Telomerase is a protein-RNA enzyme complex that adds a six-base DNA sequence (TTAGGG) to the ends of chromosomes and thereby prevents their shortening. Reduced telomerase activity is associated with cellular senescence, whereas increased telomerase activity is associated with cell transformation and immortalization. Because many types of cancer have been associated with reduced apoptosis, whereas cell differentiation and senescence have been associated with increased apoptosis, we tested the hypothesis that telomerase activity is mechanistically involved in the regulation of apoptosis. Levels of telomerase activity in cultured pheochromocytoma cells decreased prior to cell death in cells undergoing apoptosis. Treatment of cells with the oligodeoxynucleotide TTAGGG or with 3,3'-diethyloxadicarbocyanine, agents that inhibit telomerase activity in a concentration-dependent manner, significantly enhanced mitochondrial dysfunction and apoptosis induced by staurosporine, Fe3+ (an oxidative insult), and amyloid β-peptide (a cytotoxic peptide linked to neuronal apoptosis in Alzheimer's disease). Overexpression of Bcl-2 and the caspase inhibitor zVAD-fmk protected cells against apoptosis in the presence of telomerase inhibitors, suggesting a site of action of telomerase prior to caspase activation and mitochondrial dysfunction. Telomerase activity decreased in cells during the process of nerve growth factor-induced differentiation, and such differentiated cells exhibited increased sensitivity to apoptosis. Our data establish a role for telomerase in suppressing apoptotic signaling cascades and suggest a mechanism whereby telomerase may suppress cellular senescence and promote tumor formation.

Telomeres consist of repeats of the sequence TTAGGG/CCCTAA at the ends of chromosomes. These DNA repeats are synthesized by enzymatic activity associated with an RNA-protein complex called telomerase (1–3). Telomerase activity decreases dramatically during the processes of growth arrest and cell differentiation (4–6). Indeed, in most somatic cells, telomerase activity is low or nonexistent, and telomere length decreases with increasing cell divisions; telomere shortening has therefore been proposed to play a role in cellular senescence (7–9). The latter hypothesis recently gained strong support from studies showing that the life span of normal human fibroblasts can be extended if they are transfected with a vector encoding the telomerase catalytic subunit (10). The mechanism whereby telomerase activity suppresses cellular senescence has not been established.

Apoptosis is a stereotyped form of cell death that occurs in a variety of physiological and pathological settings (11, 12). Apoptosis is characterized by cell shrinkage, plasma membrane blebbing, and nuclear chromatin condensation and DNA fragmentation. Biochemical features of apoptosis include loss of plasma membrane phospholipid asymmetry, activation of one or more cysteine proteases of the caspase family, mitochondrial dysfunction, and release of factors from mitochondria that induce nuclear destruction (13–15). During the aging process, damaged senescent cells are eliminated by apoptosis (16). Abnormal apoptosis is associated with many different disease states, including cancers, in which apoptosis is suppressed (17), and neurodegenerative disorders, in which apoptosis is enhanced (18). Because cell immortalization is often associated with both increased telomerase activity (19, 20) and increased resistance to apoptosis (21), we performed experiments aimed at determining whether telomerase actively modulates the cell death process.

**EXPERIMENTAL PROCEDURES**

**PC12 Cell Cultures and Experimental Treatments**—Control vector-transfected PC12 cells (PC12-V) and PC12 cells stably overexpressing human telomerase (PC12-Bcl2) were established using methods described previously (22, 23). Cultures were maintained in plastic culture flasks and subcultured onto polyethyleneimine-coated plastic 60-mm dishes for telomerase activity assays, 22-mm2 polyethyleneimine-coated glass coverslips for analyses of apoptosis and mitochondrial transmembrane potential, or polyethyleneimine-coated 24-well plates for 3,4,5-di-methyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide assays. Cells were maintained in RPMI medium supplemented 10% with heat-inactivated horse serum and 5% with heat-inactivated fetal bovine serum (at 37 °C in a 5% CO2 atmosphere). Immediately prior to experimental treatment, the medium was replaced with RPMI medium containing 1% fetal calf serum. 3,3'-Diethyloxadicarbocyanine (DODCB) (24) was purchased from Sigma and was prepared as a 500× stock in dimethyl sulfoxide. Synthetic oligodeoxynucleotide telomeric repeat DNA sequence (5′-TTAGGG-3′) and an oligonucleotide with a scrambled sequence (5′-TGTAGG-3′) were purchased from IDT (Coralville, IA) and were prepared as 1 μM stocks in sterile deionized water. The telomerase inhibitors were administered in a 1-h pretreatment and remained in the medium during the rest of the experiment. Staurosporine (STS) was purchased from Sigma and was prepared as a 500× stock in dimethyl sulfoxide. Synthetic amyloid β-peptide 25–35 (Aβ) was purchased from Bachem (Torrance, CA) and was prepared as a 1 μM stock in sterile deionized water 2 h prior to use. Fe3+ (Sigma) was prepared as a 1 μM stock in water. In order to differentiate cells into a neuron-like phenotype, the culture medium was replaced with RPMI medium containing 0.1% bovine serum albumin and 50 ng/ml nerve growth factor (NGF).

**Quantification of Apoptosis**—Following experimental treatments, cells were fixed in 4% paraformaldehyde, and membranes were permeabilized with 0.2% Triton X-100 and stained with the fluorescent DNA-binding dye Hoechst 33342 as described previously (23). Hoechst-stained cells were visualized and photographed under epifluorescence illumination (340 nm excitation and 510 nm barrier filter) using a × 60

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oil immersion objective (200 cells/culture were counted, and counts were made in at least four separate cultures/treatment condition). Analyses were performed without knowledge of the treatment history of the cultures. The percentage of "apoptotic" cells (cells with condensed and fragmented DNA were considered apoptotic) in each culture was determined. We previously found that the method for assessment of apoptosis is superior to the terminal transferase uridyl nick-end-labeling assay (which also detects necrotic cells) and that the death of PC12 cells induced by STS, Fe^{2+}, and Aβ (at the concentrations used in the present study) can be completely blocked by caspase inhibitors and macromolecular synthesis inhibitors (23, 32–34).

Telomerase Activity—Telomerase activity was measured using a polymerase chain reaction-based telomeric repeat amplification protocol (TRAP) as described previously (25, 26) using a kit from Oncor (Gaithersburg, MD). Briefly, cells (10^10–10^11 cells/culture) were scraped in PBS, pelleted by centrifugation for 5 min at 400 × g, and resuspended in 200 μl of lysis buffer that contained 0.5% CHAPS, 1 mM MgCl₂, 1 mM EDTA, 0.1 mM benzamidine, 5 mM β-mercaptoethanol, 10% glycerol, and 10 mM Tris-HCl, pH 7.5. The lysate was incubated for 30 min at 4 °C and centrifuged at 12,000 × g for 20 min at 4 °C, and protein concentration of the supernatant was determined using a BCA kit (Pierce). Cell extract (1–200 ng of protein) was added to a reaction mixture containing 10× TRAP buffer (15 mM MgCl₂, 630 mM KCl, 0.5% Tween 20, 10 mM EDTA, 0.1% bovine serum albumin, and 200 mM Tris-HCl, pH 8.3), 50× dNTP mixture (2.5 mM each of dATP, dTTP, dCTP, and dGTP), TS primer mixture (Oncor), and 5 units/μl Taq polymerase. The mixture was incubated at room temperature for 20 min, followed by a 30-min incubation at 30 °C. The mixture was then subjected to 30 cycles of amplification (94 °C for 30 s, 53 °C for 45 s, and 72 °C for 60 s). Each reaction product was amplified in the presence of a 36-base pair internal TRAP assay standard. Samples were loaded on a 12.5% nondenaturing polyacrylamide gel, and DNA was electrophoresed through the gel. Gels were stained with SyBr (Molecular Probes), and images were captured using an AlphaImager. For quantification of relative telomerase activity in each sample, the total density of bands 1–10 of the characteristic ladder (band 1 being the band immediately above the internal standard band) was quantified using NIH Image software, and values are expressed relative to a control value as indicated in the figure legends. TRAP assays were performed on serial dilutions of extracts from untreated control PC12 cells in order to establish the linear response range of the assay and to thereby select appropriate protein concentrations for analyses of extracts from cells subjected to experimental treatments. Control reactions included tubes lacking cell extract or containing cell extract treated with 200 μg/ml RNase.

Mitochondrial Transmembrane Potential—The dye rhodamine 123 was used as a measure of mitochondrial transmembrane potential using methods described previously (27, 28). Briefly, cultures were incubated for 30 min in RPMI medium containing 5 μM rhodamine 123 and were then washed with Locke’s buffer: NaCl, 154 mM; KCl, 5.6 mM; CaCl₂, 2.3 mM; MgCl₂, 1 mM; NaHCO₃, 3.6 mM; glucose, 5 mM; HEPES, 5 mM (pH 7.2). Cellular fluorescence was measured using a confocal laser scanning microscope with excitation at 488 nm and emission at 510 nm; cells were selected randomly under bright-field optics and then scanned with the laser. Levels of cellular fluorescence were quantified from the fluorescence images using ImageSpace software (Molecular Dynamics); the fluorescence measurements were made in the entire cell body and are expressed as average pixel intensity/cell body. Measurements were made in at least 40 cells/culture (analyses were performed without knowledge of treatment history of the cultures).

RESULTS

Telomerase Inhibitors Enhance Apoptosis of Undifferentiated PC12 Cells—Because tumor cells that are generally more resistant to apoptosis than are normal cells (21, 23) and because telomerase activity is increased in tumor cells (19, 20), we determined whether inhibition of telomerase would modify the cell death process. In order to determine an appropriate concentration of cell extract that would allow reliable quantification of relative levels of telomerase activity in PC12 cells, the basal level of telomerase activity was measured in serial dilutions of PC12 cell extract (1–200 ng). An example of such an assay is shown in Fig. 1A, and values of relative telomerase activity are shown in Fig. 1B. Essentially no bands were detected in TRAP assay samples lacking cell extract, and treatment of the cell extract with RNase prior to TRAP assay completely eliminated telomerase activity, demonstrating specificity of the assay (Fig. 1C). Based upon these results, we used 20–100 ng of PC12 cell extract for subsequent assays. A comparison of relative levels of telomerase activity in HeLa cells (known to exhibit high telomerase activity) and PC12 cells indicated that PC12 cells have a quite high basal level of telomerase activity (Fig. 1C). We next examined the effects of two different putative telomerase inhibitors on telomerase activity in PC12 cells. One inhibitor was the six-base oligonucleotide TTAGGG, which corresponds to the sequence that telomerase adds to telomeres; this oligonucleotide was previously shown to be effective in suppressing telomerase activity in various cultured cell lines (29–31). The second inhibitor was the compound DODCB, an agent previously shown to bind selectively to dimeric hairpin quadruplexes (24) and to inhibit telomerase activity.2 As expected, both TTAGGG and DODCB caused decreases in telomerase activity in PC12 cell homogenates (Fig. 1, C and D). A scrambled control oligonucleotide (TGTGAG) had no effect on telomerase activity (data not shown). The IC₅₀ values for DODCB and TTAGGG were approximately 2 and 4 μM, respectively (Fig. 1D). Maximal inhibition of telomerase activity was observed with concentrations of DODCB and TTAGGG in the range of 10–50 μM. DODCB and TTAGGG, at concentrations effective in suppressing telomerase activity (1–10 μM DODCB and 2–20 μM TTAGGG), did not affect the TRAP assay when added immediately prior to the polymerase chain reaction step (data not shown).

In order to determine whether telomerase activity influenced the vulnerability of PC12 cells to apoptosis, we employed STS, Aβ, and Fe^{2+}, three agents known to induce apoptosis in PC12 cells (23, 32). Cultures were pretreated with TTAGGG, TGTGAG, or DODCB prior to exposure to STS, Aβ, or Fe^{2+}. Basal levels of apoptosis were approximately 4–5% in vehicle-treated control cultures and were not significantly affected by TTAGGG or DODCB at concentrations (10–20 μM) that inhibited telomerase activity by over 80% (Figs. 1D and 2A), although there was a trend toward an increased basal level of apoptosis in cultures exposed to the telomerase inhibitors (Fig. 2A). Apoptosis induced by each insult (STS, Aβ, and Fe^{2+}) was significantly enhanced in cultures treated with TTAGGG and DODCB but not in cultures treated with scrambled DNA (Fig. 2, A and B). These results suggested an anti-apoptotic role for telomerase activity. Alterations in mitochondria, including membrane depolarization and release of apoptotic factors, occur prior to nuclear alterations in a variety of cells undergoing apoptosis (13). We previously showed that STS, Aβ, and Fe^{2+} each caused mitochondrial membrane potential measured as a decrease in levels of rhodamine 123 fluorescence (Fig. 3). The magnitude of the decrease in mitochondrial membrane potential was significantly greater in PC12 cells treated with TTAGGG or DODCB than in cells treated with vehicle or scrambled DNA (Fig. 3), suggesting that the anti-apoptotic action of telomerase occurs at a step prior to mitochondrial alterations.

Telomerase Activity Decreases in Cells Undergoing Apoptosis—We next determined whether levels of telomerase activity changed in PC12 cells undergoing apoptosis. STS, Aβ, and Fe^{2+} each caused a time-dependent increase in the number of PC12 cells exhibiting apoptotic nuclei beginning 8 h following treatment and progressing through 24 h (Fig. 4A). Levels of telomerase activity were reduced within 4–8 h of treatment with each apoptotic insult and continued to decrease through 16 h (Fig. 4B).
Bcl-2 and a Caspase Inhibitor Protect against the Apoptosis-enhancing Actions of Telomerase Inhibitors—Expression of the proto-oncogene bcl-2 is correlated with resistance of many types of tumor cells to apoptosis, and overexpression of Bcl-2 in cultured cells confers resistance to apoptosis induced by an array of agents, including STS, Aβ, and Fe²⁺ (35). We previously generated lines of PC12 cells stably overexpressing Bcl-2 and demonstrated their resistance to apoptosis induced by a variety of insults (23, 32). In agreement with a recent study (41), we found that levels of telomerase activity were approximately 2-fold greater in PC12 cells overexpressing Bcl-2 (PC12-Bcl2) compared with vector-transfected control PC12 cells (PC12-V) (levels were 194 ± 8% of the control level; n = 3 cultures). We next quantified levels of apoptosis in PC12-V and PC12-Bcl2 cells following exposure to telomerase inhibitors (TTAGGG and DODCB) in combination with apoptotic insults (STS, Aβ, and Fe²⁺). PC12 cells overexpressing Bcl-2 were very resistant to apoptosis induced by each insult in the presence of telomerase inhibitors (Fig. 5A), as well as in the absence of telomerase inhibitors (data not shown; see Refs. 23 and 32). These results indicate that the apoptosis-enhancing action of telomerase inhibitors occurs at a stage of the apoptotic cascade prior to that at which Bcl-2 exerts its anti-apoptotic action.

Members of the caspase family of cysteine proteases play important roles in effecting apoptotic cell death in most, if not all, types of mammalian cells (36, 37). In order to determine whether caspase activation played a role in the apoptosis-enhancing actions of telomerase inhibitors, we employed the broad-spectrum irreversible caspase inhibitor zVAD-fmk (38). Apoptosis induced by STS, Aβ, and Fe²⁺ was prevented by zVAD-fmk (Fig. 5B). zVAD-fmk also prevented apoptosis in PC12 cells exposed to combinations of TTAGGG or DODCB and each apoptotic insult (Fig. 5B). Collectively, the data suggest that the anti-apoptotic action of telomerase is exerted at a relatively early stage in the cell death process, prior to the points at which Bcl-2 and caspase inhibitors act.

Telomerase Activity Is Decreased, and Vulnerability to Apoptosis Is Increased in Differentiated PC12 Cells—Primary differentiated neurons are known to be more vulnerable to apoptosis, induced by a variety of insults, than are various neural tumor cell lines (23). Because cellular differentiation is often associated with a decrease in levels of telomerase activity (4–6, 39), we sought to determine whether a relationship exists between vulnerability to apoptosis and telomerase activity in PC12 cells. We first compared the vulnerability of undifferentiated PC12 cells and PC12 cells that had been differentiated into a neuron-like phenotype by treatment with NGF to apoptosis induced by STS and Aβ. Differentiated PC12 cells were significantly more vulnerable than undifferentiated PC12 cells to apoptosis induced by STS and Aβ (Fig. 6).

Previous studies have shown that telomerase activity decreases upon differentiation of NT2 teratocarcinoma cells into a neuron-like phenotype in response to treatment with retinoic acid (39). We obtained similar results in PC12 cells following exposure to NGF, a treatment that induces differentiation of PC12 cells into a neuron-like phenotype. Following exposure to NGF, there was a progressive decrease in telomerase activity,
with levels declining to approximately 10% of control levels by day 8 (Fig. 7A). In contrast to the case of undifferentiated PC12 cells (Fig. 2), when differentiated PC12 cells were exposed to apoptotic insults in the presence of the telomerase inhibitors TTAGGG or DODCB, levels of apoptosis were not different from levels in differentiated cells exposed to the insults in the absence of telomerase inhibitors (Fig. 7B). These data demonstrate a strong correlation between reduced telomerase activity and increased vulnerability to apoptosis, and when taken together with the data showing that telomerase inhibitors enhance apoptosis in undifferentiated PC12 cells, they suggest a role for telomerase in suppressing apoptosis.

**DISCUSSION**

Several lines of evidence obtained in the present study suggest that telomerase activity plays a role in cellular resistance to apoptosis. First, inhibition of telomerase activity with TTAGGG and DODCB was associated with increased vulnerability of PC12 cells to apoptosis induced by three different insults (STS, Aβ, and Fe²⁺). These data are consistent with a very recent study showing that glioblastoma cell lines with high levels of telomerase activity exhibit reduced sensitivity to cisplatin-induced apoptosis and that overexpression of telomerase antisense increases the vulnerability of the resistant cell line.
lines (39). Second, apoptotic insults caused a relatively rapid decrease in telomerase activity that preceded nuclear manifestations of apoptosis. A decrease in telomerase activity following exposure of human testicular cells to the DNA-damaging apoptotic agent cisplatin was recently documented by Burger et al. (40). Third, stable overexpression of Bcl-2 resulted in an increase in telomerase activity and resistance to apoptosis. The latter finding is consistent with a recent study showing that overexpression of Bcl-2 in human cervical carcinoma (HeLa) cells resulted in increased telomerase activity (41). Fourth, we found that telomerase activity was decreased upon differentiation of PC12 cells and that such differentiated cells exhibited increased sensitivity to apoptosis. Although further work will be required to establish the mechanism responsible for the resistance of cells with high levels of telomerase activity to apoptosis, the ability of exogenous TTAGGG to enhance apoptosis is consistent with a role for the recognized DNA repeat elongating function in the anti-apoptotic action of telomerase. Because telomeres play a role in protecting DNA, it is possible that telomerase could prevent DNA-damaging events that trigger apoptosis. Indeed, DNA damage may be a critical event that

FIG. 3. Telomerase inhibitors exacerbate mitochondrial membrane depolarization induced by apoptotic insults in PC12 cells. Cultures of PC12 cells were pretreated for 1 h with saline (vehicle), 20 \( \mu \text{M} \) telomeric repeat DNA (TTAGGG), 20 \( \mu \text{M} \) scrambled DNA (TGTGAG), or 10 \( \mu \text{M} \) DODCB. Cultures were then exposed for 6 h to 0.2% dimethyl sulfoxide (control), 1 \( \mu \text{M} \) STS, 20 \( \mu \text{M} \) A\( \beta \), or 10 \( \mu \text{M} \) Fe\( \text{II} \). Levels of rhodamine 123 fluorescence were quantified, and values are the mean and S.E. of determinations made in four separate cultures. Values for vehicle-treated cells exposed to STS, A\( \beta \), and Fe\( \text{II} \) were significantly less than the corresponding control value (\( p < 0.01 \)). Values for cells treated with TTAGGG or DODCB and then exposed to STS, A\( \beta \), or Fe\( \text{II} \) were significantly less than corresponding values for cultures treated with STS, A\( \beta \), or Fe\( \text{II} \) alone (\( p < 0.02 \)). Analysis of variance with Scheffe’s post hoc tests was used.

FIG. 4. Telomerase activity is decreased in cells undergoing apoptosis. A, PC12 cell cultures were exposed for the indicated time periods to vehicle (0.2% dimethyl sulfoxide) (control), 1 \( \mu \text{M} \) STS, 20 \( \mu \text{M} \) A\( \beta \), or 10 \( \mu \text{M} \) Fe\( \text{II} \), and levels of apoptosis were quantified. Values are the mean and S.E. of determinations made in four separate cultures. B, PC12 cell cultures were exposed for the indicated time periods to vehicle (0.2% dimethyl sulfoxide), 1 \( \mu \text{M} \) STS, 20 \( \mu \text{M} \) A\( \beta \), or 10 \( \mu \text{M} \) Fe\( \text{II} \), and levels of telomerase activity in cell extracts (100 ng of extract) were quantified. Values are expressed as a percentage of the telomerase activity level in untreated control cultures and represent the mean and S.E. of determinations made in three cultures.
triggers apoptosis in response to a variety of stimuli (44). For example, apoptosis induced by STS is associated with early DNA damage (45), and DNA damage is a very early event in apoptosis induced by oxidative stress in human bladder tumor cells (46). Consistent with a role for DNA damage being an early and pivotal event in many apoptotic paradigms are data showing that Bcl-2 overexpression (47–49) and caspase inhibition (48, 50) can prevent apoptosis induced by DNA-damaging agents. Our data showing that Bcl-2 overexpression and caspase inhibitors protect PC12 cells against the pro-apoptotic actions of telomerase inhibitors in three different apoptotic paradigms are consistent with the latter studies and suggest that telomerase suppresses an early event in the apoptotic cascade.

Our data suggest possible roles of telomerase in modulating apoptosis that occurs in both physiological settings and pathological states. Apoptosis occurs during the normal turnover of a variety of cell types throughout the body. The relatively low level of telomerase activity in many somatic cells that turn over (e.g. fibroblasts and various epithelial cells) may be important in allowing the cells to undergo apoptosis in response to appro-
FIG. 7. Telomerase activity decreases in PC12 cells during NGF-induced differentiation into a neuron-like phenotype, and telomerase inhibitors do affect differentiated PC12 cells. A, PC12 cells were treated with NGF for the indicated time periods (see under “Experimental Procedures”), and relative levels of telomerase activity in cell extracts (100 ng) were measured. Values are expressed as a percentage of telomerase activity in control cultures not treated under “Experimental Procedures”), and relative levels of telomerase activity in cell extracts (100 ng) were measured. Values are expressed as a percentage of telomerase activity in control cultures not treated with NGF and represent the mean and S.E. of determinations made in three separate cultures. B, PC12 cells were treated with NGF for 10 days. Cultures were then pretreated for 1 h with saline (vehicle), 20 μM STS, 10 μM DODCB, or 10 μM Fe²⁺. The percentage of cells exhibiting apoptotic nuclei in each culture was calculated, and values are the mean and S.E. of determinations made in four separate cultures. Each value for cultures treated with STS, Aβ, and Fe²⁺ was significantly greater than the corresponding value in control cultures (p < 0.001).

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