Kinetics of Senescence-associated Changes of Gene Expression in an Epithelial, Temperature-sensitive SV40 Large T Antigen Model

Ola Larsson, Camilla Scheele, Zicai Liang, Jürgen Moll, Christina Karlsson, and Claes Wahlestedt

1Center for Genomics and Bioinformatics and 2Department of Cell and Molecular Biology, Karolinska Institutet, Stockholm, Sweden, and 3Department of Pharmacology, Pharmacia, Pfizer Group, Nerviano, Italy

ABSTRACT

Repetitive senescence limits the number of times primary cells can divide and is therefore regarded as a potential checkpoint for cancer progression. The majority of studies examining changes of gene expression upon senescence have been made with stationary senescent cells. We wanted to study the transition from normal growth to senescence in detail and identify early regulators of senescence by analyzing early changes in global gene expression, using Affymetrix microarrays. For this purpose, we used a murine epithelial senescence model, where senescence is abrogated by SV40 large T antigen and can be induced by using a temperature-sensitive form of SV40 large T antigen (SV40ts58). Comparisons were made to wild-type SV40 large T antigen-expressing cells and to cells expressing SV40ts58 large T antigen grown to confluence. After removal of genes that are similarly regulated in wild-type and temperature-sensitive SV40 large T antigen-expressing cells, 60% of the remaining genes were shared between cells arrested by inactivation of SV40 T antigen and by confluence. We identified 125 up-regulated and 39 down-regulated candidate genes/expressed sequence tags that are regulated upon SV40 T antigen inactivation and not during heat shock or confluence and classified these based on their kinetic profiles. Our study identified genes that fall into different functional clusters, such as transforming growth factor-β-related genes and transcription factors, and included genes not identified previously as senescence associated. The genes are candidates as early regulators of the senescence checkpoint and may be potential molecular targets for novel anticancer drugs.

INTRODUCTION

Primary cells have a limited replicative life span in vitro. Hayflick and Moorhead (1) described the phenomenon in 1961 and called the state when cells stop dividing “senescence.” One of the triggers for the onset of replicative senescence is thought to originate from the telomere, a DNA-protein structure located at the end of the chromosome. The length of the telomere decreases with each cell division cycle and reaches a critical length that induces senescence, possibly via structural changes (2). However, several other stimuli have also been found to induce senescence including oncogenic RAS, chemotherapy, and oxidative stress. Senescent cells can be identified based on several criteria in vitro. Their morphology is less polarized and more spread out, and a population of cells that is regarded as senescent has lost the ability to proliferate and is apoptosis resistant. Several markers of senescence have been described, and the most commonly used is senescence-associated β-galactosidase, although the specificity of this marker seems to be uncertain (3).

Cellular senescence has been suggested to be important for cancer surveillance, because the majority of cells derived from tumors maintain their telomeres at a stable length and thereby abrogate the induction of senescence. This is achieved either by expressing telomerase, the enzyme that extends the telomeric repeat, or by using an alternative mechanism for telomere maintenance (ALT; Ref. 4). Recent data have shown that lymphomas enter senescence upon treatment with cyclophosphamide, indicating that induction of senescence could be an important mechanism by which chemotherapy operates, and is therefore a promising strategy for new cancer treatments (5).

Many tumor suppressors (p21, p16, and p53) are activated during the induction of senescence, often in a cell type-specific manner, and are believed to be the downstream targets of telomeric erosion. However, the understanding of the pathway(s) leading to senescence is still far from complete. Isolation of putative genes regulating the senescence machinery downstream of p53, p16, and p21 is complicated by the fact that primary cells in culture enter senescence asynchronously over prolonged passages. This results in difficulties in finding key genes that are early triggers or effectors of senescence, especially because they may be active only during the initial phase of the senescence transition and then turned off. Because there are cell type-specific pathways to induce senescence and a large proportion of all cancers are of epithelial origin, there is particular interest in the regulation of epithelial cell senescence.

The SV40 large T antigen cellular model applied in this study provides the possibility of studying senescence in a highly controlled manner in epithelial cells. The use of the temperature-sensitive SV40 T antigen allows inhibition of the normal induction of senescence at the permissive temperature and induction of a proliferative block that resembles senescence by raising the temperature. The appeal of this model includes its temporal synchrony and the fact that it involves activation of pathways similar to those induced by telomere erosion (p53 and RB). We studied the transcriptome of two mouse thymic epithelial cell lines immortalized with either wild-type SV40 T antigen (Epi-A1 cells) or a temperature-sensitive mutant SV40 T antigen (Epi-A1ts58 cells; Refs. 6, 7). When Epi-A1ts58 cells are grown at the permissive temperature (33°C), the SV40 T antigen causes the cells to escape senescence. At 39°C, the SV40 T antigen is inactivated, and the cells enter a state that resembles senescence, as judged by morphological changes (enlarged and flattened shape), a proliferative block, and expression of senescence-associated β-galactosidase (7). The Epi-A1 cells (wild-type SV40 T antigen) are used to separate the effect of the inactivated SV40 T antigen from the effect of the heat shock treatment used to inactivate the temperature-sensitive SV40 T antigen.

We identified some groups of genes, e.g., genes related to transforming growth factor (TGF)-β signaling, based on their functions and time of induction during the establishment of senescence, including several previously uncharacterized genes.

MATERIALS AND METHODS

Cell Culture. Epi-A1ts58 cells and Epi-A1 cells were serially cultured in DMEM (Sigma, Stockholm, Sweden) supplemented with 10% fetal bovine serum, 2 mM L-glutamate, 100 units/ml penicillin, and 100 μg/ml streptomycin (all from Sigma) at 33°C and 5% CO2.

Flow Cytometry. Epi-A1ts58 cells and Epi-A1 cells were grown to 20% confluence. Prewarmed medium at either 39°C or 33°C was added to a set of dishes from both Epi-A1ts58 cells and Epi-A1 cells, and the plates were...
transferred to 39°C or 33°C. Cells were harvested after 0, 6, 12, 18, 24, and 32 h for further analysis. Cells were stained with propidium iodide and analyzed using a fluorescence automated cell sorter (Becton Dickinson).

BrduR Assay. Epi-A1ts58 cells were plated on glass coverslips at 50% confluence. At time 0, the medium was changed (prewarmed to 33°C or 39°C), and the cells were incubated at 33°C or 39°C. BrduR was added to the media at −2, 0, 2, 4, 6, or 8 h, and the cells were harvested 2 h later by fixation followed by staining for BrduR and Hoechst 33342, according to the instructions of the manufacturer (BrduR labeling kit; Roche). The BrduR incorporation was analyzed using a fluorescence microscope (Leica DMRXA). More than 200 cells were counted for each time point, and the experiment was repeated twice.

Heat Inactivation and mRNA Preparations. For each heat inactivation, the Epi-A1 and Epi-A1ts58 cells were plated on day 1 at 20% confluence, and the heat inactivation was started on days 2–3, when cells had reached 50% confluence except for the 72-h heat inactivation of the Epi-A1 cells for which the heat inactivation was initiated at 20% confluence to avoid high confluence after 72 h. All processes of heat inactivation were initiated by changing medium to fresh medium prewarmed to 39°C and maintained by incubating cells at 39°C and 5% CO2 for the desired time (1, 2, 4, 10, or 72 h). To avoid a possible serum bias in the study, the cells for the 72-h heat inactivation were subjected to medium change 3 h before harvest. At harvest, the mRNA was extracted using the PolyAtract 1000 kit (Promega). All experiments were performed two to four times.

Expression Analysis. Two to four independent mRNA preparations from each condition were labeled separately, resulting in independent biological replicates. After labeling, the samples were hybridized, washed, and scanned as described previously (8). The fragmented cRNA was hybridized to the mouse U74Av2 chip (Affymetrix).

Data Analysis. Chips were analyzed according to the following standards. Data were extracted using the Micro Array Suit 5 (MAS5); a linear normalization approach based on the 50th percentile of each chip and a per-gene normalization to the median was used. To identify up-regulated genes, all genes at a given time of heat shock (j) of the cell types Epi-A1 (WT) and Epi-A1ts58 (TS) were filtered according to the listed criteria (each filter is combined with AND, and the number of genes that pass each added filter is shown): 1. Select genes where ≥2 measurements were assigned “Present” or “Marginal” by MAS5 [7430 genes/expressed sequence tags (ESTs)]; 2. TS/TS0 ≥ 2 (1198 genes/ESTs); 3. TS/WT0 ≥ 2 (404 genes/ESTs); 4. TS/WT0 ≥ 2 (342 genes/ESTs); 5. TS0 ≠ TS1 (Student’s t test, P = 0.05; 303 genes/ESTs); 6. TS0 ≠ WT1 (Student’s t test, P = 0.05; 231 genes/ESTs); and 7. TS0 ≠ WT0 (Student’s t test, P = 0.05; 215 genes/ESTs).

The 215 genes (with a per-gene normalization to the median applied to get a clustering, reflecting kinetic effects on single genes rather than absolute differences between genes) were pooled and clustered using the self-organizing maps (SOM) algorithm to generate six groups (A–F). Several options were tested, and the latter turned out to generate homogeneous groups regarding expression patterns. Down-regulated genes were considered positive if they passed the following filters (each filter is combined with AND and the number of genes that pass each added filter is shown): 1. Select genes where all four measurements for TS0 were assigned “Present” or “Marginal” by MAS5 [5360 genes/ESTs]; 2. TS2/TS0 ≤ 0.5 (505 genes/ESTs); 3. TS2/WT2 < 0.25 (271 genes/ESTs); 4. TS2/WT2 < 0.5 (256 genes/ESTs); 5. TS22 ≤ TS0 (Student’s t test, P = 0.05; 231 genes/ESTs); 6. TS22 ≤ WT22 (Student’s t test, P = 0.05; 226 genes/ESTs); and 7. TS22 ≠ WT22 (Student’s t test, P = 0.05; 198 genes/ESTs).

All down-regulated genes (with a per-gene normalization to the median to obtain a clustering, reflecting kinetic effects of single genes rather than absolute differences between genes) were clustered using the SOM algorithm into two groups (G and H) as homogeneous populations of genes were generated.

We further classified all positive genes using data derived from the confluence control (con), based on the following criteria: TS0 ≠ TScon (Student’s t test, P = 0.05) and the maximum or minimum relative expression compared with the confluence control was calculated for each gene.

Quantitative Real-Time PCR. Epi-A1 cells and Epi-A1ts58 cells were heated shocked for 0, 6, 18, and 48 h according to the same scheme as above. At harvest, total RNA was isolated with RNeasy Mini (Qiagen) and treated with RNase-Free DNase (Qiagen) according to the manufacturer’s protocol. First-strand cDNA synthesis was performed according to the manufacturer’s protocol (Applied Biosystems). For a reaction volume of 80 μl, 1.6 μg of total RNA were used as template. Later, 1/10 aliquots of the cDNA reactions were analyzed by quantitative real-time PCR using an ABI PRISM 7000 (Applied Biosystems) according to the manufacturer’s protocol. Briefly, gene-specific primers for the target genes were mixed separately with SYBR Green qPCR Mastermix Plus (MedProbe) and added to a 96-well plate containing the cDNA to be analyzed. Samples were run in triplicates, and the data obtained were analyzed with ABI Prism SDS Software (Applied Biosystems). The analysis included a melting temperature-dependent dissociation curve at the amplicon.

RESULTS

Selection of Time Points. Previous studies using temperature-sensitive SV40 T antigen to identify senescence candidate genes were performed after a 72-h heat inactivation in rat cells (9, 10). At this time point, all cells were supposed to have entered senescence. In our case, the goal was not to study cells that are already senescent but rather to study the earliest changes in gene expression that occur when the senescence program is triggered. Thus, we could possibly identify genes contributing to the initiation and manifestation of the phenotype. To achieve this, the time points needed to be carefully selected. In previous experiments, SV40ts58-expressing cells were found to be irreversibly growth arrested after 2–3 days of cultivation at 39°C, the restrictive temperature for the temperature-sensitive SV40 T antigen (11). To more specifically define when the cells exit the cell cycle, we performed fluorescence-activated cell sorter analysis of cells, which were seeded at very low density (20%) to avoid effects of density-dependent growth inhibition. Cell cycle profiles of Epi-A1 cells and Epi-Alts58 cells were compared at 0, 6, 12, 18, 24, and 32 h after transferring the cells from 33°C to 39°C. Fig. 1, A–C, shows that although the Epi-A1 cells maintain stable growth at 39°C, the Epi-Alts58 cells do not. At 12 h, the Epi-Alts58 cells start to accumulate in G1, and this is more evident at 18 h. At 32 h, 74% of the cells are in the G1 phase, while only 5% are in S-phase. The percentage of cells in G2 also drops but does not decrease below a level of 20–25% at the last three time points, which may indicate that some cells are unable to exit the G2 phase.

Because the first changes in cell cycle profile of Epi-Alts58 cells were already detected at 12 h and to generate a more dynamic picture of the proliferative arrest, we looked more closely at the first 10 h by measuring BrduR incorporation. Epi-Alts58 cells were grown either at 33°C or 39°C, and BrduR was added to the medium after −2, 0, 2, 4, 6, and 8 h (the cells were shifted to 39°C at time 0). The cells were fixed and stained by anti-BrduR immunofluorescence 2 h after the addition of BrduR. As shown in Fig. 1D, the fraction of BrduR-positive cells starts to decrease at 2–4 h, and the change is continuously manifested until the end of the study (8–10 h). On the basis of these experiments, we selected 1, 2, 4, and 10 h with a clear emphasis on studying early events of the senescence induction. We also added
SENESCENCE-ASSOCIATED CHANGES OF GENE EXPRESSION

Identification of Senescence-related Genes. Epi-A1ts58 cells and Epi-A1 cells were transferred from 33°C to 39°C, and at the time points described above, mRNA was isolated for analysis using U74Av2 chips (Affymetrix). The data obtained were analyzed to identify genes/ESTs that are induced or repressed in the Epi-A1ts58 cells but not in Epi-A1 cells upon SV40 T antigen inactivation. This assured that the observed changes were related to the growth arrest and not to the heat shock. Validity of the dataset was indicated by the expression pattern of known markers of senescence. These included Ctu (Clusterin/ApoJ; Fig. 1E), a gene found previously to be induced in the SV40 T antigen senescence model in rat (10), and p21 whose expression was drastically induced within 1–4 h (Fig. 1F) and maintained slightly overexpressed at 72 h. The rapid induction of p21 mRNA is probably a consequence of a fast inactivation of the SV40 large T antigen, allowing p53 to activate downstream targets shortly after the heat inactivation is initiated. In addition, several genes related to cell cycle progression (e.g., cyclin B2, cyclin A2, and cdc2a) and replication (e.g., cdc6, rrm1, and mcml2) were down-regulated at 72 h, some of which have already been identified as differentially expressed in the SV40 T antigen senescence model, such as cyclin A2 and cdc2 (12).

After applying our filters as described in the “Materials and Methods” section, 215 genes/ESTs were identified as induced and 198 genes/ESTs as repressed after temperature shift of the Epi-A1ts58 cells. The induced genes were clustered using the SOMs algorithm into six groups (see “Materials and Methods”; Fig. 2): four groups were early-induced genes/ESTs (groups C–F), and two groups were late-induced genes/ESTs (groups A and B). The repressed genes were clustered into two groups using the SOM algorithm (groups G and H; only genes with known names are shown in Fig. 2).

Because p53 might contribute to the induction of senescence in the SV40 T antigen model, we classified the genes as being putative p53-responsive genes or not (Fig. 2) using the results from a bioinformatics approach to identify p53-regulated genes (13). Thirteen % of the identified genes/ESTs were putative p53 regulated, indicating that other responses than direct p53-mediated transcription are initiated upon SV40 T antigen inactivation.

To separate putative senescence-regulated genes from genes related to other types of growth arrest, we classified the induced and repressed genes as being confluence related or not by also analyzing the expression in Epi-A1ts58 cells grown to confluence. The maximum relative expression compared with the confluent culture is shown in Fig. 2 for the genes that show a difference using t-statistics. Sixty % of all identified genes/ESTs showed no difference compared with confluent cells and are therefore not senescence specific. Fewer of the up-regulated genes/ESTs (42%) compared with the down-regulated genes/ESTs (80%) showed an expression pattern similar to that of cells grown to confluence. A total of 125 up-regulated and 39 down-regulated genes/ESTs were specific for the SV40 T antigen model compared with cells grown to confluence, and some were isolated more than once. In support of this approach to classify genes as putative senescence related or not, Clusterin/ApoJ was found to be specifically up-regulated during SV40 T antigen inactivation, whereas cyclin A2 and cdc6, for example, were down-regulated in the confluent culture as well.

Genes That Are Induced Early, 1–4 h after SV40 T Antigen Inactivation. To get an overview of the induced and repressed genes, we have attempted to classify the identified genes based on the expression patterns we obtained and their known functions. Among the early-induced genes (Fig. 2, C–F) at least two classes can be identified, being aware that a functional overlap between the classes could occur. The first class includes Pparg (14), Nedd1 (15), Gata3 (16), M-twist (17), Dlx2 (18), Pem (19), and Ssprl1a (20), which have been associated with differentiation and are not induced in confluent cells. Five of these (Pparg, Gata3, M-twist, Dlx2, and Pem) are transcription factors, and the expression of Dlx2 is maintained also after 72 h. This initial induction of genes related to differentiation could indicate that senescence involves the onset of differentiation programs that also involves irreversible growth arrest. An alternative explanation is that SV40 large T antigen blocks differentiation and inactivation of SV40 large T antigen and therefore induces onset of a senescence-like differentiation program.

The second class of early-induced genes has been related to growth arrest (Ddit3, gadd153, Chop10) (21), Dusp6 (22), Cnn2 (23), Nab2 (24), and Akap12 (25). None of these but Dusp6 and Nab2 is induced in confluent cells. Nab2 and Chop10 act by inhibiting transcription, SScKS is a scaffolding protein that binds G1 to S signaling molecules, Dusp6 inhibits the Erk1/2 pathway, and Cnn associates to actin filaments. Nab2, Dusp6, and Cnn are overexpressed after 72 h, which may suggest that they are involved in the manifestation of the irreversibly growth-arrested phenotype, whereas the others could have a function in the initial cessation of proliferation. The lack of overlap in gene expression between senescent and confluent cultures within this class could be explained by induction of different pathways or that confluent cells have passed the initial phase of growth restriction.

A gene that does not fit into the above classes and is induced early
Fig. 2. All genes identified as up-regulated or down-regulated specifically in Epi-A1ts58 compared with Epi-A1 cells were normalized to the median expression of each gene (to get a clustering reflecting kinetic events of single genes rather than absolute differences between genes), pooled, and clustered using SOMs. The graphs show the median expression of each of the eight clusters in both Epi-A1ts58 cells and Epi-A1 cells after heat inactivation. Clusters A–F contain up-regulated genes, and G and H contain down-regulated genes. The table lists the members of each group with known names, the accession number, and maximum/minimum relative expression (fold change) compared with Epi-A1ts58 cells grown to confluence (no number indicates no difference, see "Materials and Methods"). An asterisk after the gene abbreviation indicates that the gene is a putative p53-responsive gene. The genes were colored based on gene ontology and transcription factors (red), signaling molecules (green), adhesion molecules (blue), and cell cycle regulators (pink).
is Ccn1 (cyclin G1). Cyclin G1 is the only cyclin that is induced upon SV40 T antigen inactivation, and the expression is maintained after 72 h. Cyclin G1 has been suggested to be part of a feedback loop because it is a putative p53-responsive gene known to affect the MDM2-mediated regulation of p53 (26). Another gene that is induced early and whose expression is maintained after 72 h is interleukin 6. Interleukin 6 is up-regulated in SAM mice (27), which is a model for accelerated aging. The induction of interleukin 6 (not seen in confluent cells) implies the possibility that senescence could be induced or maintained by a secreted factor.

**Genes That Are Induced Late, 10–72 h after SV40 T Antigen Inactivation.** Several secreted factors are found among the later-induced genes (Fig. 2, A and B), which suggests that secreted molecules indeed could be important for manifestation of the senescent phenotype. The induction of TGF-β3 is the best example and attributable to the complexity of the signaling downstream of TGF-β, which is so highly cell type- and cell stage-specific that it could act as an initiator of transcription important for senescence. A role for the TGF-β pathway in the SV40 T antigen senescence model is further suggested by the up-regulation of known TGF-β regulated genes. Dpt (dermatopontin; Ref. 28) has been reported as induced by TGF-β but is not specific for T antigen-mediated growth arrest in our experiment. Igfbp3 (insulin-like growth factor binding protein 3) is specifically induced in the T antigen model and can be induced by TGF-β (29) but also by p53. Igfbp3 is induced during senescence (30), and the mechanism of Igfbp3-induced growth arrest in a cancer cell line is dependent on the TGF-β pathway (31). Other identified genes modulate the TGF-β pathway: a non T antigen-specific induction is observed for Fmod (fibromodulin) and Dcn (decorin), which both can bind to TGF-β and sequester it to the extracellular matrix, but decorin may have other functions related to growth arrest mediated through the Akt/protein kinase B pathway (32).

Among the late-induced genes (after 10 or 72 h), we could identify at least two other classes in addition to the class including TGF-β-related genes; induced expression of extracellular matrix components is a hallmark of senescent cells. This is illustrated by the isolation of several different forms of collagen. Col3A1, Col6A1, Col6A2, and Col6A3 are all induced upon SV40 T antigen inactivation and confluence-mediated growth arrest, whereas Col5A1 and Col4A1 are specific for growth arrest induced by SV40 T antigen inactivation. The second class of late-induced genes contains genes related to apoptosis and/or growth arrest, but the function is sometimes also related to terminal differentiation. Stat3 and Itga5 have been described to have growth-regulatory as well as antiapoptotic effects. Cryab (B-crystallin) to have antiapoptotic functions, and AK1 (adenylate kinase) and Klf4 (Kruppel-like factor 4) to have growth arrest-related functions. Stat3 has been associated with G0 in some cells (33) and a constitutively active Stat3-inhibited apoptosis (34), but Stat3 is also induced during confluence in our experiment, indicating a function in the establishment of G0 rather than senescence-related functions. Itga5 (integrin α5) is specifically induced during SV40 T antigen-mediated growth arrest and has been shown to activate the expression of gas1 (35) as well as to suppress apoptosis in colon cancer cells (36). B-crystallin can inhibit caspase-3 (37). AK1 has been identified as a p53-responsive gene (38) but is only slightly induced in the SV40 T antigen model compared with a confluent culture (1.6-fold). Klf4 is a transcription factor that is specific for SV40 T antigen inactivation-mediated growth arrest and has been shown to mediate p53-mediated G1-S arrest (39).

A few more interesting genes that do not fit into the classes mentioned above are induced late in the SV40 T antigen model. OsMr (osostatin M receptor) is a putative p53-induced gene that is not induced during confluence. OsMr has been shown to inhibit breast cancer cell growth (40), sometimes accompanied by an induction of p21 (41). Introduction of Jup (plakoglobin) into p53−/− renal carcinoma cells suppressed tumorigenicity (42).

**Genes That Are Repressed upon SV40 T Antigen Inactivation.** There is a big overlap among the down-regulated genes/ESTs between growth arrest induced by SV40 T antigen inactivation and growth arrest induced by confluence (80%). Most of the down-regulated genes are related to the cell cycle, either as molecules important for replication (e.g., Mcm5, Mcm4, Mcm2, Rrm1, Rrm2, Top2a, and Cdc6) or controlling other stages of the cell cycle (e.g., Ccnb2 (cyclin B2), Cen2 (cyclin A2), Cdc25c, and Cdc2a). Among these, mc4m5, Ccnb2, Cdc2a, and Top2a are only repressed in the SV40 T antigen model, whereas the other genes are repressed in confluent cells as well. Several other genes are markers for proliferation: Pikl (polo-like kinase) is important for mitosis and has been described as a p21-repressed gene (together with Top2a; Refs. 43, 44), and Stk18 (serine/threonine kinase 18/5ak, another polo-like kinase family member) is important for progression of mitosis (45, 46), and which is also true for Stk5 (aurora-related kinase 2; Refs. 47, 48). Another SV40 T antigen model-specific gene is correlated to apoptosis. Tia1 is a RNA-binding protein that regulates splicing (49) and is an apoptosis-promoting factor (50).

Taken together, these results indicate that although there is a big overlap between SV40 T antigen inactivation-induced growth arrest and growth arrest induced by confluence among the down-regulated genes/ESTs, several genes with functions related to cell cycle regulation or progression are specifically repressed in the SV40 T antigen model and could therefore be important for the irreversible phenotype. However, there are several kinases that clearly associate with proliferation and are cell cycle regulated among them; hence, down-regulation is rather a consequence of growth arrest.

**Validation of Differentially Expressed Genes by Quantitative Reverse Transcription-PCR.** To validate the obtained expression patterns and to obtain an estimate of the number of false positives, we selected 10 genes that we considered biologically interesting and performed quantitative real-time PCR. We included p21 as a positive control and TGF-β1 as a negative control, because TGF-β1 was not identified in our study, although TGF signaling was implied as a possible mediator of senescence induction. The mRNA levels in both Epi-A1ts58 and Epi-AI cells after 0, 6, 18, and 48 h of heat inactivation of the SV40 large T antigen were measured. The time points were chosen to include an early, an intermediate, and a late time point. To obtain kinetic information as well as a relative comparison between the cell lines at different times of heat inactivation, the data were normalized to Epi-A1ts58 cells before heat inactivation. As shown in Fig. 3, A–L, 9 of 10 of our selected genes were verified (showing both a 2-fold induction as well as a 2-fold increase compared with the maximal Epi-A1 heat-repressed response), indicating an approximate false-positive discovery fraction of 10%. As shown in Fig. 3E, the expression pattern of TGF-β3 is verified. Interestingly, TGF-β1 (Fig. 3D) is induced in the Epi-A1ts58 but also during the heat shock in the Epi-AI cells (this is in agreement with a microarray study, data not shown). This could indicate an overlap between the heat shock response and senescence and may suggest a risk of false-negative genes appearing in the analysis that are shared between the heat shock response and senescence. Some genes seem to be increased by heat shock (Pem, Tgb3, and Cnn2) but never reach the levels seen in the Epi-A1ts58 cells, possibly indicating additive effects from both heat shock and SV40 large T inactivation. The expression levels of other genes in the Epi-A1ts58 and the Epi-A1 cells are not at the same level in the two cell types before the heat inactivation had been initiated (Tes-1, Pem, Foxd1, Ccn, Cnn2, or Igfbp3). This indicates some inefficiency of the temperature-sensitive form of SV40 large T
antigen at 33°C. A similar conclusion can be drawn by the expression pattern for p21 (higher expression at 33°C in the Epi-Alts58 cells compared with the Epi-A1 cells; Fig. 3C) and appearance of spontaneous senescence of the Epi-Alts58 cells at 33°C (<5%, data not shown), as well as the population doubling time for the Epi-A1 cells (24 h) compared with the Epi-Alts58 cells (40 h).

DISCUSSION

Identification of genes downstream of tumor suppressors such as p53 or p16 that are active as inducers of senescence could be important from a cancer treatment perspective, knowing that these classical tumor suppressors are often mutated in cancers. The possibility to induce senescence in vivo has been reported recently and highlights the potential of the strategy (5). Here we have used a highly controllable senescence model to isolate candidate genes involved in the induction of senescence. In the SV40 T antigen model, a release of p53 and RB from SV40 large T antigen contributes to the induction of senescence, and one expects to identify genes that act downstream of p53 and/or RB but also other genes whose transcription is affected by the SV40 T antigen.

In contrast to most other comparable studies, we used epithelial cells. This is important from a cancer perspective because the majority of human cancers are of epithelial origin, and induction of senescence is cell type specific. The model was established in mouse cells because mouse cells with overexpressed SV40 large T antigen do not enter crisis, which occurs when human cells that have bypassed senescence reach a critically short telomere length with genomic instability as a consequence. Using a mouse cell model to study senescence compared with a model established in human cells could affect the interpretation of the results as replicative in vitro senescence and is believed to be triggered by telomere erosion in human cells. In contrast, mouse cells have longer telomeres and normal replicative senescence in mouse cells is induced by O2 (51). However, although the initiation signals for in vitro senescence caused by serial passaging might differ between human and mouse cells, execution of mouse senescence is likely to be similar to human senescence. This notion is supported by several findings, e.g., that other stimuli than short/eroded telomeres can induce senescence in human as well as mouse cells (e.g., overexpression of Ras or chemotherapy).

We had two primary goals in our expression study of the temperature-sensitive SV40 large T antigen model. The first goal was to study early events that lead to senescence rather than the stationary senescent phenotype, and the second goal was to separate primary genes related to the induction of the senescence phenotype from secondary genes related to the growth arrest that is a consequence of senescence. To accomplish these goals we studied early time points of senescence induction and also made a comparison with cells growth arrested by high confluence. By the addition of a later time point where the growth arrest is irreversible, we were also able to classify genes as being induced early or late during our study.

In our screen for differentially expressed genes with related functions and expression kinetics, we found that several of the identified transcripts were related to TGF-β signaling. This is interesting because recent data indicate a role for TGF-β in other senescence models as well. TGF-β has been shown to be important in senescence induced by H2O2 (52) and oncogenic RAS (53) and has been correlated to the induction of Clusterin/ApoJ as well as other senescence-associated genes in H2O2-induced senescence (52). A possible mechanism of TGF-β in senescence is indicated by the ability of TGF-β to activate the p38 mitogen-activated protein kinase pathway (54) as p38 has been shown to be necessary for RAS-induced senescence (55) as well as H2O2-induced senescence (56). Interestingly, we found that although induction of TGF-β seems to be specific for senescence in the SV40 large T antigen model, TGF-β was induced both in senescent cells as well as in the cells used as our heat shock control. Although an overlap between heat shock and senescence has not been reported, it can be argued that both senescence and heat shock can be considered as stress responses, and an overlap might occur.

Two major efforts have been made previously to identify senescence-related genes using the SV40 T antigen model. Both these studies used rat embryo fibroblasts and applied either a subtraction-based approach (10) or a two-dimensional gel approach (9). Our analysis differs in three major ways from the previous studies: (a) we used cells of epithelial origin (the other studies used fibroblasts); (b) we emphasized that we wanted to identify early changes in gene expression (compared with stationary senescent cells used in the other studies); and (c) we used a global gene expression approach to find senescence-associated genes. In the previous approach applying subtractive cloning, a low number of positive genes indicates a less efficient system for finding differentially expressed genes compared with monitoring of global gene expression. The two-dimensional gel approach has the advantage of studying protein levels rather than using transcription as means to approximate protein levels. The setbacks of the two-dimensional gel approach are mainly caused by the method, which is less quantifiable and enables fewer genes to be studied because of resolution problems. The genes isolated as candi-

Fig. 3. To validate the expression patterns obtained from the microarray study, we selected 10 genes in addition to a positive control (p21) and a negative control (TGF-β1) and assessed the expression levels using quantitative reverse transcription-PCR after 0, 6, 18, and 48 h of heat inactivation of the SV40 large T antigen. The expression level was calculated as the ratio of the measured gene/Cyclophilin A (expression of which was constant for both cell lines and all time points) and normalized to the expression of Epi-Alts58 cells before heat inactivation. The experiment was performed twice in triplicates; bars, SE.
date senescence genes in these two studies overlap slightly compared with the present study. The low degree of overlap could be caused by using different cell types (species), different methods, and most probably on our unique approach to identify early senescence-associated changes in gene expression.

Senescence has been studied using microarray approaches in several other models, e.g., a model where senescence is induced by adding doxorubicin to HCT116 cells (57) or a model where a temperature-sensitive papilloma virus E2 is used to induce senescence in HeLa cells (58). Both of these models use human cells, but the induced senescence differs largely in terms of cell cycle profile in the senescent population that they generate. Senescence induced by introduction of doxorubicin leads to an arrest in G2, whereas induction of E2 activity leads to G1 arrest. Because our model involves mainly G1 arrest and, similar to the E2 model, does not involve any external stimuli except for the temperature change (in the E2 model the temperature is reduced), one would expect a larger overlap between the E2 model and our SV40 large T antigen model. To investigate whether this was the case and to study the similarities among the three studies in detail, we compared the genes identified as differentially expressed in these two studies to the genes identified as differentially expressed in the SV40 large T antigen model. To compare the studies we linked the differentially expressed genes from the doxorubicin and the E2 study to the corresponding genes on our chip using Netaffx. Of 703 differentially expressed genes/ESTs from the E2 study, we were able to link and find an overlap compared with our identified genes for 47 genes (represents 11% of the genes identified in the SV40 large T antigen model). Thirty of 47 genes/ESTs were down-regulated, and as expected, this group included mainly genes related to DNA replication or G2-M phase progression such as cdc2, cdc2a, Tbk, and Mad2l1. In our experiments, only Ptk, Ssk5, Prss1, RalGsp1, and Cdc2a from the list of genes/ESTs shared with the E2 study were more repressed during senescence compared with confluence, and the remaining genes are therefore not senescence specific but rather a consequence of growth arrest. Seventeen genes were up-regulated in both the E2 model and the SV40 large T antigen model. Thirteen of these were senescence specific (not induced in confluent cells in our study), and among these were a few genes with possible regulatory functions [Osmr (oncostatin M receptor), Jup (plakoglobin), and Stat3]. Fifteen of the 17 shared up-regulated genes were found in clusters A and B in Fig. 2 (representing 13% of all genes in clusters A and B). Interestingly, there is an enrichment of senescence-specific genes (compared with the confluent culture) from 56% for all genes to 87% for the shared genes in clusters A and B. The overrepresentation of shared genes in clusters A and B is expected, because these clusters contain genes that are induced late in our SV40 large T antigen model and because a 72-h induction of senescence was used in the E2 study. The big overlap of senescence-specific genes (not regulated during confluence) among the induced genes could indicate that when a gene is shared between two models, it is more likely to have a senescence-specific role in either model. When we linked the genes from the doxorubicin study with our identified genes, we found an overlap for 19 genes (~5% of our genes). The lower degree of overlap could be expected from the differences in methods to induce senescence and from the cell cycle profile generated (as discussed above). Interestingly, all of these overlapping genes were down-regulated in both studies, which indicates that there might be a difference between senescence induced by the physiological stimulus used in both the E2 and the SV40 large T antigen model compared with doxorubicin. The down-regulated pool that is shared between the SV40 large T antigen model and the doxorubicin model is characterized by genes that are associated with replication and cell cycle progression, such as Mctd, Top2a, and Rrm1 as well as to DNA damage responses, such as Rad51. Only 8 genes/ESTs were differentially expressed (down-regulated) in all three studies [those known are Ssk5, Mad2l1, Cdc2a, Bab1, Tp (thymiylate synthase), and Mcmd2], and they probably reflect that the cells are no longer dividing in any of the models because only Ssk5 and Cdc2a were repressed more during senescence compared with confluence in our study (9.9-fold and 2.7-fold, respectively). The overlap between the SV40 large T antigen model and the E2 model or the doxorubicin model can be summarized in two major points: (a) The overlap is greater compared with the E2 model than with the doxorubicin model (11% compared with 5%). This probably reflects the different methods used to induce senescence, but the percentage of shared genes is also affected by the data analysis approaches. (b) The genes that are shared with the doxorubicin model are all down-regulated; hence, no similarities in the possible activating genes were found, which is in contrast to the E2 model where several of the shared up-regulated genes were of signaling nature and represent potentially senescence-inducing genes. The overlap of induced senescence-specific genes that are shared between the E2 model and the SV40 large T antigen model indicates coregulation of senescence in mouse and human cells.

The genes that are shared in several studies and not associated with growth arrest induced by confluence could be genes with a general role in senescence. However, further studies are needed to test these candidate genes as well as other genes identified in our study functionally for their contribution to the senescent phenotype.

REFERENCES


Kinetics of Senescence-associated Changes of Gene Expression in an Epithelial, Temperature-sensitive SV40 Large T Antigen Model

Ola Larsson, Camilla Scheele, Zicai Liang, et al.

Cancer Res 2004;64:482-489.