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BASIC–ALIMENTARY TRACT

Growth Arrest, Apoptosis, and Telomere Shortening of Barrett's-Associated Adenocarcinoma Cells by a Telomerase Inhibitor

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Background & Aims: **Barrett's esophageal adenocarcinoma (BEAC) is a complication of gastroesophageal reflux disease, with no effective chemotherapy and poor prognosis. BEAC cells, like many other types of cancers, may reactivate telomerase to achieve unlimited proliferative potential, making telomerase a unique therapeutic target. The purpose of this study was to evaluate effects of telomerase inhibition on BEAC.** ***Methods:*** **We examined the effect of a selective G-quadruplex intercalating telomerase inhibitor, 2,6-bis[3-{N-Piperidino} propionamido]anthracene-9,10-dione (PPA), on telomerase activity, telomere length, colony size distribution, and proliferative potential in 2 BEAC cell lines, BIC-1 and SEG-1.** ***Results:*** **Telomerase activity was >10-fold and >600-fold elevated in the adenocarcinoma cells as compared with normal gastric/intestinal cells and normal diploid fibroblasts, respectively. Telomeres were short, being less than 4 kilobase pair in both tumor cell lines. Exposure to PPA effectively inhibited telomerase activity and shortened telomeres. PPA also arrested cell proliferation and reduced colony number and size after a lag period of about 10 cell generations, consistent with the attrition of telomeres. The growth arrest was not due to senescence but was due to apoptosis. Expression analysis of the cells following PPA treatment did not show significant change in the expression of genes involved in cell-cycle proliferation and apoptosis. Exposure to PPA had no effect on proliferative potential of normal intestinal cells.** ***Conclusions:*** **We conclude that telomerase inhibition by PPA induces cell growth arrest in BEAC cells and demonstrate the potential of telomerase inhibitors in chemoprevention and treatment of Barrett's-associated esophageal adenocarcinoma.**

Esophageal adenocarcinoma has a dismal outcome, with a 5-year survival rate of only 10% in the early 1990s because of the early spread of the tumor before the onset of clinical symptoms.[1] Moreover, incidence of this cancer appears to be rising at an alarming rate. It has been reported that the incidence of esophageal adenocarcinoma in white men has increased almost 350% since the mid-1970s.[2] The reason for this increased incidence is not known. Most if not all esophageal adenocarcinomas are derived from specialized intestinal metaplasia of the Barrett's esophagus. Barrett's esophagus is a complication of gastroesophageal reflux disease in which normal esophageal squamous epithelium is replaced by metaplastic columnar epithelium.[3] It has also been shown that the progression of the specialized intestinal metaplasia to invasive carcinoma occurs gradually, passing through changes of worsening dysplasia.[3] Telomerase activity is very low in the normal squamous epithelium[4] and is increased in the Barrett's mucosa and even higher in the adenocarcinoma.[5] It has been suggested that the immortal clones produced by high telomerase activity in the Barrett's esophagus may progress from metaplasia to dysplasia and from dysplasia to Barrett's-associated adenocarcinoma (BEAC) through accumulation of other mutations.[6–8] Therefore, BEAC may be an excellent candidate for chemoprevention and anticancer therapy with telomerase inhibitors.

Telomeres are nucleoprotein complexes that are present at the ends of chromosomes. Human telomeric DNA in different cell types comprises several hundred to several thousand repeats of TTAGGG sequence.[9–11] Telomeres protect chromosomes from nucleolytic degradation and fusion.[12] DNA replication mechanisms lack the ability to copy chromosomal DNA distal to the

Abbreviations used in this paper: BEAC, Barrett's esophageal adenocarcinoma; PPA, 2,6-bis[3-{N-Piperidino}propionamido]anthracene-9,10-dione.

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primase site.[13] Therefore, a small amount of telomeric DNA (approximately 50–100 bp) is lost with each cell division so that somatic cells have limited potential of less than ~100 doublings, after which they undergo replicative senescence. Severe telomeric disruption may also lead to apoptotic cell death.[14,15] Telomerase is a ribonucleoprotein with reverse transcriptase activity that can add short TTAGGG repeats at the free 3′ end of telomeres. By adding the short TTAGGG repeats at the free 3′ end of telomeric DNA, telomerase helps maintain telomere length.

Telomerase activity is found in 80% to 95% of the cancers, including those of the gastrointestinal tract such as colon, pancreas, and Barrett's-associated esophageal adenocarcinoma.[5,7,16,17] Although some normal cells such as germline, hemopoietic, and actively proliferating gastrointestinal epithelial cells also express telomerase, most normal tissues do not express this enzyme. Telomerase plays a key role in cancer cells in which it has been shown to protect cells from replicative senescence, DNA damage, and apoptosis,[18] contributing to cell "immortality." By allowing unlimited proliferation, telomerase permits cancer cells to accumulate mutations leading to invasive phenotype. The unique role that telomerase plays in cancer cells makes it an excellent target for anticancer therapy for the following reasons.[19–21] (1) Telomerase is present primarily in cancer and not in normal somatic cells; telomerase inhibitors may have selective effects on cancer cells without untoward side effects on the somatic cells. (2) The expression of telomerase is increased in precancerous dysplastic tissues[5,7,16]; therefore, telomerase inhibitors may be important in chemoprevention. (3) Moreover, telomerase expression has been reported to correlate with adverse clinical outcome and tumor recurrence.[17,22–24] Consequently, these inhibitors have considerable potential in the treatment of cancer and particularly in inhibiting its invasive behavior.

Although clinical trials of telomerase inhibitors as cancer treatment are not yet available, studies in several cancer cells have shown that telomerase inhibitors cause replicative senescence[25] or apoptosis.[18,26–28] Such studies are not available in Barrett's-associated adenocarcinoma, which is known to express high levels of telomerase. We report here that a novel G-quadruplex-intercalating telomerase inhibitor, 2,6-bis [3-{N-Piperidino} propionamido]anthracene-9,10-dione (PPA)[29] causes growth arrest of Barrett's adenocarcinoma cells (BIC-1 and SIG-1). The growth arrest is evidenced by a reduction in colony number and size that is accompanied by apoptotic cell death. These effects are correlated with inhibition of telomerase activity and shortening of telomeres. The apoptosis is due to activation of DNase-1 that causes DNA cleavage. Moreover, the effects of PPA appear to be primarily due to suppression of telomerase activity in that PPA treatment did not significantly affect gene expression profile of the cancer cells. These studies suggest that telomerase inhibitors are important potential candidates for the chemoprevention and treatment of Barrett's-associated adenocarcinoma.

Materials and Methods

Cell Lines

Two esophageal adenocarcinoma cell lines (SEG-1 and BIC-1), derived from Barrett's-associated adenocarcinomas of the distal esophagus, were obtained from Dr. David Beer, University of Michigan, Ann Arbor, MI. Normal cell strains from stomach and intestine (CRL7869) and from intestine (CRL7820) were purchased from American Type Culture Collection (Rockville, MD). Human diploid fibroblasts (GM01662) were obtained from the Genetic Mutant Cell Repository (National Institute for General Medical Sciences), maintained by the Coriell Institute for Medical Research, Camden, NJ.

Telomerase Inhibitor

Telomerase inhibitor 2,6-bis[3-{N-Piperidino}propionamido]anthracene-9,10-dione (PPA), a nonnucleoside 2,6-diaminoanthraquinone derivative that inhibits telomerase activity through formation of discrete binary complex with G-quadruplex structure was purchased from Oncogene Research Products, San Diego, CA.

Treatment and Growth of Cells

The cells were maintained in monolayer culture at 37°C in humidified air with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) (Sigma Chemical Co., St. Louis, MO) containing 10% fetal bovine serum, as described previously.[26,27,30]

Experimental Design

BEAC cell lines (BIC-1 and SEG-1) were treated with inhibitor PPA at 1, 5, or 10 μmol/L in a relatively dark room to reduce exposure to light. On day 7, the cells were harvested and evaluated for telomerase activity to determine the minimal inhibitor concentration required for inhibition of >90% enzyme activity. This concentration was subsequently used to study its effect on telomere length and survival of BEAC cells. Constant numbers of cells were plated in multiple 100-mm dishes (~5×10^5 cells per dish) and treated with inhibitors. The cells were harvested, counted, and replated weekly at the same cell number (~5×10^5 cells per dish) in the presence or absence of inhibitor at the same concentration. Remaining cells were washed with PBS and aliquoted. Cells aliquoted for telomere length assay were stored at −150°C, whereas those

aliquoted for analyses of gene expression changes and apoptosis were processed immediately.

Assay of Telomerase Activity

Telomerase activity was assayed using the "TRAPeze XL" telomerase detection kit (Intergen, Purchase, NY), as described by the manufacturer. Briefly, the cells were lysed, and the 1000-cell equivalent of lysate was mixed with primers and TRAPeze reaction mixture. The reaction mixture was incubated 30 minutes at 30°C to allow telomerase-dependent elongation of "TS" primer. Elongated and amplified telomerase products were quantitated with a fluorescence plate reader (Spectra Max Gemini XS, Molecular Devices). Telomerase activity (in TPG units) was calculated by comparing the ratio of telomerase products to an internal standard for each lysate, as described by Intergen.

Colony Size Reduction Assay

We evaluated colony formation and distribution of colony sizes for both BEAC cell lines (BIC-1 and SEG-1) following treatment with telomerase inhibitor. One day before treatment, cells were plated at low density (to aim for 50 to 100 colonies/flask) in triplicate T-150 cm^2 tissue culture flasks. After 24 hours, the inhibitors were added to the concentration, which inhibited >90% telomerase activity. Fresh medium was added each week with supplementation of inhibitor at the same concentration. The colonies were fixed and stained by a 30-minute incubation in 0.2% methylene blue in 70% methanol, dried and photographed under the same magnification (200×). For colony size distribution analyses, 50 to 100 colonies representing random microscopic fields were measured.

Estimation of Telomere Length

Genomic DNA, from treated and untreated cells, was isolated using "Puregene" DNA isolation reagents (Gentra Systems, Minneapolis, MN), and median telomere lengths were determined utilizing "TeloTAGGG Telomere Length Assay" (Roche Diagnostics Corp, Indianapolis, IN). In brief, genomic DNA (4–6 μg) was digested with restriction enzymes *Hinf*I and *Rsa*I, twice in succession. The digested DNA was electrophoresed on 0.8% agarose gel, transferred to "Hybond-N^+" nylon membrane (Amersham Biosciences Corp, Piscataway, NJ) and hybridized to a Digoxigenin (DIG)-labeled telomere-specific probe. Telomeric DNA was detected by labeling with anti-DIG alkaline phosphatase. Hybridized membrane was exposed with BioMax film (Eastman Kodak, Rochester, NY), and the film was photographed using a digital camera (Alpha Innotech Corp, San Leandro, CA). Telomere length was analyzed using NIH image software.

Apoptosis

Apoptotic cells, following treatment with telomerase inhibitor, were detected using "Annexin V-BIOTIN Apoptosis Detection Kit" (Oncogene Research Products, San Diego, CA). Briefly, 0.5 mL of cells (1×10^6 cells/mL) were mixed with annexin V-BIOTIN, incubated for 15 minutes at room temperature (RT), washed, resuspended in "Binding Buffer," and treated sequentially with streptavidin conjugated to fluorescein isothiocyanate (FITC) and propidium iodide (PI). Apoptotic cells within the same microscopic field were viewed and photographed by phase contrast (PC), by fluorescence emitted at 518 nm (FITC filter), and by fluorescence emitted at 620 nm (PI filter). Apoptotic cells were analyzed using a fluorescence microscope.

In Situ Oligo Ligation

BEAC cells were treated with inhibitor for 2–3 weeks, and apoptotic cells with specific DNase I-type cleavage were detected by ApopTag Peroxidase In Situ Oligo Ligation kit (Intergen), as described by the manufacturer. Briefly, cells were fixed in methanol-free formaldehyde (1% in PBS; pH 7.4), dried, and postfixed in ethanol/acetic acid 2:1 (vol/vol). Fixed slides were incubated with a mixture of DNA ligase and a unique synthetic biotinylated oligo for 18 hours at 16°C. After washing, the slides were sequentially treated with streptavidin-peroxidase and peroxidase substrate, mounted under a glass coverslip in mounting medium "permount," and viewed under microscope.

Gene Expression and Data Analysis

BIC-1 cells with or without 10 μmol/L inhibitor PPA treatment for 7 days were harvested, and total RNA was isolated utilizing "RNeasy kit" (Qiagen Inc.). RNA was reverse transcribed using the "Superscript II RT kit" (Life Technologies), and the resulting cDNA was used in an in vitro transcription reaction to synthesize biotin-labeled cRNA utilizing ENZO RNA labeling kit (Enzo Diagnostics, Farmingdale, NY). Labeled cRNA was purified and hybridized to Human Genome U133 arrays (Affymetrix, Santa Clara, CA), representing approximately 33,000 human genes. Normalization of arrays and calculation of expression values were performed using the DNA-Chip Analyzer computer program (dChip).[31–32]

Results

Telomerase Activity in Barrett's Esophagus-Associated Adenocarcinoma Cell Lines

We assayed telomerase activity in BIC-1 and SEG-1 (BEAC) cell lines, normal diploid fibroblasts, normal intestinal epithelial cells (ATCC; CRL-7820), and normal mixed stomach and intestinal cells (ATCC; CRL-7869) using the TRAPeze telomerase detection kit (Intergen). Whereas telomerase activity in normal cells from stomach intestine (CRL-7869) was 20 ± 2 total product generated (TPG) units and small intestine (CRL-7820) was 25 ± 8 TPG units, it was 257 ± 40 TPG units in BIC-1 and 245 ± 18 TPG units in SEG-1 (Figure 1). Telomerase activity in normal fibroblasts was only 0.004 TPG units. Thus, the activity in normal

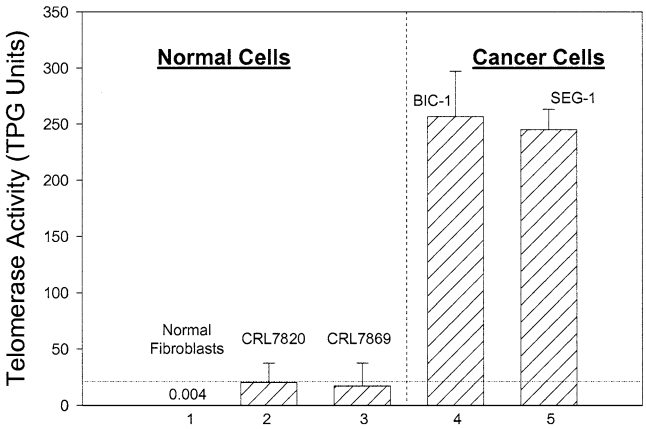

Figure 1. Assay of telomerase activity in normal and BEAC cells. Telomerase activity in BEAC cell lysates was determined using the TRAPeze XL telomerase detection kit (Intergen). Lysate (1000 cell equivalents) was mixed with TRAPeze XL reaction mix containing Amplifuor primers and incubated for 30 minutes at 30°C. Telomerase products were quantitated using a fluorescence plate reader. *Lane 1:* normal fibroblasts; *Lane 2:* normal cells from intestine (CRL7820); *Lane 3:* normal cells from stomach and intestine mixed (CRL7869); *Lane 4:* BIC-1 cells; *Lane 5:* SEG-1 cells.

gastrointestinal cells (stomach and intestine) was ~10-fold lower ($P < 0.001$) than BEAC cells but significantly higher ($P < 0.05$) than the activity in diploid fibroblasts.

Inhibition of Telomerase Activity in BEAC Cell Lines by Telomerase Inhibitors

Telomerase inhibitors that intercalate in G-quadruplex DNA effectively inhibit telomerase activity in vitro.[33] Two BEAC cell lines (BIC-1 and SEG-1) were treated with telomerase inhibitor at different concentrations for 7 days, harvested, and assessed for telomerase activity. Telomerase inhibitor PPA was required at 10 μmol/L for nearly complete (>90%) inhibition of telomerase activity in both the cell lines (Figure 2*A*). Both BIC-1 and SEG-1cells were treated with telomerase inhibitor PPA at 10 μmol/L for 1–7 days, and telomerase activity was measured. Although inhibition of telomerase activity started to appear on day 1, complete inhibition required 5 days in BIC-1 and 7 days in SEG-1 cells following treatment (Figure 2*B* and *C*).

Growth Inhibition Following Telomerase Inhibitor Treatment of BEAC Cells

Both BIC-1 and SEG-1 cells were treated with telomerase inhibitor PPA at the minimal concentration that blocked >90% of telomerase activity (10 μmol/L), and viable cell number was counted. A marked arrest of cell proliferation was observed in both cell lines following treatment with the inhibitor. Viable cell number did not change for the first 7 days and then gradually declined by 75%–99% by 21 days. Treatment with PPA at 5 μmol/L and 10 μmol/L concentrations induced 75% and 95% cell death in BIC-1 cells, respectively (Figure 3*A*). Similar induction of cell death was observed following treatment of SEG-1 cells with PPA (Figure 3*B*). Exposure of normal (CRL7820) cells to 10 μmol/L PPA for 3 weeks had no effect on live cell number (Figure 4*C*, panel IV).

Colony Formation Assay Following Treatment of BIC-1 and SEG-1 Cells With Telomerase Inhibitors

We measured the distribution of colony sizes following treatment of normal intestinal cells (CRL-7820) or cancer (BIC-1 and SEG-1) cells with telomerase inhibitor. Colony size distribution is a robust and sensitive measure of replicative potential of a cell.[26,34–35] Cells were plated at low cell density in the presence or absence of inhibitors, and colonies were stained and analyzed at weeks 1 and 3. Colony sizes in BIC-1 and SEG-1 cells were not affected by the drug treatments at 1 week (Figure 4*A*). However, following 3 weeks treatment with PPA, a marked reduction in number and size of colonies was observed in these cell lines (Figure 4*B*). Colony size (mean ± SEM, in mm) in untreated and treated BIC-1

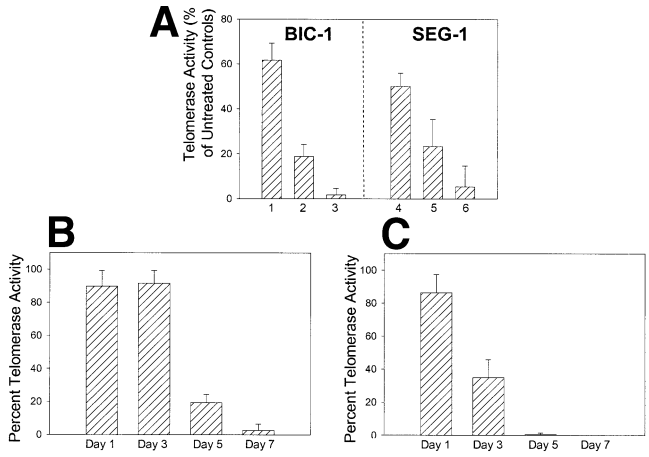

Figure 2. Effect of telomerase inhibitors on telomerase activity in BEAC cells. Telomerase activity in BIC-1 and SEG-1 cell lysates (1000 cell equivalents) was determined by fluorometric detection as described previously. (*A*) Telomerase activity in BIC-1 and SEG-1 cells following 1-week exposure to various concentrations of telomerase inhibitor PPA is presented as percentage of activity in the untreated cells. *Lane 1:* BIC-1 cells treated with 1 μmol/L inhibitor PPA; *Lane 2:* BIC-1 cells treated with 5 μmol/L PPA; *Lane 3:* BIC-1 cells treated with 10 μmol/L PPA; *Lane 4:* SEG-1 cells treated with 1 μmol/L PPA; *Lane 5:* SEG-1 cells treated with 5 μmol/L PPA; *Lane 6:* SEG-1 cells treated with 10 μmol/L PPA. (*B*) Telomerase activity following exposure of BIC-1 cells to PPA (10 μmol/L) for 1–7 days, presented as percentage of activity in untreated cells. (*C*) Telomerase activity following exposure of SEG-1 cells to PPA (10 μM) for 1–7 days, presented as percentage of activity in untreated cells.

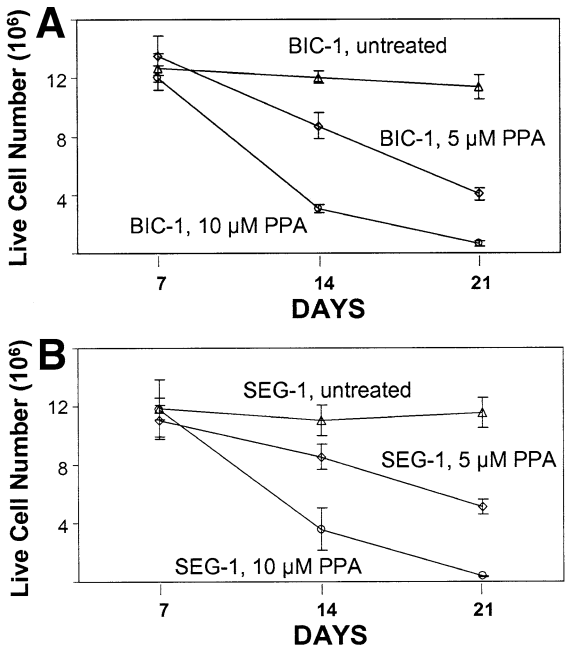

Figure 3. Limited replicative potential of BEAC cells treated with telomerase inhibitor. (*A*) BIC-1 cells were cultured in medium containing no inhibitor (*triangles*), 5 μmol/L PPA (*rectangles*), or 10 μmol/L PPA (*circles*). At the end of each 7-day-treatment cycle, cells were harvested, and the number of viable cells was counted. (*B*) SEG-1 cells were cultured in medium containing no inhibitor (*triangles*), 5 μmol/L PPA (*rectangles*), or 10 μmol/L PPA (*circles*), and viable cell number was determined as described previously.

cells at 3 weeks of culture was 25.0 ± 0.96 and 2.40 ± 0.11, respectively ($P < 0.001$), whereas, in untreated and treated SEG-1 cells, it was 26.0 ± 0.63 and 2.00 ± 0.14 mm, respectively ($P < 0.001$) (Figure 4*B*). These data indicate a marked impairment in maximal replicative potential of BEAC cells following exposure to telomerase inhibitor. Importantly, both the colony size and the number remained unchanged following exposure of normal intestinal cells (CRL-7820) to PPA (Figure 4*C*).

Effect of Telomerase Inhibitor PPA on Telomere Length in BEAC Cells

We next analyzed telomere length following exposure of BIC-1 and SEG-1 cell lines to inhibitor PPA for 3 weeks. "Telomere length," the mean size of telomeric restriction fragments generated by digesting the DNA with telomere-sparing restriction endonucleases, was estimated using genomic DNA isolated for each cell sample. The median telomere length was 3.3 kbp in BIC-1 and 2.4 kbp in SEG-1 cells. Exposure of BEAC (BIC-1 and SEG-1) cells to telomerase inhibitor PPA was associated with marked reduction in telomere length (Figure 5*A* and *B*). Telomere length in BIC-1 was reduced by 1000 bp (i.e., by 30% of the initial median value), whereas that in SEG-1 was reduced by 500 bp (i.e., by 21% of the initial median value) (Figure 5*B*).

Apoptotic Cell Death Following Telomerase Inhibition

We utilized annexin V labeling and DNA fragmentation assay to assess apoptosis. Treatment with the inhibitor resulted in induction of apoptosis in the majority of BIC-1 and SEG-1 cells. Treatment with PPA (10 μmol/L) for 2 weeks led to >80% of BIC-1 and SEG-1 cells in early apoptosis, as detected by annexin V staining (Figure 6*B*, panels I to VI). Less than 2% cells were labeled with annexin in untreated BIC-1 and SEG-1 samples (Figure 6*A*). Necrotic or late apoptotic (PI positive) cells were not detected at 2 weeks after treatment with telomerase inhibitor PPA.

We further evaluated specific DNase-I type cleavages, activated by caspase 3, in BEAC cells following 3-week treatment with PPA, utilizing ApopTag Peroxidase In Situ Oligo Ligation kit (Intergen, Purchase, NY). Cells were fixed and incubated with a mixture of DNA ligase and a unique synthetic biotinylated oligonucleotide, which specifically ligates genomic DNA carrying DNase-I type cleavage. The ligation complexes were identified by sequential treatments with streptavidin-peroxidase and peroxidase substrate and visualized under microscope. As seen in Figure 7, BIC-1 and SEG-1 cells treated with inhibitor PPA showed >85% cells with DNase-I-type cleavage.

Gene Expression Profile Following Treatment With Telomerase Inhibitor PPA

Gene expression profile following a 7-day treatment of BIC-1 cells with inhibitor PPA showed a ≥2-fold change in only 28 genes out of 33,000 genes surveyed (Figure 8). None of the important genes involved in cell cycle, cell proliferation, DNA repair, recombination, and apoptosis showed major change in the expression. The genes that changed ≥2-fold included *MAD1* (mitotic arrest deficient), a mitotic checkpoint gene,[36] *P57* (cyclin-dependent kinase inhibitor 1C), a candidate cell cycle control gene,[37] and *HSP70-1* (heat shock 70-kilodalton protein 1A)[38] (Figure 8).

Discussion

This study shows that (1) telomerase activity is markedly increased in Barrett's-associated adenocarcinoma cell lines BIC-1 and SEG-1, and telomeres are short (less than 4 kbp); (2) a G-complex-intercalating telomerase inhibitor (PPA) causes growth arrest and inhibits colony formation; (3) growth arrest is associated with apoptosis; (4) apoptosis is due to DNase-1 activation; and (5) effects of PPA are associated with reduction

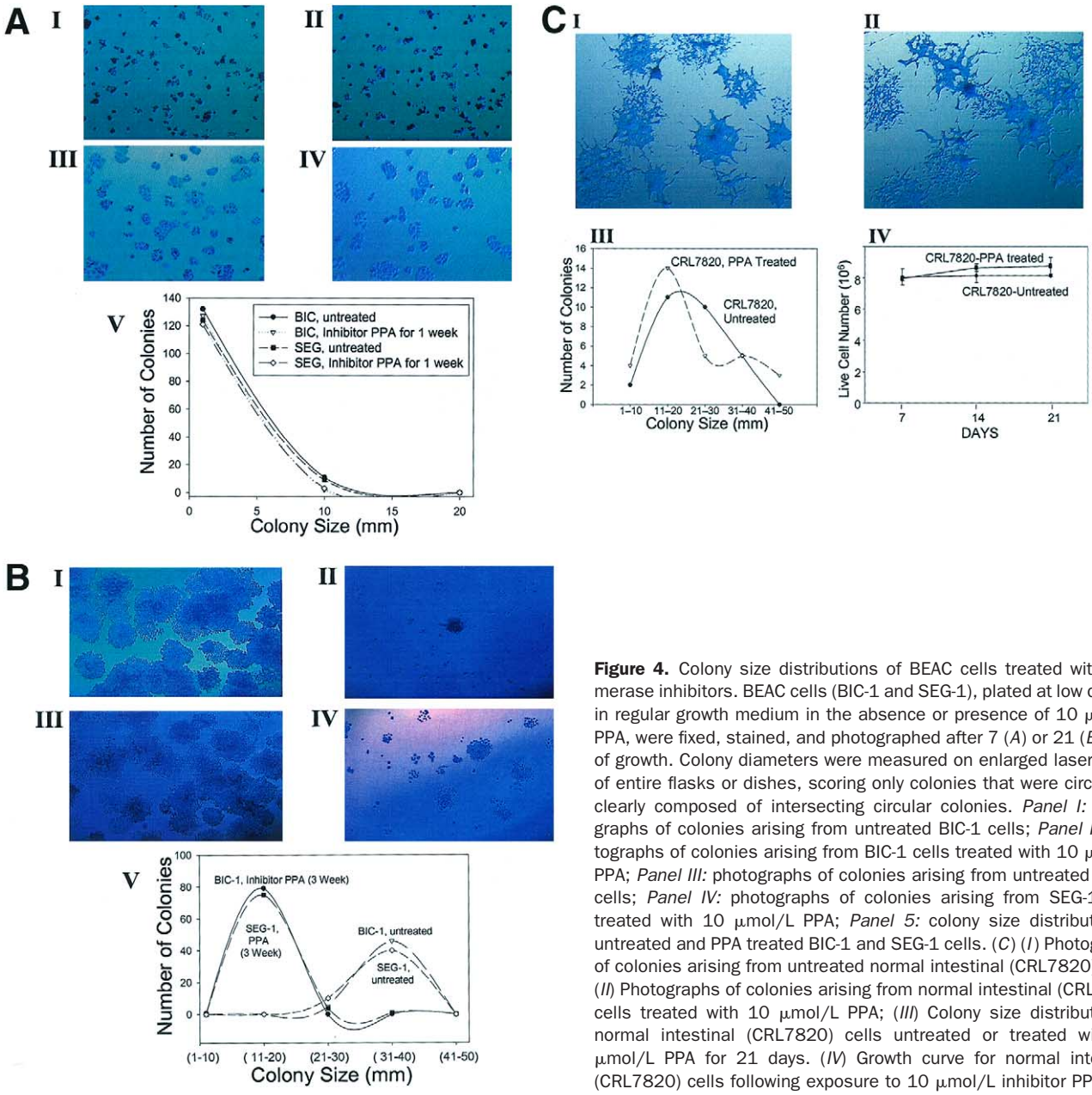

Figure 4. Colony size distributions of BEAC cells treated with telomerase inhibitors. BEAC cells (BIC-1 and SEG-1), plated at low density in regular growth medium in the absence or presence of 10 μmol/L PPA, were fixed, stained, and photographed after 7 (*A*) or 21 (*B*) days of growth. Colony diameters were measured on enlarged laser prints of entire flasks or dishes, scoring only colonies that were circular or clearly composed of intersecting circular colonies. *Panel I:* photographs of colonies arising from untreated BIC-1 cells; *Panel II:* photographs of colonies arising from BIC-1 cells treated with 10 μmol/L PPA; *Panel III:* photographs of colonies arising from untreated SEG-1 cells; *Panel IV:* photographs of colonies arising from SEG-1 cells treated with 10 μmol/L PPA; *Panel 5:* colony size distribution of untreated and PPA treated BIC-1 and SEG-1 cells. (*C*) (*I*) Photographs of colonies arising from untreated normal intestinal (CRL7820) cells; (*II*) Photographs of colonies arising from normal intestinal (CRL7820) cells treated with 10 μmol/L PPA; (*III*) Colony size distribution of normal intestinal (CRL7820) cells untreated or treated with 10 μmol/L PPA for 21 days. (*IV*) Growth curve for normal intestinal (CRL7820) cells following exposure to 10 μmol/L inhibitor PPA.

in telomerase activity, shortening of telomere length, and without any significant changes in the gene expression profile of these cells.

We found that the telomerase activity was over 60-fold higher in normal cells from stomach and intestine than the diploid fibroblasts. This elevated activity may be required for maintenance of ever-renewing gastrointestinal tract epithelial cells and confirms the observations made in primary patient cells by Bachor et al.[39] Telomerase activity in both BEAC cell lines was further 10-fold higher than the actively proliferating normal cells from stomach and intestine. High telomerase activity has also been previously reported in primary cells from patients with Barrett's esophagus and esophageal adenocarcinoma.[5] Our studies also show that the median telomere length of the BEAC cells was short, being only 3.3 kbp in BIC and 2.4 kbp in SEG-1 cells. This is in contrast to human somatic tissues and nonneoplastic Barrett's mucosa that have ~10-kb[40] and 11.8 ± 1.2-kb telomeres,[41] respectively. The short telomeres may increase the susceptibility of the BEAC cells to the telomerase inhibitors. These results provided further rationale for investigating the importance of telomerase as a target for the treatment of Barrett's-associated adenocarcinoma. Moreover BEAC cells are unique in that the origin of the cancer cell is from a premalignant condition in which other mechanism(s) besides telomerase may be operative in maintaining telomere length. This provides a unique

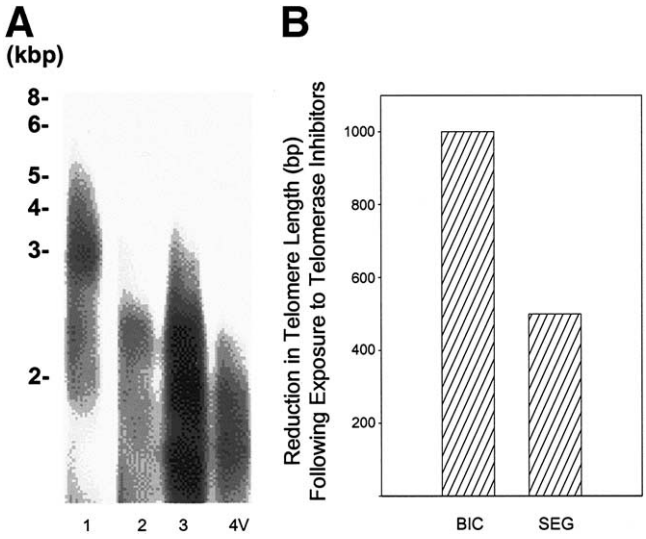

Figure 5. Reduction in telomere length following PPA treatment of BEAC cells. BIC-1 and SEG-1 were treated with 10 μmol/L inhibitor PPA for 3 weeks. Genomic DNA was isolated, and median telomere length was determined as described. (*A*) Telomere terminal restriction fragments of (1) untreated BIC-1 cells; (2) BIC-1 cells treated with 10 μmol/L PPA for 21 days; (3) untreated SEG-1 cells; (4) SEG-1 cells treated with 10 μmol/L PPA for 21 days. (*B*) Reduction in median telomeric DNA restriction fragments, 50th percentile lengths, calculated from BIC-1 and SEG-1 telomere fragment size distributions in scanned lanes as in A.

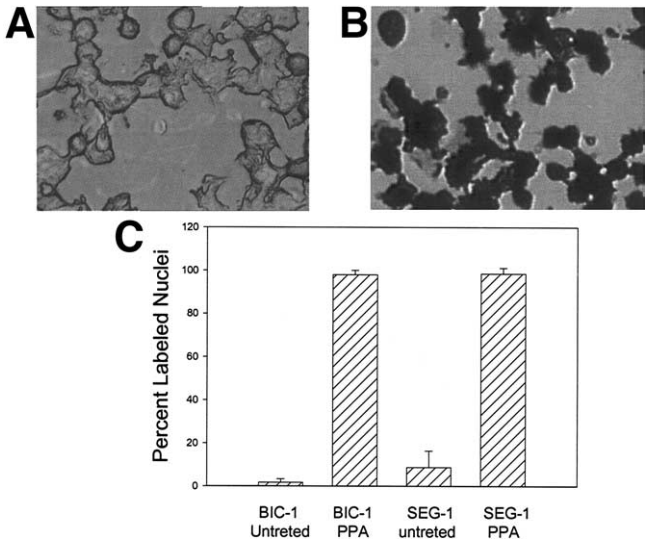

Figure 7. Identification of specific DNase I-type cleavages in PPA-treated BEAC cells. Cells were fixed and incubated with a mixture of DNA ligase and a unique synthetic biotinylated oligo. The ligation complexes were identified by sequential treatments with streptavidin-peroxidase and peroxidase substrate and visualized under microscope. (*A*) Untreated BIC-1 cells. (*B*) BIC-1 cells treated with 10 μmol/L PPA for 21 days. (*C*) Percentage labeled nuclei, indicative of specific DNase I cleavage, in BIC-1 and SEG-1 cells following 21-day exposure to PPA.

scenario in evaluating efficacy of the telomerase inhibitors in BEAC.

Several approaches have been used for inhibiting telomerase.[42] However, recently, most interest has been focused on the agents that react with G-quadruplex DNA[43] and a large variety of other unrelated compounds with multiple actions.[44] The TTAGGG motif of telomeric DNA allows formation of unusual DNA conformation based on guanine quadruplex (G-qudruplex). G-quadruplex ligands can block the action of telomerase. Tumor cells naturally take up these agents, and transfection is not needed. However, G-quadruplex intercalators can bind to both intra- and intermolecular G-quadruplex structures and may thus alter cellular transcriptional activity leading to cell death by mechanisms other than telomerase inhibition.[45]

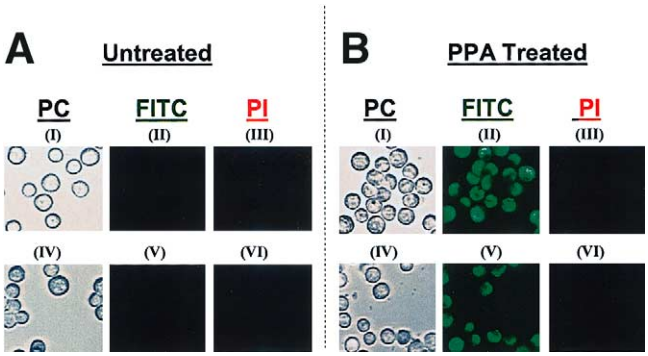

Figure 6. Apoptosis following treatment of BEAC cells with telomerase inhibitors. BIC-1 and SEG-1 cells untreated and treated with PPA (10 μmol/L) for 14 days were mixed with annexin V-BIOTIN, incubated for 15 minutes at room temperature, and treated sequentially with streptavidin conjugated to fluorescein isothiocyanate (FITC) and propidium iodide (PI). Apoptotic cells within the same microscopic field were viewed and photographed by phase contrast (PC), by fluorescence emitted at 518 nm (FITC filter), and fluorescence emitted at 620 nm (propidium iodide or PI filter). Using the FITC filter, early apoptotic cells (positive for Annexin V-Biotin-FITC staining) appear bright green, and, using PI filter, the late apoptotic or necrotic cells (positive for PI) appear reddish. (*A: I–III*) Untreated BIC-1 cells; (*IV–VI*) Untreated SEG-1 cells. (*B: I–III*) BIC-1 cells treated with PPA. (*IV–VI*) SEG-1 cells treated with PPA.

We used a 2,6-diaminoanthraquinone derivative, 2,6-bis[3-{N-Piperidino}propionamido]anthracene-9,10-dione (PPA) that is thought to be a selective inhibitor of telomerase.[29] This agent showed no acute cytotoxicity in either the cancer or normal cells. Following 7-day treatment of BIC-1 cells with PPA, expression of only 28 out of 33,000 genes was altered. The importance of these changes is not fully understood. However, the occurrence of minimal change in gene expression, prolonged exposure required to induce cell death, and reduction of telomere length associated with PPA exposure suggest that effects were predominantly because of its action on telomerase.[33]

Treatment of BIC-1 and SEG-1 cells with PPA resulted in the loss of telomerase activity in 5–7 days. It

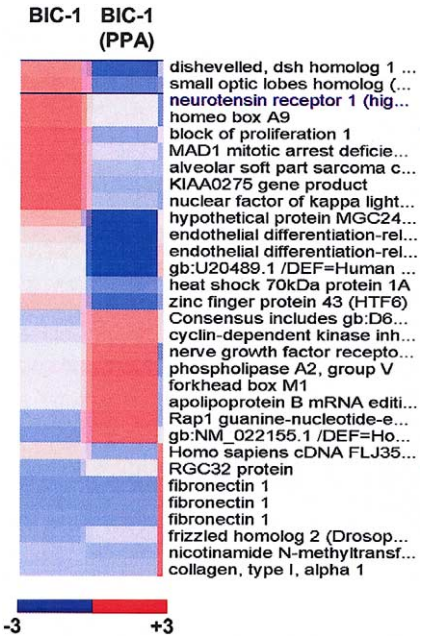

Figure 8. Gene expression profile following exposure of BEAC cells to PPA. Total RNA was isolated from BIC-1 cells treated with 10 μmol/L PPA for day 7 and hybridized to human genome U133 (Affymetrix) representing approximately 33,000 human genes. Cluster image of genes with ≥2-fold change in expression is shown. BIC-1, untreated BIC-1 cells; BIC-1 PPA, BIC-1 cells treated with PPA (10 μmol/L) for 7 days.

has been reported that, in cervical cancer cells, inhibition of telomerase activity alone without telomere shortening may cause caspase-associated apoptosis that occurs within a week of telomerase inhibition.[46] However, in the BEAC cells, apopotosis was delayed, correlating with reduction in telomere length. The telomerase inhibition was associated with reduction of 500 to 1000 bp (21% to 30% of the control) in the telomere lengths. Although the effect of PPA on telomere length has not been reported in any cancer cells, other telomerase inhibitors have been shown to cause similar reduction in telomere length. The median telomere shortening was considerably more pronounced in BIC-1 cells that have relatively longer telomeres. BIC-1 cells lost 1000 bp of telomeres in the same time frame in which SEG-1 lost only 500 bp, reflecting higher proliferative rate of BIC-1 cells. In both the cell lines, PPA treatment resulted in maximal cell death of >80% cells in 3 weeks. A lag phase of 7–14 days and requirement for a total of 3 weeks to induce maximal cell death suggest gradual telomere shortening reaching a critical telomere length that leads to growth arrest.[47,48] The critical minimal median telomere length required for survival of cells in culture is ~2 kbp.[49] SEG-1 cells, with 2.4-kbp median telomere length, may undergo apoptotic cell death with relatively smaller reduction in telomere length.

Telomere disruption may lead to either replicative senescence or apoptosis. In our study, the BEAC cells did not show evidence of necrosis (PI positivity). Instead, the telomerase inhibitor induced apoptosis in the tumor cells as shown by a positive annexin V labeling. These finding are similar to those reported in other cancers in which telomerase inhibitors cause apoptotic cell death.[18,28] Apoptosis may be related to severe telomere disruption. It has been proposed that, with severe reduction in telomere length referred to as M2, telomeres are no longer able to protect chromosomal integrity and apoptosis ensues.[50,51]

Caspases that are serine proteases activate specific DNases that cause DNA fragmentation and apoptotic cell death.[52] We found that, in BEAC cell lines, apoptosis was associated with DNase-1 type cleavage of the DNA as evidenced by the in situ ligation (ISOL) technique that selectively labels DNase-1 type cleavage. It is unclear how the telomerase inhibitor causes DNase-1 activation. DNase-I is normally activated by caspase 3-mediated cleavage of poly (ADP-ribose) polymerase (PARP) and actin.[53] It has been suggested that telomerase may act to suppress mitochondrial dysfunction and caspase activation and, therefore, telomerase inhibitors might promote mitochondrial dysfunction and caspase 3 activation.[18]

In summary, these studies show that BEAC cells have relatively short telomeres and markedly elevated telomerase activity. Moreover, a G-quadruplex ligand, 2,6-bis[3-{N-Piperidino}propionamido]anthracene-9,10-dione, selectively inhibits telomerase and shortens the telomere length. The inhibitor also causes replicative arrest and apoptosis of the cancer cells after prolonged exposure. Importantly, proliferative potential (as assessed by growth kinetics and colony forming ability) of normal intestinal cells remains unaffected following exposure to the inhibitor at the same concentration and for the same duration. The effects of this ligand are similar to those caused by introduction of peptide nucleic acid (PNA) oligonucleotides targeted to the RNA component of telomerase, which inhibited telomerase activity, shortened telomere length, reduced colony size, and induced proliferation arrest of immortal human cell lines.[26] We have also observed similar growth inhibitory effects by other telomerase inhibitors including another G-quadruplex interacting agent QQ98 (fluroquinoanthroxazine) and SiRNA specifically directed against hTERT (not shown) in BEAC cells. Telomerase inhibition in BEAC cells, whether mediated by RNA interference, QQ98, or PPA, leads to reduction in colony number and size and apoptotic cell death in a period of 3 weeks.

These studies provide a strong rationale for the potential use of telomerase inhibitors in chemoprevention and treatment of Barrett's-associated adenocarcinoma.

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