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Senescent Human Fibroblasts Resist Programmed Cell Death, and Failure to Suppress *bcl*2 Is Involved[1]

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ABSTRACT

Programmed cell death (apoptosis) is an active process by which cells initiate their own self-destruction. Growing evidence shows that this event is controlled by the activation of unique gene expressions; some function as survival genes, such as *bcl2*, and others as killer genes, such as *ced3* or interleukin converting enzyme. Likewise, external factors, such as the presence or absence of stimuli in the microenvironment of a cell, play a key role in ushering it towards survival or suicidal fate. Previously, I and others have reported that withdrawal of serum from culture medium can induce contact-inhibited quiescent mouse 3T3 fibroblasts to undergo rapid programmed cell death, as evidenced by the presence of massive DNA fragmentation within 24 h. I now report that, although the same process of serum withdrawal is capable of inducing apoptotic death in quiescent young human fibroblasts, the process takes as long as 2 weeks. Repeated attempts at the same serum withdrawal with cultures of senescent human fibroblasts show that phenotypic signs of apoptosis, such as DNA fragmentation and loss of cell viability, are not observed for up to 4 weeks; I suggest that *in vitro* aged human fibroblasts are resistant to undergoing programmed cell death. I have investigated the level of *bcl2* presence as a possible protector of senescent human fibroblasts from apoptotic death; biochemical characterization shows that in mouse as well as human fibroblasts, *bcl2* is present as an easily extractable (0.1% Triton) cytoplasmic protein. *bcl2* level is in inverse relationship with the ease of induction of apoptotic death between young and senescent human fibroblasts. Immunofluorescence staining shows that, in senescent human fibroblasts, *bcl2* is present not only in the cytoplasmic punctate spots seen in both mouse and young human fibroblasts but also in the nuclei as well as large granules surrounding the nuclei. Upon serum deprivation, the *bcl2* level is reduced to undetectable in mouse 3T3 fibroblasts within 24 h and in young and intermediate aged human fibroblasts within 2 weeks; however, it remains unchanged in senescent human fibroblasts after the deprivation of serum for 2 weeks. These findings lead me to conclude that senescent fibroblasts are resistant to the induction of apoptotic death by serum deprivation. Furthermore, I suggest that repeated serial passaging during the *in vitro* aging process has inadvertently instituted a molecular mechanism whereby the *bcl2* level cannot be repressed upon serum deprivation, which may subsequently allow senescent fibroblasts to be long-lived and protected from self-destruction.

INTRODUCTION

In 1961, Hayflick and Moorhead (1) reported that normal diploid human fibroblasts lose their replicating ability after serial passaging in culture; the final phenotype of nonreplicating cells has since been termed senescent fibroblasts (reviewed in Ref. 2). Further characterization showed that the inability to proliferate is dominant, *i.e.*, in a cell hybrid fused between young growing and senescent fibroblasts, the DNA synthesis ability is turned off even in the younger nuclei (3, 4). Recent work shows that this DNA synthesis inhibitory activity is attributed to specific senescence-related genes located on chromosomes 1, 4, and 6 (5, 6) and specifically to the expression of *Sdi1*, a gene product of M_r 21,000 also known as *Pic1* or *Cip1*, a kinase inhibitor for cdks[3] enzyme activity, associated with the p53 protein (7–11).

The specific presence of *Sdi1* in senescent fibroblasts provides the link to another series of findings showing that c-*fos* expression is repressed (12) and RB protein remains unphosphorylated (13) in these cells. Furthermore, senescent fibroblasts are also found to lack the expression of *cdc*2 and cyclin A and B genes (14), as well as the accumulation of inactive *cdk*2-cyclin D1 or E complexes (15). These findings on either the repression or the functional impairment of key cell cycle gene expressions suggest that senescent fibroblasts truly exemplify the permanently "growth-arrested" cell type and lead me to ponder whether fibroblast senescence is the manifest destiny of "antiproliferation" when cell cycle traverse is irreversibly blocked. Based upon this logic, providing a strong promoter such as the SV40 T-antigen to senescent cells allows c-*fos* repression to be lifted, overcomes the blockage to DNA synthesis, and extends cellular life span to more rounds of cell cycle traverse until the loss of replication is again observed permanently (16, 17).[4]

In this report, I describe that senescent human fibroblasts not only cannot proliferate but are also resistant to undergoing programmed cell death. This resistance to enter the self-destructive mode is characterized by the ability of long-term survival in serum-deprived conditions, which with other cell types, such as mouse 3T3 fibroblasts, activates the rapid events of programmed cell death. A high degree of viability, and the absence of typical apoptotic phenotypes such as DNA fragmentation, are observed in senescent fibroblasts when serum is withdrawn for as long as 4 weeks. This resistance to apoptotic death is also related to the persistent high-level presence of *bcl*2. *bcl*2 levels in young and senescent fibroblasts seem to be in inverse relationship to the ease of being induced to undergo apoptosis. Furthermore, upon withdrawal of serum, the *bcl*2 level is seen to be down-regulated to an undetectable level, except in senescent fibroblasts, where it remains high. Therefore, the resistance to apoptosis seen in senescent fibroblasts may be related to the ability to maintain *bcl*2 at high levels in the prolonged absence of serum. These findings lead me to suggest that the senescent phenotype seen in senescent fibroblasts is not only "antiproliferation" but also "antiprogrammed cell death" or "antiapoptosis."

MATERIALS AND METHODS

Cell Cultures. Swiss mouse 3T3 fibroblasts were cultured in DMEM containing 10% FBS and antibiotics (50 units/ml penicillin and 50 μg/ml streptomycin). WI38, a cell strain of normal human fibroblasts, was obtained from the American Type Culture Center (Rockville, MD). The *in vitro* life span of this cell strain, expressed in CPDL, has been assessed previously (1). Cultures of human fibroblasts with less than 15 CPDL were considered young cells with high proliferative ability; at approximately 38 CPDL, cultures were used as intermediate, with less than 50% of their *in vitro* life span remaining.

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[3] The abbreviations used are: cdk, cyclin-dependent kinase; FBS, fetal bovine serum; CPDL, cumulative population doubling levels.

[4] J. Campisi, personal communication.

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When cultures reached 58 CPDL, they were considered as senescent; during a 3-week period, there was no detectable incorporation of [^{3}H]thymidine, reflecting DNA synthesis activity, nor doubling in cell numbers by direct counting of culture population. The details for this determination were published previously (18, 19).

Cultures of human fibroblasts were grown on Petri dishes of 100-mm diameter in MEM supplemented with 10% FBS and 1% nonessential amino acids, as well as 50 units/ml penicillin and 50 μg/ml streptomycin. All cultures were maintained at 37°C in humidified air with 5% CO_2. In general, mouse 3T3 fibroblasts were seeded at 6×10^3 cells/cm^2 and allowed to grow to confluency, with cell density at 6×10^5 cells/cm^2. For the normal human fibroblast, cells were seeded at 3×10^3 cells/cm^2 and allowed to grow to a contact-inhibited confluent state at 1.2×10^7 cells/100-mm dish for the young fibroblast cultures, and for the intermediate age cultures, at $6-8 \times 10^6$ cells/100-mm dish. For senscent cultures, the cell density was around $1-2 \times 10^6$ cells/100-mm dish.

Induction of Programmed Cell Death by Serum Deprivation. Confluent cultures of mouse 3T3 fibroblasts and WI38 at young, intermediate, and senescent stages of their *in vitro* life span, maintained for at least 24 h in the quiescent stage to ensure no detectable DNA synthesis activity, were induced to undergo programmed cell death by serum deprivation according to a previously described procedure (20). In brief, medium containing 10% FBS was removed from confluent cultures of quiescent mouse or human fibroblasts and was replaced by serum-free medium. These cultures were then subjected to washing six times in serum-free medium to remove residual cell-associated FBS, and after the final rinse, the same serum-free medium was then placed onto the monolayer cultures of fibroblasts, which were kept in incubation at 37°C for various periods of time and assayed for viability as described below. For control experiments, medium was replaced with fresh DMEM containing 10% FBS.

Viability Tests of Serum-deprived Cultures. Survivability after serum deprivation was determined by harvesting fibroblast cultures at designated times after replacing FBS-containing with FBS-free medium. Cells in individual monolayer cultures were harvested and made into single-cell suspension by trypsinization. The cell suspension was then incubated with trypan blue dye, followed by counting those cells which do not take up the dye as viable and those cells taking up the dye as dead; the details of this trypan blue exclusion test were described earlier (20). Each viability test by trypan blue exclusion measurements was performed in triplicate; the number of viable cells was estimated as the percentage of cells remaining negative to the blue dye staining after trypan blue exclusion.

DNA Fragmentation Assays by Oligonucleosome Ladder Display. Cultures were harvested at different time intervals after activation of programmed cell death by withdrawal of FBS. Cells were initially washed in PBS and scraped to collect the cell pellet by low-speed centrifugation, followed by resuspension in lysis buffer containing 10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, RNase A (20 μg/ml), and 0.5% SDS. The cell suspensions were incubated at 37°C for 1 h, after which proteinase K (Boehringer-Mannheim, Mannheim, Germany) was added to a final concentration of 100 μg/ml, and samples were incubated for another 3 h at 50°C. This incubation step was followed by subsequent triple extraction with phenol saturated with 100 mM Tris-HCl buffer (pH 7.4). The final precipitation of DNA was obtained from the aqueous phase with ammonium acetate and ethanol at −20°C. The DNA precipitate was washed with 75% ethanol and finally dissolved in Tris-EDTA (TE) buffer [10 mM Tris-HCl (pH 8.0)-1.0 mM EDTA]. The pellets of DNA precipitates were again digested with 20 μg/ml RNase A for one h at 37°C and extracted twice again with phenol to obtain RNA-free DNA samples. Ten μg of DNA collected from individual cell cultures were analyzed on a 1% agarose gel containing ethidium bromide by electrophoresis in TE buffer at 20 V for 14 h at room temperature; a further incubation in 50 μg/ml ethidium bromide solution was performed in some cases to increase detectability under UV light.

***In Situ* DNA Fragmentation Assays by End-labeling Biotin UTP via Terminal Transferase Binding Activity.** Density-arrested mouse 3T3 fibroblasts and young and senescent human fibroblasts were cultured on poly-L-lysine-coated glass coverslips and subjected to serum deprivation as described above. These coverslip cultures were processed for serum deprivation by rinsing them in sterile serum-less medium, followed by transferring them to new cultureware containing the same type of serum-less medium. At different time points afterwards, these coverslip cultures were fixed with acetone: methanol (1:1) at −20°C for 10 min. *In situ* analysis of DNA fragmentation was performed using end-labeling of nicked DNA with biotin-conjugated UTP by terminal deoxynucleotidyl transferase (Pharmacia Biotech, Inc., Baie d'Urfé, Québec, Québec, Canada) as described by Gavrieli *et al.* (21). The coverslip specimens were then rinsed twice in PBS and once with distilled water. After the coverslips were mounted in PBS containing 50% glycerol, the results of cell-bound UTP were detected by a secondary reaction with FITC-tagged streptavidin and evaluated with a Nikon fluorescence microscope or Bio-Rad MRC600 Confocal microscope.

Immunofluorescence Microscopy. Coverslip cultures of fibroblasts similar to those used for *in situ* DNA fragmentation assays were fixed with 3% paraformaldehyde in PBS for 30 min at room temperature. After this fixation step, the cultures were extracted with 0.01% Triton in the same buffer for 15 min at room temperature. After rinsing with PBS to remove the detergent, the coverslip cultures were incubated with mouse monoclonal antibody to *bcl2* overnight at room temperature. Mouse antibody to *bcl2* was purchased commercially from DAKO company (Santa Barbara, CA); the antibody is in supernatant form and used in 1:10 dilution. After overnight incubation, the coverslip cell specimens were processed by further rinsing with PBS and incubation with FITC goat antimouse immunoglobulin (IgG; Cappel Research Reagents, Turnholt, Belgium). The details for processing cell specimens for monoclonal antibody staining were according to the procedure published previously (22). In some cases, whole nuclear morphology was examined by propidium iodide staining. After rinsing with PBS and mounting in 50%

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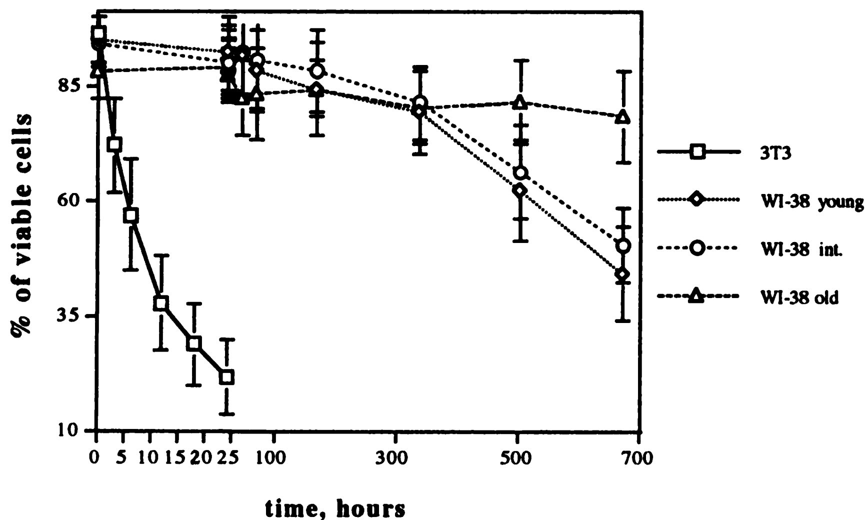

Fig. 1. Viability assays of density-arrested quiescent mouse 3T3 fibroblasts, young and intermediate life span human fibroblasts, and their senescent culture counterparts during serum deprivation. Mouse 3T3 fibroblasts, young (CPDL, 12) and intermediate life span (CPDL, 38) human fibroblasts were cultured to 100% confluency and left at this contact-inhibition growth state for another 24 h before serum deprivation was performed, as described in "Materials and Methods." Senescent human fibroblasts obtained via serial passaging were at CPDL = 58 and were verified to be growth arrested by the criteria of no incorporation of [^{3}H]thymidine nor population doubling after culture time of 3 weeks. Cultures were harvested at designated hours as indicated on the X-axis, and cells suspended in 10 μl sterile PBS were mixed with 10 ml trypan blue (0.4% stock solution from GIBCO, Grand Island, NY) and incubated for 5 min. Direct counting of cells excluding and taking up the dye was performed with a hemocytometer. The proportion of viable cells in any given harvest was the average of three independent sets of experiments: *bars*, 1 SD for each time point. The percentage of viable cells after total withdrawal of serum is shown here for mouse 3T3 fibroblasts (□), young WI38 human fibroblasts (◇), intermediate life span human fibroblast cultures (○), and senescent human fibroblasts (△).

glycerol, cell specimens were evaluated by a Nikon Labophot fluorescence microscope equipped with epiillumination capability.

Protein Extraction, SDS-PAGE, and Immunoblotting Assays. Extraction and fractionation of proteins from cultured fibroblasts were carried out as described earlier (23). Cultures at various time points after total withdrawal of serum, as described above, were harvested by scraping in cold (4°C) PBS; cell pellets were collected by centrifugation at 1000 × *g*. After a subsequent washing with cold PBS, the cell pellets were resuspended in RIPA buffer minus Triton detergent or with Triton present in low concentration [0.01%; 10 mM Tris-HCl, 150 mM NaCl, 0.1% SDS, and 1 mM EDTA at pH 7.4 containing 0.5 mM phenylmethylsulfonyl fluoride (Sigma Chemical Co., St. Louis, MO), 10 μg/ml aprotinin, and 2 μg/ml each of pepstatin and leupeptin (Boehringer-Mannheim, Mannheim, Germany)]. After incubation at 4°C for 10 min, the cell suspensions were sonicated in an ice bath for 5 min; the resulting protein mixture was used as the source of total protein. Protein concentration was assayed with a standard Bio-Rad reagent (Bio-Rad Laboratories, Richmond, CA) using bovine plasma-globulin as a standard.

For determination of *bcl2*s detergent solubility, cell pellets of various cultures collected after harvesting by scraping and low-speed centrifugation were resuspended in PBS buffer containing 0, 0.01, 0.1, or 1% Triton or 1% Triton plus 0.1% SDS. These cell mixtures were incubated at 4°C for 10 min, followed by subsequent centrifugation at 15,000 rpm for 30 min; the supernatants and pellets were then collected and used as the source for detergent-soluble *versus* detergent-insoluble fractions and processed for further protein extraction as described above.

For each assay of protein profile determination, 100 μg of either total protein or detergent-soluble or -insoluble fractions were loaded onto each lane for SDS-PAGE, according to the procedure described by Laemmli (24). For identification of *bcl2* presence in each of the above samples, after extracted proteins were separated on SDS-PAGE gels, they were transferred to nitrocellulose membrane blots (25). These blots were then blocked in Tween 20-Tris buffer solution [10 mM Tris-Cl (pH 7.4), 300 mM NaCl, and 0.5% Tween 20] for 1 h at room temperature and further incubated with mouse anti-*bcl2* monoclonal antibody overnight at 4°C. The *bcl2* antibody was used at 1:50 dilution in 0.05% Tween 20 in the same Tris buffer. Afterwards, the blots were washed four times with Tween 20 containing Tris buffer again and further incubated with rabbit anti-mouse IgG antibody (Cappel Research Reagents, Turnholt, Belgium). The rabbit anti-mouse IgG was used at 1:2000 dilution and incubated for 1 h at room temperature, followed by further incubation with a final antibody, goat anti-rabbit IgG conjugated with peroxidase (Cappel Research Reagents), for 1 h at room temperature. After washing again with the Tris buffer, these blots were then processed for chemiluminescence detection with the commercially available kit (ECL; Amersham Life Science) according to the manufacturer's suggested protocol.

Densitometry and Quantitation of bcl2 Protein Level. Densitometric scans were performed with immunoblots of bcl2 protein using a GS-300 densitometer (Hoefer Scientific, San Francisco, CA). The amount of protein from different cell specimens loaded onto each lane was standardized to 100 μg/lane, and the eventual determination of bcl2 amount was quantified per μg total protein, rather than per cell. To control for loading variation, a known amount of BSA was added to each lane; after transferring to the blots, the remaining gel was processed for silver staining, and densitometric scanning was performed to quantitate the BSA band intensity in each lane. These measurements of BSA bands were used for standardizing loading accuracy. In addition, the densitometric scanning program also includes the measurement of background intensity on several different areas of the blots; averages of these measurements were subtracted to derive valid *bcl2* immunopositive band intensity measurements.

RESULTS

The survival of density-arrested mouse 3T3 fibroblasts depends upon the continued presence of growth factors (26, 27). Upon deprivation, these cells are induced to undergo programmed cell death, with the typical phenotype of DNA fragmentation, and final demise occurs within 24 h. The precipitous drop of live cells in the cultures is evaluated by the viability test *via* trypan blue exclusion assay. As shown in Fig. 1, after withdrawal of serum from density-arrested confluent cultures, the percentage of mouse 3T3 fibroblasts remaining viable declines rapidly and reaches 20% by 24 h. In contrast, this rapid reduction of viable cells was not observed either in similarly density-arrested young, intermediate, or senescent human fibroblasts within the same time frame. After total withdrawal of serum, a small percentage (5%) of cell loss was observed in the first week with all three types of human fibroblast cultures, followed by an equal loss in the second week. After this time, a noticeable drop in the percentage of viable cells was observed in density-arrested cultures of young and intermediate stages of human fibroblasts, while the viability of senescent cultures remained unchanged. After 4 weeks of serum deprivation, young fibroblast cultures have only a 40% survival rate, while cultures of intermediate life span will have about a 50% survival rate

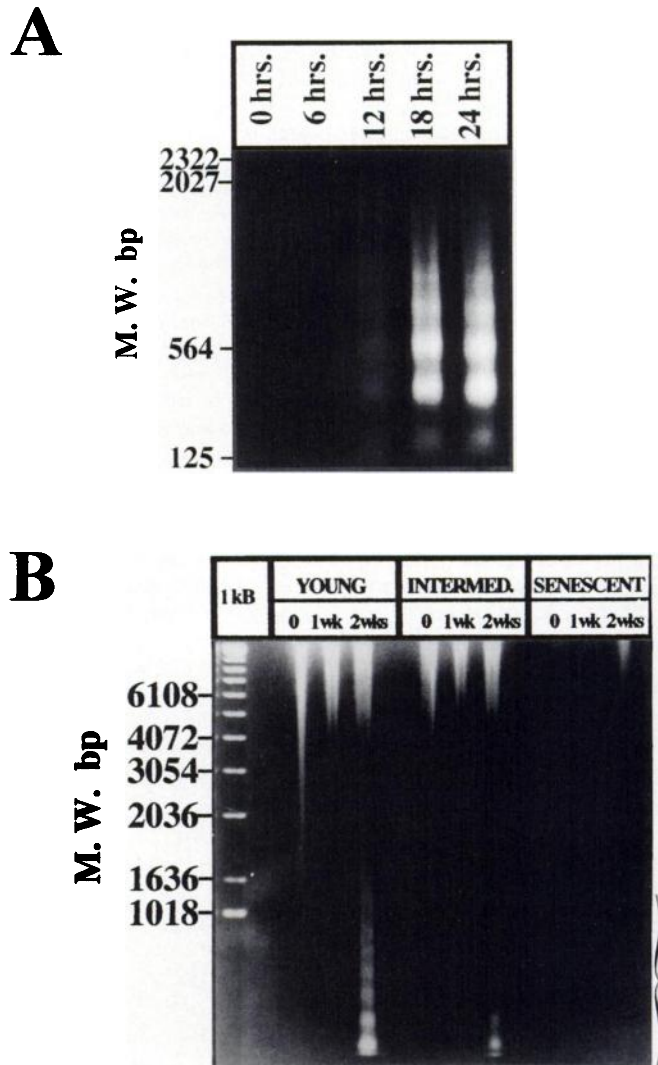

Fig. 2. Biochemical assays for fragmentation of DNA to oligonucleosomal ladder following total withdrawal of serum from cultures of contact-inhibited, growth-arrested mouse 3T3 fibroblasts (*A*) and cultures of WI38 human fibroblasts at young (CPDL, 12), intermediate (CPDL, 38), and senescent (CPDL, 58) stages of their *in vitro* life span (*B*). In (*A*), duration of serum deprivation for mouse 3T3 fibroblasts is indicated in hours (descending from *left* to *right*) above each lane, and molecular weight (*M.W.*) standards are shown in the extreme *left lane*. In (*B*), duration of serum deprivation is labeled as 0, 1, or 2 weeks and shown for young, intermediate, and senescent culture specimens. The molecular weight standard in kilobase divisions is shown in the extreme *left lane*. Equal amounts (10 μg) of DNA samples from each time point for each of the cell cultures were loaded on each lane; the DNA specimens were separated electrophoretically on agarose gel and identified by UV illumination after reaction with ethidium bromide. Notice that fragmentation of DNA into smaller molecular weight positions (indicated by *bracket, lower right side*) is observed only in young and intermediate cultures, in lanes showing 2 weeks after serum deprivation, not in senescent cultures treated in the same fashion.

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(Fig. 1). On the other hand, almost 80% of fibroblasts in senescent cell cultures remain viable, even after 4 weeks, in conditions of no serum.

Induction of cell death was further characterized as programmed and apoptotic by the display of internucleosomal cleavage in cultures subjected to serum deprivation. As shown in Fig. 2*A*, the typical ladder pattern of DNA is seen in mouse 3T3 fibroblast cultures harvested at 18 and 24 h after total withdrawal of serum. DNA degradation into oligonucleosomal fragments in the lower molecular weight range was not seen with cultures harvested at 0 or 6 h after serum removal from the cultures; a very faint band of lower molecular weight is seen at 12 h after withdrawal of serum from cultures of mouse 3T3 fibroblasts. When the same analysis of DNA integrity was performed with samples isolated from young and intermediate life span cultures of human fibroblasts, degradation to lower molecular weights, reflecting the presence of oligonucleosome fragments, was only seen with DNA samples collected at the end of 2 weeks after total withdrawal of serum (Fig. 2*B*). With DNA samples from senescent fibroblasts subjected to the same period of serum deprivation,

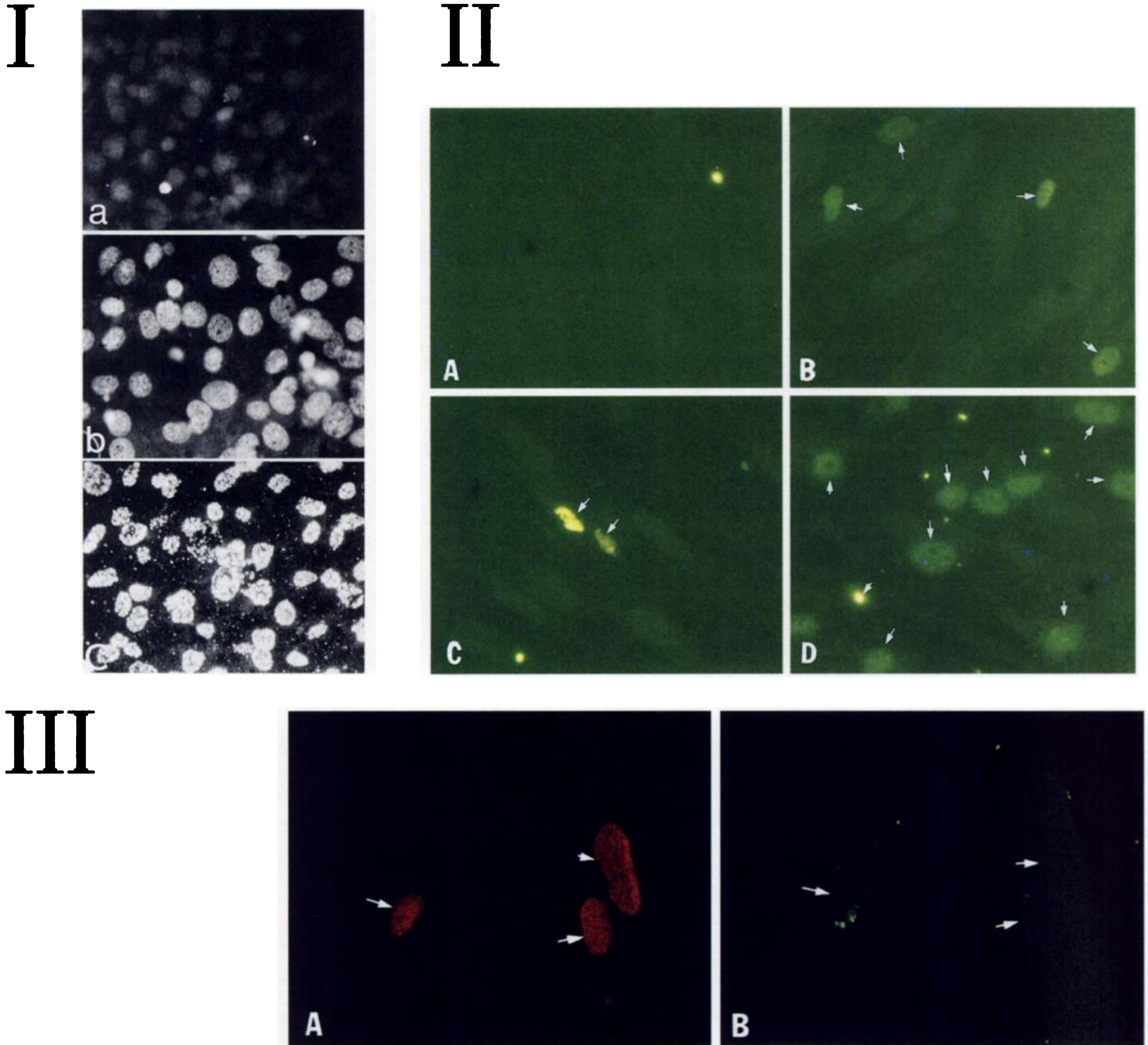

Fig. 3. *In situ* assay for DNA fragmentation by the end-labeling technique. Density-growth arrested confluent cultures of mouse 3T3 fibroblasts (*panel I*) and confluent cultures of WI38 young human fibroblasts (CPDL, 12; *panel II*) grown on coverslips were processed for serum deprivation by total withdrawal of FBS from the culture medium, as described in "Materials and Methods." Similarly, senescent cultures of the same WI38 strain were processed for deprivation of FBS at CPDL = 58. These coverslip cultures were fixed at different time intervals subsequent to the removal of FBS and processed for end-labeling by reaction with terminal transferase and biotin-UTP to evaluate the presence of nicked DNA. *Panel I* shows density-arrested mouse 3T3 fibroblasts fixed at 0 (*a*), 12 (*b*), and 24 (*c*) h after the removal of FBS. Notice that the weak background staining for nuclei at 0 h increases to significant levels of positive staining in virtually all cells in the field at 12 h; this staining intensity continues to increase to show the nuclei containing fragmented condensed chromosomal structures at 24 h. *Panel II* shows density-arrested young human fibroblasts fixed at 0 (*A*), 1 (*B* and *C*), and 2 (*D*) weeks after serum deprivation. Notice the absence of nuclei with bound UTP staining at the time of serum deprivation (*panel II, A*). A few nuclei show bound UTP staining at 1 week after serum deprivation, as indicated by *arrows* in *panels B* and *C* (*panel II*). By 2 weeks after serum deprivation, a significant number of nuclei show positive staining for nicked DNA (*arrows, panel II, D*). *Panel III* shows double-labeling for propidium iodide (*A*) and incorporated dUTP activity (*panel B*) of the same nuclei. Positive staining in *panel III A* reveals the normal chromatin phenotype in these WI38 senescent cell nuclei (*arrows*), which show no detectable fragmented DNA by the lack of staining for nuclear incorporated dUTP (*arrows, panel B*). *Panel I*, × 780; *panel II*, × 920; *panel III*, × 1020.

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there was no detectable presence of any oligonucleosome profile on the ethidium bromide-stained agarose gel (Fig. 2*B*). Even up to 4 weeks after serum deprivation, I could not detect any oligonucleosomes to reflect DNA degradation in these senescent fibroblast cultures (data not shown).

Since this agarose-gel analysis of DNA integrity provides results on total cell population based on culture mass, I performed further characterization using *in situ* DNA fragmentation assays, whereby the evaluation can be obtained on an individual cell basis. Coverslip cultures of both mouse and human fibroblasts were fixed at different times after total withdrawal of serum and then were processed for end-labeling reaction of incorporated dUTP, according to the procedure described in "Materials and Methods." As shown in Fig. 3, *panel I, a,* at 0 time point, density-arrested mouse 3T3 fibroblasts show no staining activity, reflecting that DNA is intact, with little or no nicking ends to incorporated dUTP. In contrast, after 12 and 24 h of serum deprivation, a significant number of nuclei (up to 80% at 24 h) show intense positive presence of incorporated dUTP, reflecting the abundant presence of nicked DNA ends in the nucleus (Fig. 3, *panel I, b* and *c*). A similar analysis was performed with density-arrested young fibroblasts; the end-labeling reaction for incorporated dUTP was found to be of background intensity at 0 time point (Fig. 3, *panel II, A*) but increased to about 5% at 1 week after serum deprivation (Fig. 3, *panel II, B* and *C*). By 2 weeks after serum was removed from the cultures, a large portion of the cell population showed intense nuclear staining for incorporated dUTP. Some nuclei are small with condensed and refractile appearance, a typical phenotype of cells undergoing programmed cell death (Fig. 3, *panel II, D*). During the same time period, senescent cultures were processed for end-labeling for the presence of nicked DNA; as shown in Fig. 3, *panel III,* there was no staining for incorporated dUTP in the nuclei of cells deprived of serum for up to 2 weeks. Nuclear morphology, as evaluated by propidium iodide staining, shows normal appearance with no suggestion of any chromosomal condensation, as seen in the serum-deprived mouse 3T3 fibroblasts or young human fibroblasts.

I reason that the failure to undergo programmed cell death in senescent fibroblasts after serum deprivation may be due to the intracellular presence of survival factors at levels facilitating a mechanism protecting the cells from self-destruction. Further characterization of the death of the fibroblasts was then performed to investigate bcl2 regulation as the obvious candidate for the survival factor in senescent fibroblasts. Before I initiated quantitative evaluation of bcl2 levels in cells, I first performed crude cell fractionation studies in the presence of different amounts of detergent, so as not to lose any bcl2 proteins during the extraction procedure. As shown in Fig. 4, most of the bcl2 protein is retained in the cell pellets of density-arrested young fibroblasts when protein samples are prepared with no or 0.01% Triton-100. A small amount of bcl2 is extracted by 0.1% Triton; and with 1% Triton or 1% Triton combined with 0.1% SDS, the majority of bcl2 protein is extracted and present only in the supernatant. When the cell pellets are subjected to sonication, about three-fifths of the bcl2 is lost. This analysis allows me to set up conditions such as extraction with 0.01% Triton with fixed samples for preparation for immunofluorescence microscopy and to exclude detergent in protein preparation for samples used for SDS-PAGE and immunoblotting analysis.

Immunofluorescence microscopy was performed to evaluate *bcl2* localization and distribution in different cultured cells used in this study. As shown in Fig. 5, *a* and *b*, density-arrested mouse 3T3 fibroblasts contain *bcl2* in small dots throughout the entire cytoplasm; the dotted appearance increases in abundance in confluent cultures of young fibroblasts (Fig. 5, *c* and *d*). In senescent fibroblasts, however, *bcl2* is found not only in the small dots but also in larger

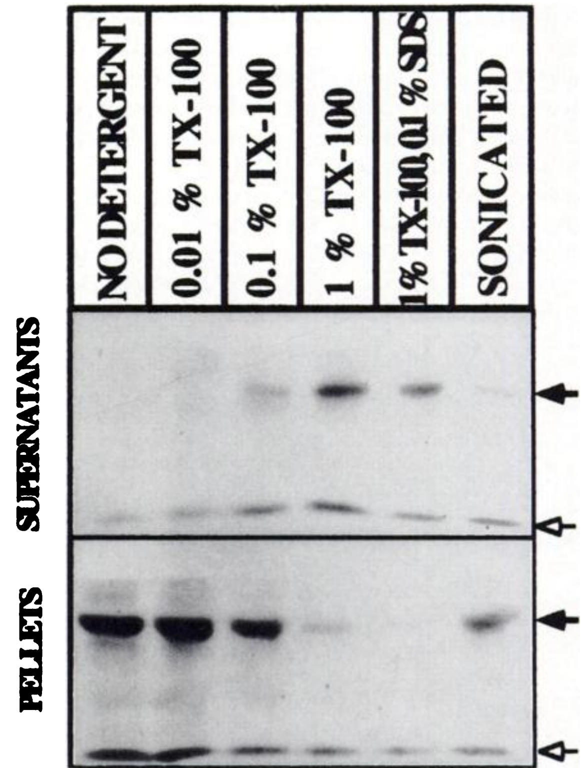

Fig. 4. Biochemical characterization of detergent solubility of *bcl2* in density-growth arrested young human fibroblasts. Cultures of WI38 human fibroblasts were grown to confluency at a CPDL of 12 and processed for protein extraction as described in "Materials and Methods." Crude fractionation by detergent was performed with Triton (TX-100) treatment at concentrations of 0, 0.01, 0.1, and 1%, as well as other variations including 1% Triton plus 0.1% SDS or sonication. Extracted cell materials were centrifuged into pellets as the detergent-insoluble fraction, and supernatants as the detergent-soluble fraction. *Closed arrows,* the position of the *bcl2* band; *open arrows,* the position of the SDS-gel front. These protein extracts were then processed for SDS-PAGE and immunoblotting assay to evaluate the presence of *bcl2* in the different fractions. Notice that most of the *bcl2* is retained in the pellets when Triton extraction strength is at or below 0.1%; this indicates that *bcl2* remains associated with the cell specimens in the weak (<0.1%) detergent treatment conditions.

cytoplasmic granules as well as the nuclei (Fig. 5, *e* and *f*). There seems to be more intense staining with *bcl2* antibody/cell in senescent cultures than in young human cells, which in turn show more than mouse 3T3 fibroblasts.

Biochemical quantitation of *bcl2* presence was performed by immunoblotting assays of density-arrested young human fibroblast cultures and their senescent counterparts. When these two cultures were subjected to crude fractionation with 0.01% Triton extraction, as observed in Fig. 4, most of the *bcl2* in both cell types was present in the pellet fraction, with the young human fibroblasts showing proportionally less *bcl2* quantity per μg protein (Fig. 6*A*). This finding verifies that the immunofluorescence staining seen with cell specimens extracted with 0.01% Triton indeed reflects the distribution of most, if not all, bcl2 proteins in the cells and confirms the observation made by the immunofluorescence staining assays, *i.e.,* senescent cultures contain more bcl2/μg protein than their young counterparts. Densitometric analysis of bcl2 protein levels in the two cell types, young and senescent human fibroblasts, shows a significantly higher amount of bcl2 present in the latter (Fig. 6*B*).

To establish whether the *bcl2* level is indeed related to the onset of programmed cell death in cultured fibroblasts, I analyzed the *bcl2* level before and after serum deprivation. As shown in Fig. 7*A*, the presence of *bcl2* is rapidly lost in mouse 3T3 fibroblasts after serum is removed from density-arrested cultures. This loss of *bcl2* is not due to the overall protein degradation by cells that are dying, since as

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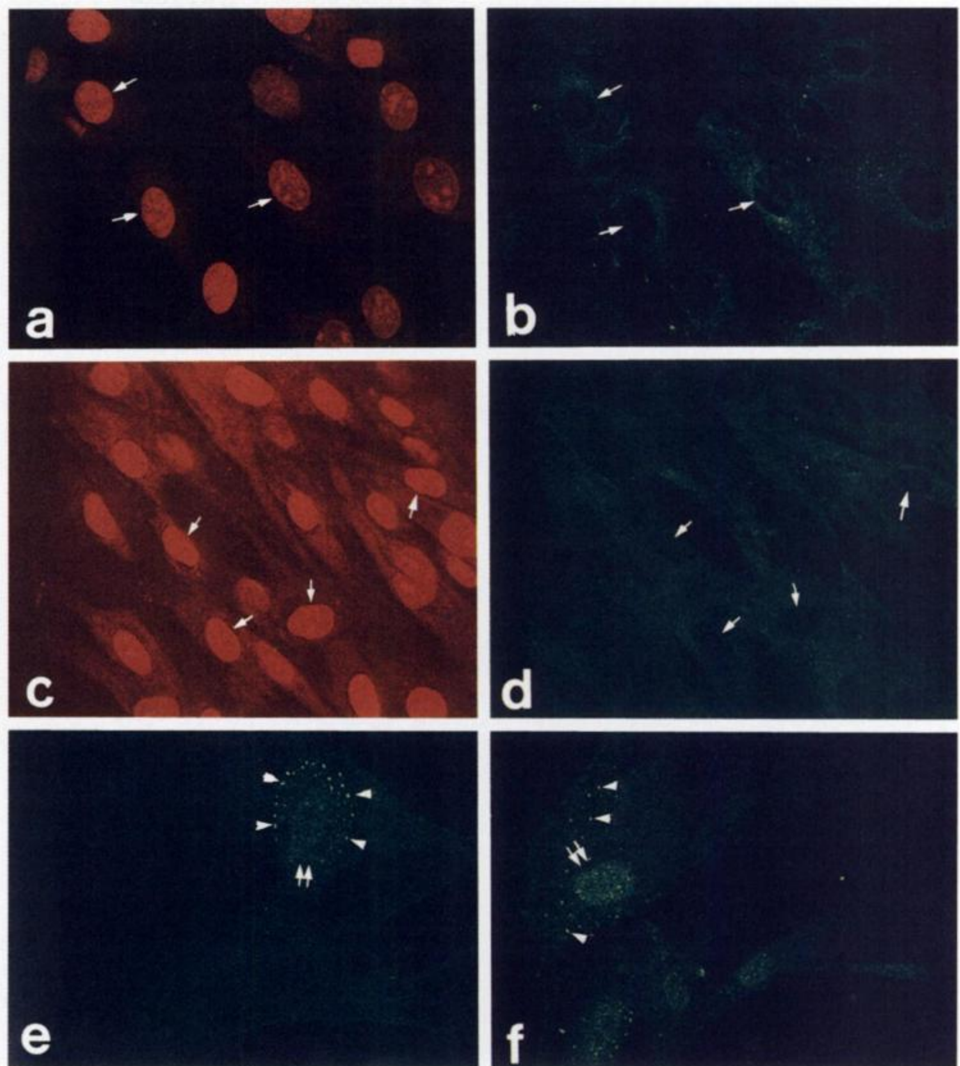

Fig. 5. Immunofluorescence microscopy of *bcl2* distribution in mouse and human fibroblasts. Confluent cultures of mouse 3T3 fibroblasts (*a* and *b*) and WI38 young human fibroblasts (CPDL, 12; *c* and *d*) were fixed and processed for staining of the nuclei by propidium iodide (*a* and *c*) and for staining of *bcl2* antibody reaction (*b* and *d*), as described in "Materials and Methods." Similar staining for *bcl2* distribution was also performed for WI38 senescent human fibroblasts (CPDL, 58). *Arrows*, the positions of nuclei in *a-d*. *a* and *b*, the same field of mouse 3T3 fibroblasts, bearing *bcl2* in dotted appearance throughout the cytoplasm. Similarly, the cytoplasmic dotted appearance of *bcl2* is found in the young cells shown here of the same field by propidium iodide staining and *bcl2* localization (*c* and *d*). In two representative fields of staining in senescent cells (*e* and *f*), *bcl2* is seen not only in the cytoplasmic dotted presence but also in large granules (*arrowheads*) surrounding the nuclei as well as nuclear staining (*double arrows*), which may reflect antibody reaction on the nuclear surface. × 1080.

shown in Fig. 7*B*, the total protein profile of parallel cultures harvested at the same time points exhibits no noticeable differences. Furthermore, the loss of *bcl2* is a precipitous decline occurring at very early time points (6 h), when no detectable DNA fragmentation is even observed (Fig. 2*A*). When the same human fibroblast specimens used for Fig. 2*B* were processed for total protein extraction and SDS-PAGE followed by immunoblotting, the level of *bcl2*, as shown in Fig. 7*C*, is seen at normal level at 0 week, again with young cells possessing less *bcl2* than senescent cells per μg protein. However, the *bcl2* level in the protein samples obtained from cultures deprived of serum for 2 weeks and prepared in the same fashion is reduced significantly and became almost undetectable in samples of young and intermediate aged human fibroblast cultures; in contrast, the level of *bcl2* protein in senescent fibroblast cultures treated similarly shows no change at all. This lack of reduction in *bcl2* level seems to be true only with senescent cultures (Fig. 7*C*). When the *bcl2* bands seen in all these conditions were evaluated by densitometric tracing, this change in *bcl2* quantitation is seen in a more dramatic way. For example, although cultures at intermediate stages of their life span contain the same level of *bcl2*, if not slightly more, than the senescent fibroblast specimens, the reduction of *bcl2* after 2 weeks of serum deprivation seems to behave like young rather than senescent cell cultures. This result suggests that the lack of reduction in the *bcl2* level observed in senescent cultures is due to the loss of ability to down-regulate *bcl2* presence after serum deprivation, rather than *bcl2* overload. Therefore, the results of densitometric measurements allow me to suggest that, although the quantitative presence of *bcl2* is regulated to some extent (~2-fold) by the *in vitro* age of the culture, the inability to down-regulate its quantitative presence after withdrawal of serum is more the key to the apoptosis-resistance phenotype seen with senescent cells. This is further supported by the fact that although intermediate- and late-passaged cultures have similar levels of bcl2 protein, the former behave like early passaged cells, *i.e.*, they die in the same time frame as the young cells.

DISCUSSION

Senescent human fibroblasts obtained by *in vitro* serial passaging are characterized by the unique feature of a permanently growth-arrested state (1, 2). The ability to reenter cell cycle traverse and replicate seems to be irreversibly lost; this loss is considered to be the hallmark of reaching the end of the *in vitro* replicating life span. In this report, I describe that senescent fibroblasts lose not only their ability to replicate, but they also cannot undergo programmed cell death (or apoptosis), an event by which cells commit self-destruction. Using the typical mode of serum withdrawal to induce programmed cell death, I found that while density-arrested mouse 3T3 fibroblasts can be easily activated to undergo apoptotic cell death, similar treat-

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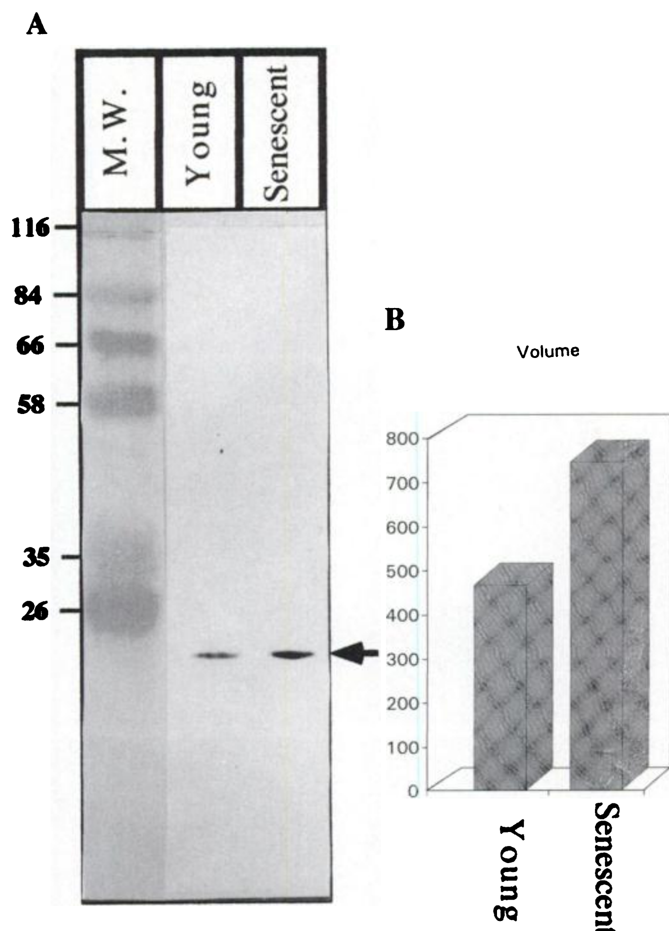

Fig. 6. Biochemical characterization of *bcl2* presence in young and senescent human fibroblasts of the WI38 strain. Cultures of density-arrested young (CPDL, 12) and senescent (CPDL, 58) cells were processed for protein extraction with 0.01% Triton, as described in "Materials and Methods." Protein extracts (100 μg) of the pellet fraction from each of the two cell types were loaded on the two lanes shown in (*A*); after SDS-PAGE, the separated protein samples were electrophoretically transferred to nitrocellulose paper and processed for reaction with a monoclonal antibody to *bcl2*. The positive antibody reaction product is represented here by a single band as indicated by the *arrow*. Quantitative measurements of the *bcl2* level in each of the two samples were obtained by densitometric tracing of three similarly-prepared Western blots; the averages of triplicates are presented in *B*. Notice that per μg protein, senescent fibroblasts contain more than two-fifths of the amount of *bcl2* seen in their young counterparts.

ment could only induce the same type of cell demise after a prolonged period (2 weeks) with quiescent young and intermediate life span human fibroblasts and failed to induce death at all in senescent cultures, even up to 4 weeks. The failure of activation of programmed cell death in these cells was demonstrated by high survival rate as well as little or no detectable DNA fragmentation, one of the hallmarks of programmed cell death. Further characterization as to why senescent fibroblasts are resistant to undergo programmed cell death after serum deprivation was performed by examining *bcl2* levels in these cells. Examination of cell extracts showed that most of the *bcl2* is retained in cell pellets treated with 0.01% Triton detergent. Both immunofluorescence microscopy and immunoblotting assays show that there is some quantitative increase (2-fold) in *bcl2* in senescent fibroblasts compared with their young counterparts, per cell or per μg protein. Upon serum deprivation, cultures of both young and intermediate life span lose their *bcl2* to undetectable levels, but in senescent fibroblasts, the *bcl2* level remains unchanged. This observation leads me to suggest that *bcl2* remaining quantitatively constant in senescent fibroblasts may be one of the molecular factors contributing to the resilient power to survive after prolonged deprivation of serum.

Senescent fibroblasts by definition are cells that have gone through a long period of cell division and eventually arrived at a state where further replication is no longer possible, except through forced induction of DNA synthesis by SV40 T-antigen (16, 17).[4] Therefore, the senescent state in these cells may also be viewed as "anti-replication", otherwise termed "replicative senescence" (2). Findings such as the repression of c-*fos* expression (28) and RB phosphorylation (13), as well as the absence or abnormal expression of genes such as *cdc2*, cyclin A and B absence, (14) and accumulation of inactive cyclinE-*cdk2* and cyclin D1-*cdk2* complexes (15) all support the notion that the loss of necessary factors by senescent cells allows them to participate in cell cycle traverse (14, 15). The recent finding (7–11, 28) of the unique presence of p21 kilodaltons, otherwise known as *Sdi1*, *Pic1*, or *Cip1*, as a p53-associated protein functioning as a *cdk* kinase inhibitor, leads me to further ponder the possibility that, in senescent fibroblasts, there may occur not simply the passive event of losing replicating ability, but the active gain of suppression to prevent cells from ever entering cell cycle traverse again; this reentrance to cell cycle traverse may be detrimental to cells, leading to death. Therefore, the antireplicative phenotype seen in senescent fibroblasts may also be viewed as "antideath", as a self-protective mode to keep them in the long-lived phenotype.

Increasing evidence shows that the functions of various early cell cycle genes are related to the apoptotic event in many cells. Among them, the most noted is the finding that if the proliferation-controlling gene c-*myc* continues to be present under conditions of growth arrest such as serum deprivation, it can also induce programmed cell death (29). Another example is the continued presence of c-*fos* preceding apoptosis in tissues of fos-lacZ transgenic mice (30); others include c-*ras* as a strong factor promoting apoptosis and the tumor suppressor function of p53, which is also an inhibitor for programmed cell death (31–33). Thus, it seems that expression of a single gene can stimulate both proliferation and apoptosis. The decision between promoting or inhibiting proliferation or apoptosis may be based on the balance between the presence or absence of growth factors or other survival factors (review in Ref. 34). Nevertheless, emerging from this picture is the fact that some early cell cycle genes may be required for programmed cell death, *i.e.*, as suggested by work done in rat prostate tissue (35) and my own work (36), the ability to reexperience some part of early cell cycle traverse may be needed for this self-demise program. With senescent fibroblasts, one might then reason that their inability to participate in cell cycle traverse may serve as a double-edged sword, excluding them from both proliferation and apoptosis. Therefore, the findings of the repression of c-*fos* expression and RB phosphorylation, and the unique presence of *Sdi1*, may be viewed as the protective mode of senescent cells not supporting entry into cell cycle traverse, which may be abortive and result in death.

The present finding that the high level of *bcl2* in senescent fibroblasts persists after prolonged deprivation of serum provides additional reasoning to explain the enduring survivability of these cells. One often forgets that senescent fibroblasts are not only nonproliferative but also long-lived. After reaching the permanent nonproliferative state, cultures of senescent fibroblasts may be maintained in cultureware in good health for as long as 2 years.[5] Therefore, these cells are endowed with additional machinery to maintain them in this prolonged healthy state. The inability to down-regulate *bcl2* upon serum deprivation may be part of this machinery acquired during serial passaging to help senescent fibroblasts survive and become less dependent on environmental factors, such as the presence of growth factors or serum.

[5] J. R. Smith and O. M. Pereira-Smith, personal communication.

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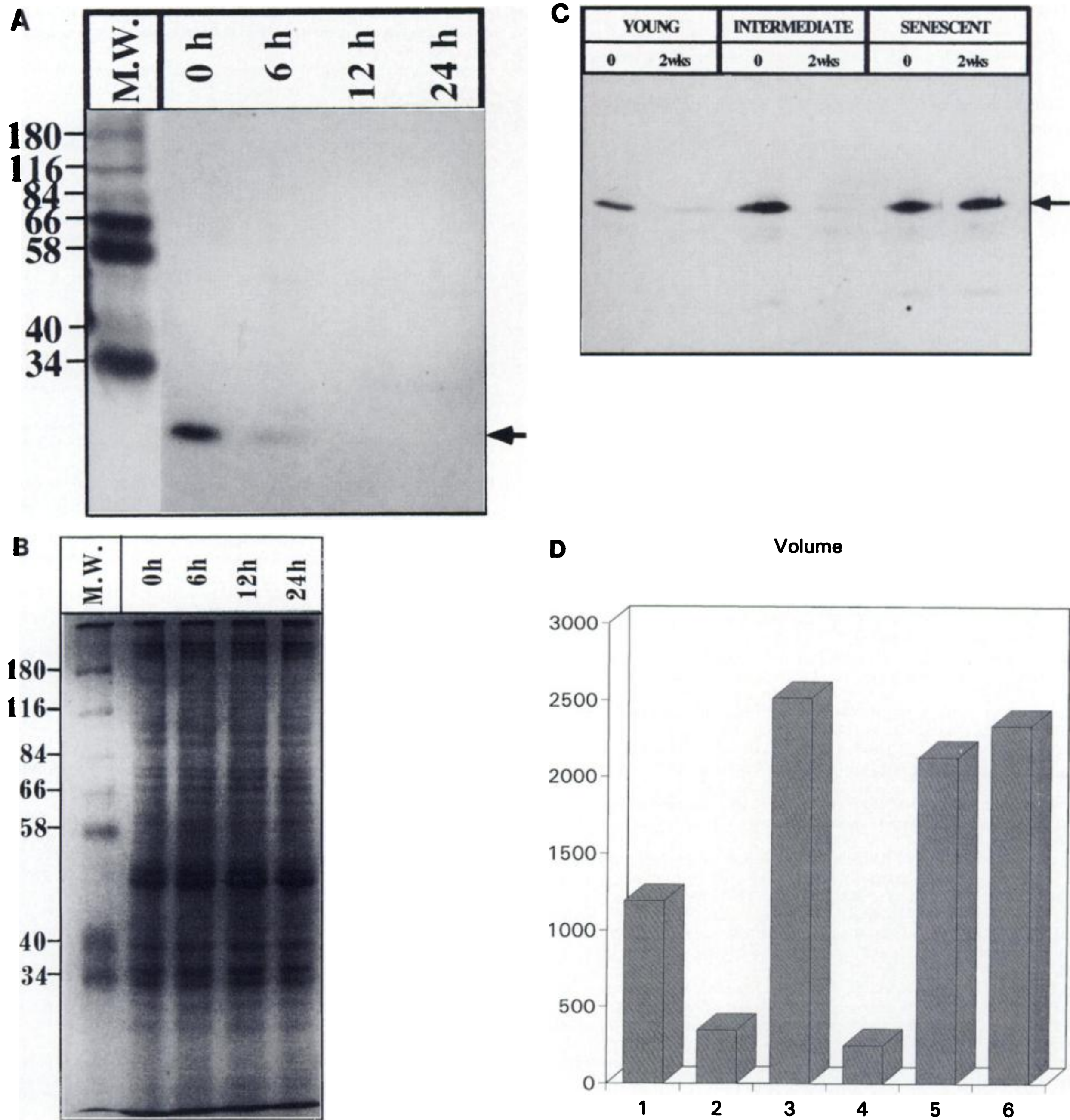

Fig. 7. Biochemical characterization of loss of *bcl2* in mouse 3T3 fibroblasts, and in young and intermediate fibroblasts but not their senescent counterparts, after serum deprivation. *A*, the loss of *bcl2* in density-arrested mouse 3T3 fibroblasts after serum deprivation. Total protein samples were prepared from cells harvested at 0, 6, 12, and 24 h after removal of serum; 100 μg of proteins were loaded onto each lane before immunoblotting with *bcl2* antibody. The presence or absence of bcl3 protein is represented here by the positive or negative antibody reaction product in the form of a specific protein band at the position indicated by the *arrow*. *B*, the total protein profile of parallel cultures harvested at the same time points after the deprivation of serum is initiated. *C*, the loss of *bcl2* protein (*arrow*) at 2 weeks after serum deprivation in confluent young (CPDL, 12) and intermediate (CPDL, 38) cultures of WI38 human fibroblasts but not in their senescent counterparts (CPDL, 58) treated in the same fashion. *D*, the densitometric quantitation of the *bcl2* levels in triplicate samples, using the same column sequence as in *B*. Comparison of *bcl2* quantities per μg protein, as shown in Fig. 6, shows that, in control samples (at the time of serum deprivation), senescent fibroblasts contain almost twice as much *bcl2* protein as young cells; in addition, a similarly elevated level of *bcl2* is also observed in cultures of intermediate life span. Upon serum deprivation for 2 weeks, the *bcl2* level is reduced to the same low level in cultures at early and intermediate life span. In contrast, after the same interval of serum deprivation, this loss of *bcl2* level is not observed in their senescent counterparts; if anything, the protein level shows a slight increase.

Depriving cells of serum may activate a cascade dictating the damage leading to death; the presence of *bcl2* may somehow counteract this trend until the reservoir of this protein is exhausted and it cannot neutralize the damage any more, when it is finally overcome by the death program. The recent findings linking the action of *bcl2* in one report to reduction in lipid peroxidation (37) and in another to maintaining glutathione level (38) certainly illustrate one of the possible ways that *bcl2* may function as a counteracting force to minimize or reduce damage. I am at present investigating whether this action of *bcl2* is indeed the reason explaining the resistance to programmed cell death in senescent fibroblasts. Future experiments, such as microinjection of antisense cDNA or specific antibody into serum-deprived senescent fibroblasts, will certainly be a way to study the cause and/or consequence relationship between the persistent high expression of *bcl2* and the resistant nature of senescent fibroblasts to death.

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