). We then examined whether stable Apo J overexpression in immortalized fibroblasts could inhibit or retard the growth arrest induction observed after treatment of cells with moderate H_2O_2 concentrations. Cells were seeded in clonal density, exposed to 100 μM H_2O_2 for 2 h, allowed to recover from the shock, proliferated for 7 days and the formed colonies were recorded as described in Section 2. As demonstrated in Fig. 4D, no significant difference was seen among the Apo J-overexpressing clones and the control cells. Similar results were also obtained when cells were treated with 400 μM H_2O_2, which induces a permanent growth arrest (data not shown). Finally we also treated WI-38, WI-$38^{pBabe-puro}$ and WI-38^{ApoJ} cells at CPD 28 with 400 μM H_2O_2 for 2 h. All cells exhibited similar survival rates and they reached a state of irreversible growth arrest after 5–7 CPD. It is thus evident that Apo J overexpression in either primary or SV40 T Ag-immortalized WI-38 fibroblasts does not confer increased resistance to oxidative stress induced by H_2O_2. In support of the reported data, in an independent study by the Toussaint laboratory, it was also found that overexpression of the canine homologue of human Apo J, the gp80 gene, in WI-38 or WI-38/SV40 T Ag cells did not confer increased resistance to H_2O_2 (Dumont et al., submitted). Interestingly, in a tumor-derived cell line (LLC-PK_1 cells), Apo J does protect against H_2O_2 [28]. This discrepancy regarding the cytoprotective function of Apo J reported in tumor-derived cell lines as opposed to Apo J cytoprotective inability found in primary or SV40 T Ag-immortalized WI-38 fibroblasts is further documented by additional data from our laboratory. Treatment of two clones (WI-38/SV40 T $Ag^{ApoJ2.1\ and\ 2.5}$) with the chemotherapeutic drug doxorubicin does not result in higher survival rates as compared with the parental cell lines (data not shown). However, U2-OS and KHOS osteosarcoma cell lines stably transfected with the Apo J construct used in this study (pcDNA3.1^{ApoJ}) have a significantly increased resistance against the same drug (Trougakos et al., submitted).

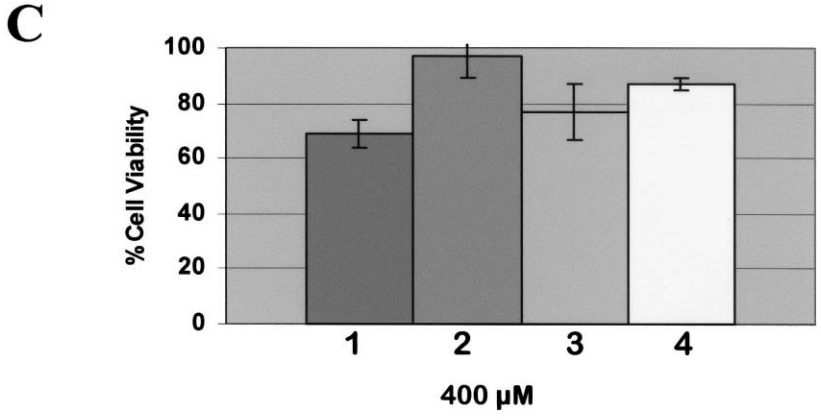
4. Discussion

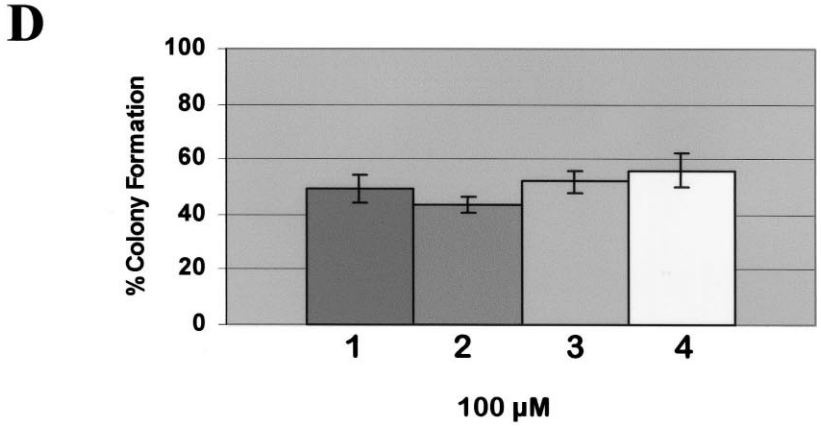
In this article we have shown the senescence-related induction of Apo J RNA levels and two protein forms and we provided evidence for no Apo J-altered biogenesis, partner binding, or intracellular distribution between cellular proliferation and senescence. Furthermore, we demonstrated that stable human Apo J overexpression in primary or SV40 T Ag-immortalized human fibroblasts did not affect their proliferative capacity and did not protect them from H_2O_2-mediated cytotoxicity.

Two Apo J forms with apparent molecular weights of 80 and 68 kDa were detected, both found up-regulated during senescence. Of these forms, the 80 kDa is constitutively secreted and can be dissociated into two subunits under reducing conditions indicating that it has been appropriately cleaved at the Asp^{227}-Ser^{228} residues [14]. The 68 kDa Apo J form is not reduced and is retained intracellularly, with no altered binding partner profile or intracellular distribution during senescence. Our observations are in agreement with those of Humphreys et al. [17] who showed the existence of a 80 kDa and an uncleaved 68 kDa Apo J form in L929 cells. Similar forms have been found in HepG2 [36] and MDCK cells [37] as well as in normal and regressing rat prostate cells [25]. In these studies it has been suggested that the high molecular weight Apo J form represents the mature glycosylated protein, while the low molecular weight form is a partially


A
400 μM
600 μM
800 μM
1000 μM


B
% Cell Viability
100
80
60
40
20
0
1
2
3
4
800 μM


C
% Cell Viability
100
80
60
40
20
0
1
2
3
4
400 μM


D
% Colony Formation
100
80
60
40
20
0
1
2
3
4
100 μM

glycosylated uncleaved precursor that is retained intracellularly [25]. Therefore the two forms we found in HDFs should correspond to the Apo J polypeptides previously described suggesting that the Apo J post-translational processing is neither cell type-dependent nor differentiated among normal and cancer cells. In addition, the absence of an altered binding profile and protein–protein interaction of Apo J during senescence is in accordance with previous reports (no protein coimmunoprecipitates specifically with Apo J in heat-shocked A4321 cells) [19] and indicates a rather conserved panel of proteins that can interact (at least intracellularly) with Apo J. Although apoptosis-related truncated Apo J forms that either remain in the cytoplasm or accumulate in the nucleus have also been reported [17,24,25,27], we did not detect any senescence-specific Apo J form or translocation of Apo J to the nucleus. Thus, we propose that Apo J biogenesis is not altered following the induction of HDF senescence. Overall, it is obvious that these two distinct Apo J processing pathways related to post-translational protein maturation and to Apo J constitutive secretion do not depend on whether cells are actively proliferating or whether they enter a state of irreversible growth arrest.

Numerous genes have been cloned up to now on the basis of their differential expression during cellular senescence in various mammalian models [5–8]. In fact, using oligonucleotide-based arrays, it was found that about 1% of the examined genes are differentially regulated upon aging in vitro or in vivo [9,10]. The identified senescence-related genes are involved in a variety of different cellular functions; they encode extracellular matrix (ECM) proteins, cell cycle control proteins, chaperones, proteins involved in DNA synthesis and repair, in chromosomal processing and assembly, in protein processing, etc. [9,10,38]. These data demonstrate the functional heterogeneity of senescence-related genes and they strongly suggest that senescence (and most likely mammalian aging) is unlikely to be controlled by a single gene family (reviewed in [39]). An important issue that we need to address in full is whether the differential expression of the verified genes is directly related to the appearance of the senescent phenotype or not. However, for only a few of the isolated genes there is, so far, evidence supporting their direct implication in the induction or inhibition of senescence. These genes can be classified in two main categories. First, those that when overexpressed promote growth arrest, such as the cyclin-dependent kinase inhibitors, $p21^{Waf1/Cip1/Sdi1}$, $p16^{INK4A}$ and $p15^{INK4B}$ [40,41], the promyelocytic leukemia protein, PML [42], the HSP70 family member, mortalin-1 [43], the nuclear localized protein, Hic-5 [44], the *ras* oncogene [45] and the transcription factor, MORF4 [46]. Second, those that extend the proliferative lifespan; the only known examples are the human telomerase gene, hTERT [47] and the helix-loop-helix protein, Id-1 [48]. For all the remaining genes either there is no experimental evidence up to now or they cannot exclusively account for the phenotypic changes that occur during senescence (for example, the ECM genes). Since Apo J overexpression does not appear to induce any of these cellular responses we suggest that Apo J falls in the category of the numerous genes that are not a prerequisite for the appearance of the senescence phenotype but are induced due to a secondary response and as a result of cell adaptation to a new 'situation'.

There is an association between Apo J increased expression and a wide variety of physiological disturbances, diseases as well as 'stress' conditions (see Section 1). It is clear that Apo J is an oxidative stress-responsive gene and is up-regulated following cell exposure to H_2O_2 [12,35]. Moreover, exogenously added purified Apo J can protect kidney LLC-PK1 cells from cell death mediated by H_2O_2 or aminotriazole and 1-chloro-2,4-dinitrobenzene exposure [28] suggesting that in these cells Apo J acts as an anti-oxidant. It has thus been proposed that the main site of Apo J action is extracellular [14,15] and that Apo J represents a new class of very efficient chaperones similar to the small heat shock proteins (HSP) [49]; the enhanced secretion rate of Apo J in senescent cells favors this hypothesis. Interestingly, microarray analysis showed that the HSP27 gene is up-regulated about 3–4-fold during aging [9], while HSP27 can also prevent apoptotic cell death [50]. In addition, it has been proposed that Apo J might act as a molecular adapter that targets the cellular debris of dead cells to specific cell surface receptors for internalization [23] thus participating in a cellular 'clearance' system. On the basis of these observations, the inability of Apo J to confer protection against H_2O_2-mediated oxidative stress in normal or immortalized human fibroblasts seems puzzling. A possible explanation could be that as might be expected the effects of many stimuli, whether redox-active or not, depend on the cellular machinery that translates the signal into a biological response. Because of differences in cellular composition, a single stimulus can exert profoundly different effects in different cell types on a dose-dependent basis. This assumption is apparently enhanced when cancer and normal cells are compared and is evident in the elegant studies of Collart et al. [51] who showed that the effects of H_2O_2 and IR on c-*jun* induction in various cell types ranged from dramatic increases to no effect at all. Thus, in the case of human fibroblasts the endogenous Apo J up-regulation achieved following exposure to a certain H_2O_2 amount might be enough to saturate the cell and confer the necessary protection, since cells normally will not experience a concentration of 100 μM of H_2O_2 or higher. Alternatively in tumor-derived cell lines a specific signaling pathway that regulates Apo J function may be altered thereby resulting in the enhanced cytoprotective action of the molecule in these cells. Current work in the laboratory is focusing to unravel this very intriguing issue.

Senescent cells are relatively oxidized as compared to early passage proliferating cells [52]. In addition, stress-induced premature senescence of HDFs that can be induced by H_2O_2

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Fig. 4. Effect of H_2O_2 treatment in WI-38/SV40 T Ag and WI-38/SV40 T Ag clones overexpressing Apo J. A: Determination of H_2O_2 (400, 600, 800 and 1000 μM)-induced cellular apoptosis using TUNEL assay in WI-38/SV40 T Ag cells. In each optical field presented about 28–32 cells occur; however, only the fluorescent-positive apoptotic nuclei can be seen. The percentage of apoptotic nuclei to the cells seeded was found to be ∼10% at 400 μM, ∼17% at 600 μM, ∼75% at 800 μM, and almost 90% at 1000 μM of H_2O_2. B,C: Cell viability assessment according to the trypan blue exclusion method, following treatment of WI-38/SV40 T Ag (1), WI-38/SV40 T $Ag^{pcDNA3.1}$ (2), WI-38/SV40 T $Ag^{ApoJ2.1}$ (3), and WI-38/SV40 T $Ag^{ApoJ2.5}$ cells (4) with 800 (B) or 400 μM (C) H_2O_2 for 2 h and cell recovery for 24 h in complete medium. No significant difference in viability is seen between the different cell types. D: Colony formation ability after cell treatment with 100 μM H_2O_2 for 2 h and cell proliferation in complete medium for 7 days. Bars (see Section 2) correspondence is as in B and C.

treatment [53] is a state reminiscent of replicative senescence that among other changes also induces Apo J accumulation [35]. Thus it is plausible that during senescence Apo J accumulation and oversecretion might correlate with either a response to a higher cellular oxidation state and an increased necessity to control and refold partially unfolded molecules [54] or to the cells' attempt to establish a 'surveillance' and/or a 'clearance' system. Even if this hypothesis regarding Apo J function during senescence is correct, a more fundamental issue remains partially addressed: what exactly replicative senescence assays for? Despite the important data from the elaborate work by the Cristofalo group, regarding the lack of correlation between donor age and maximum life-span of the derived skin fibroblast cultures [55], the 'Hayflick replicative senescence model' remains a very valuable system for studying mammalian aging in vitro. Among the various achievements, after using such in vitro systems during the last four decades, several aging biomarkers have been identified (reviewed in [3]). However, we should also bear in mind that culturing cells in vitro, under routinely applied conditions, is per se a stress-inducing phenomenon as cells are experiencing high oxygen, etc. It is, therefore, not surprising that: (a) senescence-associated genes are also up-regulated in stress conditions (for example culturing established cell lines at high temperatures [12]) and (b) several stress-induced genes, such as the HSPs, have also been identified in aging models [9]. Further studies are needed to unravel fully the exact link between these biological processes.

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