Beryllium Induces Premature Senescence in Human Fibroblasts

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ABSTRACT

After cells have completed a sufficient number of cell divisions, they exit the cell cycle and enter replicative senescence. Here, we report that beryllium causes proliferation arrest with premature expression of the principal markers of senescence. When young presenescent human fibroblasts were treated with 3 μM BeSO₄ for 24 h, p21 cyclin-dependent kinase inhibitor mRNA increased by >200%. Longer periods of exposure caused mRNA and protein levels to increase for both p21 and p16(Ink4a), a senescence regulator that prevents pRb-mediated cell cycle progression. BeSO₄ also caused dose-dependent induction of senescence-associated β-galactosidase activity (SA-β-gal). Untreated cells had 48 relative fluorescence units (RFU/μg/h) of SA-β-gal, whereas 3 μM BeSO₄ caused activity to increase to 84 RFU/μg/h. In chromatin immunoprecipitation experiments, BeSO₄ caused p53 protein to associate with its DNA binding site in the promoter region of the p21 gene, indicating that p53 transcriptional activity is responsible for the large increase in p21 mRNA elicited by beryllium. Forced expression of human telomerase reverse transcriptase (hTERT) rendered HFL-1 cells incapable of normal replicative senescence. However, there was no difference in the responsiveness of normal HFL-1 fibroblasts (IC₅₀ = 1.9 μM) and hTERT-immortalized cells (IC₅₀ = 1.7 μM) to BeSO₄ in a 9-day proliferation assay. The effects of beryllium resemble those of histone deacetylase-inhibiting drugs, which also cause large increases in p21. However, beryllium produced no changes in histone acetylation, suggesting that Be²⁺ acts as a novel and potent pharmacological inducer of premature senescence.

After normal somatic cells have completed a sufficient number of replication cycles, they cease dividing and enter replicative senescence (Itahana et al., 2001). As cells age, telomere repeats and telomere overhangs become progressively shorter until the change in telomere structure is enough to trigger entry into senescence (Herbig et al., 2004). In human fibroblasts, forced expression of the catalytic subunit of human telomerase is sufficient to prevent senescence and cause immortalization (Bodnar et al., 1998).

The major cell cycle regulatory proteins that are associated with replicative senescence are p53, p21, and p16. The p53 tumor suppressor is a transcription factor that coordinates senescence and other cellular responses such as apoptosis. p21(Waf1/Cip1/Sdi1) is a powerful broad spectrum cyclin-dependent kinase (CDK) inhibitor that blocks the activity of cyclin-CDK2 and cyclin-CDK4/6 complexes that are required for cell cycle progression. The expression of p21 is controlled by the transcriptional activity of p53, although it can also be regulated by p53-independent processes. An increase in the abundance of p21 is a central feature of senescence (Noda et al., 1994). The other major effector of cell cycle arrest in human senescent cells is p16(Ink4a). p16 inhibits cyclin D1-CDK4/6, which is the kinase complex responsible for pRb phosphorylation. In its hypophosphorylated form, pRb sequesters E2F transcription factors that are required for initiation of S phase. p16 siRNA knockdown studies indicate that p16 is needed to maintain senescence in human fibroblasts (Bond et al., 2004). In addition to these cell cycle regulatory proteins, senescence-associated β-galactosidase (SA-β-gal) activity has become widely recognized as an enzy-
matic biomarker that correlates with senescence (Dimri et al., 1995). The increase in p21 occurs early in senescence, whereas increases in p16 and SA-β-gal develop somewhat later (Stein et al., 1999).

Several treatments can induce a senescence-like state prematurely in young cells. Ectopic expression of the Ras oncogene causes premature senescence in fibroblasts via kinase cascade-dependent activation of the p53/p21 and pRb/p16 response pathways (Serrano et al., 1997; Lin et al., 1998). Smurf2, an E3 ubiquitin ligase, is produced by normal fibroblasts upon telomere shortening, and expression of Smurf2 from a retroviral vector causes premature senescence through p53 and pRb pathways (Zhang and Cohen, 2004). Premature senescence can also be elicited by forced expression of the Leo1-like domain of the RDL protein (Zha et al., 2005). An alternative approach that does not require transfection is to transiently expose cells to hydrogen peroxide (Chen and Ames, 1994). This method has become widely used to induce premature senescence, but a near-lethal dose (100–500 μM) is needed for good induction, and peroxide can cause appreciable apoptosis in this dose range (Chen et al., 2000).

Drugs that trigger senescence could potentially be therapeutically useful, but small molecule pharmacological agents that induce premature senescence are limited. Histone deacetylase (HDAC) inhibitors are the major drug class with this property. Sodium butyrate and trichostatin A, two structurally diverse HDAC inhibitors, produce a senescent-like state in human fibroblasts (Ogryzko et al., 1996). HDAC inhibitors cause global increases in histone acetylation, resulting in changes in chromatin structure that may enhance transcription of some genes. The p21 promoter is particularly sensitive to the effects of these drugs (Richon et al., 2000), but microarray and differential display studies have estimated that HDAC inhibitors affect the expression of 2 to 4% of all genes (Van Lint et al., 1996; Moore et al., 2004), including many that are irrelevant to cytostasis.

Here, we report a new kind of senescence-inducing drug, beryllium sulfate. The efficacy of beryllium salts in inhibiting cell growth was first noted for cultured chick embryo fibroblasts and skeletal muscle cells (Chevremont and Firkef, 1951). This simple agent is surprisingly potent because the growth of IMR-90 human lung fibroblasts can be inhibited by concentrations as low as 10 ng/ml Be (approximately 1 μM), with either BeSO₄ or BeCl₂ being equally effective (Hart et al., 1982). Subsequent studies of rat liver cells showed that BeSO₄ arrests the cell cycle at the G₁ phase (Skilletter et al., 1983). Similar cell cycle effects were seen in primary cultures of human skin fibroblasts and in human lung fibroblasts (Lehnert et al., 2001). The only molecular effect of Be²⁺ reported thus far is an increase in p53 and p21 protein levels after treatment with relatively high doses (10 and 100 μM) of BeSO₄ (Lehnert et al., 2001). Because p53 and p21 are important regulators of senescence, in the present study, we sought to investigate whether exposing cells to low concentrations of beryllium can elicit pharmacologically induced senescence.

Materials and Methods

Cells and Reagents. HFL-1 cells, a normal diploid human lung fibroblast line, were received from ATCC (Manassas, VA) at passage number 10 and population doubling (PD) 19 and propagated in RPMI 1640 with GlutaMAX and 25 mM HEPES (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (HyClone, Logan, UT), 100 U/ml penicillin G sodium, 100 μg/ml streptomycin sulfate, and 0.25 μg/ml amphotericin B (Invitrogen). Exponentially growing cells at passage number 15 or 16 (PD = 32–34) were used at the time of initial treatment for all experiments on young, presenescent cells, except for one experiment designed to test the effects of advanced passage number, which used HFL-1 cells at passage number 26 (PD = 52). Cells were seeded from a common cell stock, then assigned randomly as either test (BeSO₄ added) or control (no drug) and maintained in parallel until harvesting for analysis. In long-term studies, cells were split as needed to maintain subconfluency, and fresh regular or Be²⁺-containing media were provided every 3 days. Replicatively senescent HFL-1 cells were prepared by serial propagation until complete cessation of proliferation (PD = 62 to 63), then these cells were maintained in the senescent state with periodic media replacement for at least 1 additional week before use in experiments. BeSO₄-4H₂O was from Fluka (a division of Sigma-Aldrich, St. Louis, MO). Sodium butyrate, trichostatin A, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), and 4-methylumbelliferyl-β-D-galactopyranoside (MUG) were from Sigma-Aldrich.

Assay of Senescence-Associated β-Galactosidase Activity. Histochemical staining of intact cultured HFL-1 cells using X-gal to show SA-β-gal activity was done as described previously (Dimri et al., 1995). Quantitative assay of protein-normalized SA-β-gal activity in HFL-1 cell lysates using MUG was done as described previously (Gary and Kindell, 2004).

mRNA Quantification. Unamplified gene-specific mRNAs were quantified by cooperative hybridization (Canales et al., 2006) according to the QuantiGene protocol (Genospectra, Fremont, CA). In this method, total cell lysates are mixed with chimeric capture oligos. One half of the capture oligo is complementary to a segment of the target mRNA, and the other half is complementary to an oligo that has been coated into a 96-well plate. A pool of five to six distinct capture oligos is used to target each mRNA of interest, so that the captured mRNA binds to the plate in a polyanivalent manner. The attachment of up to six capture oligos per target transcript molecule permits a stringent wash condition that removes nontarget mRNA. Selectivity is further enhanced by including additional target-specific oligos that hybridize to the mRNA regions in between the capture oligos, so that a contiguous 300- to 500-nucleotide region of the mRNA target becomes heteroduplex. This cooperative hybridization promotes the coil-helix transition and serves to inhibit the formation of competing secondary structures and to stabilize the specific associations between the mRNA and the DNA oligo probe set. Some of the noncapture oligos serve as points of attachment for branched oligos bearing alkaline phosphatase, which is used to catalyze a luminescence reaction. Commercially available oligo probe sets were used to quantify human β-actin (accession no. NM_0011101), human p53 (accession no. NM_0014564), human bax (accession no. NM_004324), and human p21 (accession no. NM_00389) mRNA (Genospectra). For human p16/INK4a (accession no. NM_000077), a custom probe set was designed. p16 has three exons. Exons 2 and 3 are shared with ARF, an alternatively spliced mRNA that uses a different reading frame than p16. Therefore, capture oligos must target exon 1 to ensure that only p16 can be captured. Within exon 1, five capture oligo annealing sites were selected on the basis of sequence uniqueness with respect to p15/INK4b, a homolog of p16/INK4a. A probe set specific for p16/INK4a and incapable of hybridizing to ARF or p15/INK4b was created by incorporating the probe sequences 5′-GGACCGGATTATTCTTCTTAC-3′, 5′-GCCGCCCTCCTCCTTTCTC-3′, 5′-GGCTCCATGCTGCTCCCC-3′, or 5′-TAACTATTCGGTGCGTGGT-3′ into the five capture oligos. These capture oligos are complementary to nucleotides 36 to 54, 112 to 129, 181 to 197, 222 to 244, and 324 to 343 of p16/INK4a mRNA, respectively. QuantiGene reagents (Genospectra) were used for cell lysis, cooperative hybrid-
ization, and enzymatic detection, then luminescence was measured using a GENios plate reader (Tecan, San Jose, CA) set to 37°C.

**Western Blots.** Treated HFL-1 cells were collected by trypsinization and centrifugation, washed twice with PBS, and extracted with radioimmunoprecipitation assay buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.5 mM benzamidine, 0.25 mM phenylmethanesulfonyl fluoride). Total protein concentrations in the radioimmunoprecipitation assay extracts were measured by BCA assay (Pierce, Rockford, IL). For actin and p53 Westerns, samples were loaded onto 10% SDS-PAGE gels at 3 and 6 μg/ lane, respectively. For p21 and p16 Westerns, samples were loaded onto 11 and 12% SDS-PAGE gels at 30 (24-h experiment) or 45 μg/lane (3-week experiment). Proteins were transferred to polyvinylidene difluoride membranes (Invitrogen) and probed with the following primary and secondary antibody pairs: mouse IgG mAb anti-human p53 (sc-126; Santa Cruz Biotechnology, Santa Cruz, CA) and HRP-conjugated goat anti-mouse IgG (A-9917; Sigma-Aldrich), goat pAb raised against human/rat/mouse C-terminal actin peptide (sc-1615; Santa Cruz Biotechnology) and HRP-conjugated bovine anti-goat IgG (sc-2350; Santa Cruz Biotechnology), mouse anti-mouse/human p21 mAb (sc-6246; Santa Cruz Biotechnology) and HRP-conjugated goat anti-mouse IgG, and goat anti-human p16INK4a pH (sc-468-G; Santa Cruz Biotechnology) and HRP-conjugated bovine anti-goat IgG. Blots developed with ECL-Plus fluorescent/chemiluminescent reagent (GE Healthcare, Piscataway, NJ) and imaged by laser scanning (fluorescence mode, excitation = 457 nm; emission = 520 nm) on a Typhoon Variable Mode Imager (GE Healthcare) or by direct exposure to BioMax X-ray film. For histone acetylation assays, HFL-1 cells were treated with drugs for 24 h, trypsinized, washed in PBS, pelleted at 5 × 10⁶ cells/sample, and stored frozen at −70°C. Cell pellets were resuspended in Triton extraction buffer (PBS containing 0.5% Triton X-100, 2 mM phenylmethylsulfon fluoride, 0.02% NaN₃) at a cell density of 1 × 10⁶ cells/ml. Cells were lysed on ice for 10 min with gentle mixing, then centrifuged at 4800g for 10 min at 4°C. The supernatant was discarded, and the cell pellet was washed with Triton extraction buffer a second time. The pellet was resuspended in 0.2 N HCl at a cell density of 4 × 10⁷ cells/ml, and the histones were acid extracted overnight at 4°C. The supernatant containing the histones was collected at 4800g for 10 min at 4°C, and protein concentration was determined using the BCA assay (Pierce). Ten microliters of the histone extract was run on a 4 to 20% SDS-PAGE gel, transferred to 0.2-μm nitrocellulose, probed with anti-acetyl histone H3 antibody (06-599 from Upstate) and goat antibody (sc-6246; Santa Cruz Biotechnology) and imaged by laser scanning. For histone acetylation screening, HFL-1 cells were treated with puromycin without loss of puromycin resistance or immortalized within 4 days (data not shown). The retroviral vector became stably integrated (Ouellette et al., 1999). PA317 cell supernatants containing young presenescent HFL-1 cells. Retrovirus was produced by the retroviral vector. For discerning telomere repeat amplification protocol (TRAP) assay using the TRAPEze XL Telomerase Detection Kit (Chemicon International, Temecula, CA). Cell lysates were incubated with a telomere-like single-stranded oligo substrate. Telomerase activity adds a series of telomeric repeat sequences, resulting in variable length extensions of the oligo. The extended product was amplified with PCR primers that anneal at six-nucleotide intervals along telomeric repeat sequences, and the resulting PCR product ladder was analyzed by gel electrophoresis. Heat sensitivity of the activity was evaluated by heating the cell extract to 85°C for 10 min. TRAPEze PCR products were run on 10% polyacrylamide Tris borate-EDTA nondenaturing gels, stained with SYBR Green dye, and photographed.

**Chromatin Immunoprecipitation.** Control and beryllium-treated cells were fixed with 1% formaldehyde to cross-link DNA- associated proteins to DNA, then chromatin was sheared enzymatically using ChIP-IT Express (Active Motif, Carlsbad, CA). Chromatin immunoprecipitations used protein G-coated magnetic beads and either p53 DO-1 Ab (sc-126; Santa Cruz Biotechnology) or RNA pol II Ab (Active Motif). After a 12-h incubation at 4°C, beads were washed with ChIP-IT Express reagents, supplemented with additional washes with 100 mM Tris, pH 8.0, 500 mM LiCl, 1% Nonidet P-40, and 1% sodium deoxycholate. Cross-links were reversed by 4-h incubation at 65°C, the protein in the chromatin immunoprecipitation (ChIP) samples was digested with proteinase K, and then the DNA in the ChIP samples was used as template for quantitative real-time PCR. ChIP PCR was done using the forward primer 5’-GGGGCTGCTT-GATTGCTTCTTCG-3’ and the reverse primer 5’-CTGAAACAGGCGGCCCAAG-3’, which flank a p53-binding site in the promoter region of the human p21 gene (Kaeser and Igo, 2002). The primer binding sites are located at nucleotides 2290 to 2270 and 2205 to 2186 to the 5’ side of the p21 gene transcription start site, respectively. Real-time PCR was conducted using a BioRad iCycler with HotStar Taq polymerase (IQAGEN, Valencia, CA) and SYBR Green fluorescence quantitation. All PCR reactions yielded a single 105-bp product with a melting temperature of 83.0 ± 0.5°C, corresponding to the p21 gene amplicon. Initial experiments were also done using a TaqMan probe 5’-6-FAM;CTCTGGCATAAGAGGCTTGTTGGA-TATTT-BHQ1)-3’ that anneals to the region of nucleotides 2236 to 2208 to the 5’ side of the transcription start. This TaqMan probe gave similar results to those obtained by SYBR Green quantitation, but the latter was slightly more sensitive, so the data produced with SYBR Green are shown. Some PCR assays used a glyceraldehyde-3-phosphate dehydrogenase (gapdh) primer set (Active Motif).

The amount of p21 gene (p53-response element) sequence present in ChIP immunoprecipitates was expressed relative to the amount of input DNA, which was also measured by real-time PCR using the same p21 primers. Input DNA represents the total amount initially added to each immunoprecipitation, before antibody capture and washing. PCR efficiency was determined from the slope of the standard curve made by serial dilution of template, using the equation: efficiency (E) = [10(−1/slope)] − 1. Relative amounts of p21 gene sequence in the input and ChIP samples were determined from real-time PCR threshold cycle (Ct) values according to the equation: relative starting quantity = (1 + E)mean input Ct − ChIP Ct. This result expresses the starting quantity of template in the ChIP PCR reaction relative to that in the input PCR reactions. Volume corrections were applied to account for the fact that each PCR reaction used a 5-μl aliquot of template solution, which represents only a portion of the total input and total ChIP volumes.

**Results**

**Development of SA-β-Gal in Beryllium-Treated Fibroblasts.** Beryllium sulfate caused a gradual increase in SA-β-gal activity over time, as judged by histochemical stain-
Young presenescence HFL-1 cells treated with 3 μM BeSO₄ for 1 week displayed a perceptible increase in SA-β-gal staining (Fig. 1C), whereas untreated cells grown in parallel were negative (Fig. 1B). Staining increased considerably by 2 weeks (Fig. 1D). After 3 weeks of exposure to BeSO₄ (Fig. 1E), the staining intensity appeared to be comparable with that of authentic replicatively senescent cells, which had been cultured continuously for 8 weeks to attain senescence (Fig. 1A). Positive staining was not evident in young HFL-1 cells grown for 3 weeks in the absence of BeSO₄ (data not shown). The majority of cells treated with BeSO₄ displayed light staining after 1 week, moderate staining after 2 weeks, and intense staining by 3 weeks. To quantify the effect of BeSO₄, SA-β-gal activity in cell extracts was measured using a fluorescence-based assay (Gary and Kindell, 2005). A dose-dependent increase in SA-β-gal activity was observed in HFL-1 cells after 12 days of treatment with BeSO₄ (Fig. 2). The initial reaction rate was determined for each treatment group using data from the linear phase of the reaction. In senescent human fibroblasts, SA-β-gal activity was 135 ± 4 relative fluorescence units (RFU)/μg total protein/h ± S.D. The basal activity of early passage fibroblasts was 48 ± 1 RFU/μg/h. This basal activity was not affected by 1 μM Be²⁺ (51 ± 1 RFU/μg/h), but 3 and 10 μM Be²⁺ caused SA-β-gal to increase to 84 ± 2 and 85 ± 3 RFU/μg/h, respectively.

Effects of Be²⁺ on the Expression of p53-Regulated Genes. HFL-1 cells were used to examine the effects of Be²⁺ on transcriptional targets of p53. p21 is a p53-regulated gene that promotes cell cycle arrest and senescence, and bax is a p53-regulated gene that promotes apoptosis. After 24 h of treatment with 10 μM BeSO₄, p21 mRNA increased to 262% of control levels, whereas bax mRNA was unaffected by exposure to the drug (Fig. 3). There was no change in p53 mRNA as a result of BeSO₄ treatment. This is a typical result because p53-inducing agents generally act by preventing p53 protein degradation without affecting the rate of p53 mRNA synthesis. The amount of β-actin mRNA was the same in the treated and untreated samples. Actin is a housekeeping gene used for sample normalization whose expression is expected to remain relatively constant on a per cell basis.

Although both p21 and bax are transcriptionally regulated by p53, only p21 transcription was stimulated by BeSO₄. These effects of BeSO₄ differ from H₂O₂-induced premature senescence, which causes enhanced transcription of both of these genes. In normal human diploid fibroblast BJ cells, peroxide stress-induced senescence causes a 2.1-fold increase in p21 mRNA and a 2.4-fold increase in bax mRNA (de Magalhães et al., 2004).

![Fig. 1. Beryllium causes expression of SA-β-gal. X-gal staining of senescent fibroblasts (A), presenescence fibroblasts (B), or presenescence fibroblasts treated with 3 μM Be²⁺ for 1 (C), 2 (D), or 3 weeks (E).](image-url)
Changes in Cell Cycle Regulators Caused by Low Doses of Beryllium. Early passage HFL-1 cells (PD32) grow rapidly, with a doubling time of 24 h. The doubling time increases to 48 h by about passage 26 (PD52), and the cells cease dividing entirely after an additional 10 population doublings with the arrival of replicative senescence. We investigated whether the basal level of p21 mRNA in untreated cells changes as HFL-1 cells age and also whether the ability of BeSO$_4$ to induce p21 expression is affected by the age of the presenescent cells. Slower growing PD52 cells had 42% more p21 mRNA, normalized relative to actin mRNA, than the younger, faster growing PD32 cells (Fig. 4A). However, both kinds of cells showed large inductions of p21 in response to BeSO$_4$ (Fig. 4B). After 24-h treatment with 3 $\mu$M BeSO$_4$, young fibroblasts increased p21 mRNA content by more than 200% (to 337% of control), with similar increases seen for 10 and 30 $\mu$M BeSO$_4$. Even though untreated PD52 cells contain higher basal levels of p21 mRNA, these older cells were nearly as responsive to BeSO$_4$ as the younger cells.

In contrast to the results for p21, BeSO$_4$ at 1, 3, or 10 $\mu$M produced no change in p16 mRNA at 24 h (data not shown). Because p16 accumulates gradually over time as cells become senescent, we examined expression after longer periods of BeSO$_4$ treatment. After 14 days of culture in the presence of 10 $\mu$M BeSO$_4$, young fibroblasts showed a 7.6-fold increase in actin-normalized p21 mRNA compared with untreated control cells, attaining p21 mRNA levels very similar to those of replicatively senescent cells (Fig. 5). The treatment also caused a 71% increase in p16 mRNA, indicating that BeSO$_4$ can cause up-regulation of p16 with longer exposure time. However, BeSO$_4$-induced p16 mRNA levels did not approach the high levels seen in late-stage senescent cells. In contrast to p21 and p16, actin-normalized p53 mRNA levels were virtually identical in untreated, Be$^{2+}$-treated, and senescent cells.

The results with 10 $\mu$M BeSO$_4$ prompted a time course study of regulator induction at even lower concentrations to identify the dose threshold for this response. Three micromolar BeSO$_4$ was sufficient to cause a more than 200% increase in p21 mRNA at 1, 2, and 3 weeks of exposure (Fig. 6A). However, 1 $\mu$M BeSO$_4$ had little effect. With regard to p16 mRNA, 3 $\mu$M BeSO$_4$ caused a 75% increase at 2 weeks and an 83% increase at 3 weeks (Fig. 6B). However, no increase was seen after 1 week of treatment, indicating that the p16 response takes time to develop. There was no significant change in p53 mRNA at any of the doses or times examined, showing that p53 mRNA levels are remarkably constant in the presence or absence of BeSO$_4$ (Fig. 6C).

Western blots were used to examine p53, p21, and p16 protein levels in beryllium-treated cells. In young fibroblasts, a 24-h exposure to 3 or 10 $\mu$M BeSO$_4$ caused up-regulation of p53 and p21 protein, whereas 1 $\mu$M BeSO$_4$ was insufficient to cause induction during this time frame (Fig. 7A). In these cells, p16 protein levels were very low and were unchanged by BeSO$_4$ over 24 h. The actin levels were similar in all samples, confirming that equal amounts of protein were used.

Fig. 3. Up-regulation of p21 mRNA by BeSO$_4$ is a gene-specific effect. Young HFL-1 cells (PD33) were grown in the presence (gray bars) or absence (black bars) of 10 $\mu$M BeSO$_4$ for 24 h; then, the cells were collected and counted. Cell lysate equivalent to 10,000 (p21, p53, and bax) or 1000 ($\beta$-actin) cells was analyzed for specific mRNA content by cooperative hybridization and luminescence. mRNA values are expressed as percent of control for $n = 3$ plates per group.

Fig. 4. Be$^{2+}$ stimulates p21 expression in fast-growing young fibroblasts and in slow-growing older fibroblasts. A, basal level of p21 mRNA in young fibroblasts at PD32 and older fibroblasts at PD52. B, expression of p21 mRNA in young and old fibroblasts after 24-h treatment with 0, 3, 10, or 30 $\mu$M BeSO$_4$. 
loaded. With a 3-week exposure, 3 μM BeSO₄ caused increases in p53, p21, and p16 protein levels (Fig. 7B). The ability of 3 μM BeSO₄ to increase p16 protein levels after 3 weeks is consistent with its effects on p16 mRNA levels at this time point, as noted earlier (Fig. 6B). With 3 weeks of exposure, 1 μM BeSO₄ was able to increase p53 and p21 protein levels (Fig. 7B).

**BeSO₄ Is an Effective Cytostatic Agent in Telomerase-Positive Cells.** Normal human fibroblasts express little or no telomerase activity. Ectopic expression of hTERT, the catalytic subunit of human telomerase, is sufficient to immortalize WI-38, BJ, and other human fibroblast cell types. The potential of hTERT to immortalize HFL-1 cells has not been reported previously. Stable transfection with hTERT allowed HFL-1 cells to be propagated indefinitely, and no end of replicative lifespan has yet been observed (data not shown). The hTERT-HFL cells can be regarded as immortal, as defined by established lifespan extension criteria (Morales et al., 1999). Lysate prepared from the hTERT-transfected cells confirmed the presence of telomerase activity (Fig. 8A). The telomerase enzyme is heat-labile. PCR products arising from telomerase activity were abolished when the reactions were done using heat-treated lysate. The HFL-1 and hTERT-immortalized HFL-1 cells were grown in the presence of various concentrations of BeSO₄ for 9 days, with passage every 3 days to maintain subconfluent cell density. Cell counts after 9 days showed that regular HFL-1 cells and their immortalized counterparts were equally sensitive to BeSO₄ (Fig. 8B). The IC₅₀ was 1.9 μM for the normal fibroblasts and 1.7 μM for the hTERT-immortalized fibroblasts.

**BeSO₄ Does Not Affect Histone Acetylation.** The expression of p21 can be influenced by changes in histone acetylation produced by HDAC inhibitors. Because BeSO₄ causes effects that are similar to those caused by HDAC-inhibiting drugs, the potential of BeSO₄ to affect histone acetylation was assayed directly. A 24-h treatment with 1 to 100 μM BeSO₄ had no effect on histone acetylation in HFL-1 cells (Fig. 9), indicating that BeSO₄ does not act as an HDAC inhibitor and does not act to increase global histone acetylation by another mechanism. Authentic HDAC inhibitor drugs used as positive controls did show activity in this assay. A 5 μM dose of trichostatin A produced a large increase in histone H3 acetylation, and 5 mM sodium butyrate produced a moderate increase.
BeSO₄ Causes p53 to Bind to the p21 Promoter. ChIP was used to assess whether p21 induction in response to beryllium is due to the action of p53 as a transcription factor. After 24-h exposure to 10 μM BeSO₄, HFL-1 fibroblasts displayed a 4.1-fold increase in the occupancy of the p53-binding site of the p21 promoter, as measured by ChIP with real-time PCR quantitation based on cycle thresholds (Fig. 10A). As an additional measure, gel electrophoresis of the PCR product after a limited number of cycles gave an independent visual confirmation that there was indeed 4 times as much product in the ChIPs from Be-treated cells as in ChIPs from untreated cells (Fig. 10B). As shown by PCR of the input DNA, the treated and control chromatin samples were well matched for total DNA, and 2-fold differences in template were easily differentiated after PCR.

The hTERT-immortalized HFL-1 cells were also studied using ChIP (Fig. 10C). After treating these cells for 24 h with 10 μM BeSO₄, a 3.0-fold increase in p53 binding at the p21 promoter was observed. This increase was not the result of generalized changes in transcription, as assessed by ChIP using an antibody against RNA polymerase II (RNAP). The RNAP ChIP samples, which are expected to contain a broad range of transcriptionally active gene fragments, were probed with PCR primers targeting the promoter region of the gapdh gene. gapdh is a constitutively expressed housekeeping gene that codes for an enzyme that is required for glycolysis. It is not regulated by p53. The levels of gapdh DNA in the RNA pol II ChIPs from untreated and Be-treated cells were similar, which indicates that the basal transcription rate is not affected by BeSO₄. The gapdh PCR primers were also used on the p53 Ab ChIP samples, but the abundance of gapdh DNA sequence in the p53 ChIPs was below the PCR detection limit (data not shown). This shows that p53 did not bind to the gapdh promoter.

Discussion

Beryllium (atomic number 4) possesses distinctive physicochemical properties. Be²⁺ is the only divalent cation that is smaller than the physiological cations Mg²⁺ and Ca²⁺, and its small ionic radius gives Be²⁺ exceptional charge density. Because of its unique properties, Be²⁺ could bind tightly to clefts in proteins that are inaccessible to other cations. These considerations make it possible for beryllium to exert biological effects that are unlike those of any other metal salt.

Three distinct biological effects of beryllium have been described in the literature: low-dose antiproliferative effects, high-dose carcinogenic effects, and immune system effects relevant to chronic beryllium disease, a lung affliction caused by occupational exposure to airborne material. Very low concentrations of beryllium salt (i.e., 1–3 μM) are sufficient to inhibit cell proliferation. At high concentrations, beryllium is somewhat carcinogenic to mammalian cells (Gordon and Bowser, 2003). For example, 560 μM BeSO₄ causes a 14-fold increase in BALB/c-3T3 cell trans-
formation frequency compared with the spontaneous transformation rate (Keshava et al., 2001). The effects of Be\(^{2+}\) on the immune system are complex. Be\(\text{SO}_4\) at 1 to 10 \(\mu\)M is mitogenic in human lymphocytes isolated from people who have beryllium hypersensitivity (Rossman et al., 1988). Antigen-presenting cells from individuals with uncommon HLA-DP alleles can display Be\(\text{SO}_4\)-containing class II complexes that may stimulate T cell proliferation (Fontenot et al., 2000). This immune reaction has been studied intensively because workers in the beryllium industry who have this particular MHC polymorphism are at greatest risk of developing chronic beryllium disease.

The most potent general effect of Be\(\text{SO}_4\) is production of cell cycle arrest. As shown here, this arrest appears to result from activation of some facet of the senescence signaling pathway. Decreased proliferation was evident at 1 \(\mu\)M Be\(\text{SO}_4\) in long-term growth assays (Fig. 8), and Be\(\text{SO}_4\) at 1–3 \(\mu\)M and higher produced increases in senescence effectors and biomarkers. All changes in p21 and p16 were reflected in their levels of mRNA and protein. In contrast, actin-normalized levels of p53 mRNA always remained constant (Figs. 3, 5, and 6), even when increases in p53 protein levels were observed (Fig. 7).

In other systems, p53 is usually regulated by protein stabilization and not via changes in transcription and that appears to be the case with beryllium as well.

Be\(\text{SO}_4\) induced rapid increases of p53 and p21, and both remained elevated over 3 weeks of treatment. Up-regulation of p16 and SA-\(\beta\)-gal were late events that developed only after 2 to 3 weeks of treatment. The kinetics of Be\(\text{SO}_4\)-inducible changes parallel those seen in telomere-initiated senescence. Newly senescent cells that have just ceased dividing have high levels of p21 but little p16 and SA-\(\beta\)-gal, which require several additional weeks to reach maximum levels (Stein et al., 1999).

The effects of beryllium resemble those of histone deacetylase inhibitors, which stimulate large increases in p21 that spark premature senescence (Ogryzko et al., 1996; Munro et al., 2004). However, 1 to 100 \(\mu\)M Be\(\text{SO}_4\) produced no changes in histone acetylation (Fig. 9), showing that beryllium exerts its effects by a different mechanism than HDAC-inhibiting drugs. The ChIP data indicate that Be\(\text{SO}_4\) causes p53 to associate with the p21 promoter (Fig. 10). The recruitment of p53 to the p21 promoter also occurs during replicative senescence, as seen by ChIP (Jackson and Pereira-Smith, 2006). Immortalizing cells with hTERT did not affect their sensitivity to Be\(\text{SO}_4\) (Fig. 8), indicating that beryllium acts later in the senescence pathway than the signals that originate from telomeres.

Based on its potency, Be\(\text{SO}_4\) may bind to a senescence signaling molecule with a dissociation constant in the low micromolar range. Presumably, such interaction would alter the function of the target, either inhibiting or activating it, leading to up-regulation of senescence effectors. The identity of this putative signaling molecule is not known, but in vitro studies have shown Be\(\text{SO}_4\) to be a potent competitive inhibitor of two enzymes: myoinositol monophosphatase (Faraci et al., 1999) and myoinositol 1-phosphatase (Faraci et al., 1999).


