Abstract. Background: Indole-3-carbinol (I3C) is a phytochemical with anticarcinogenic properties. Telomerase activity is key in carcinogenesis. We investigated the effect of I3C on telomerase in human prostate cancer cell lines LNCaP and PC3. Materials and Methods: Cells were treated with I3C at 100 and 250 μM with and without 10-50 μM diethylstilbestrol (DES). Telomerase activity was performed using TRAPaze Telomerase Detection Kit, and hTERT gene expression by real time quantitative RT-PCR. Results: I3C (250 μM) inhibited telomerase activity and mRNA expression of hTERT in LNCaP and PC3 cells. I3C at 250 μM combined with any concentration of DES was cytotoxic to LNCaP. Telomerase activity in PC3 cells with 250 μM of I3C and 25 or 50 μM of DES was significantly reduced or inhibited, respectively. I3C combined with DES reduced PC3 viability and eliminated LNCaP cells. Conclusion: I3C significantly inhibited telomerase activity and hTERT mRNA expression in LNCaP and PC3 cells. Combination of I3C and DES enhanced the inhibitory effect on telomerase activity, gene expression, and cell viability. These results implied that I3C and DES combined might help in prostate cancer treatment.

Telomeres are found at the ends of linear chromosomes with highly conserved sequences (5'-TTAGGG-3') in the majority of eukaryote. They protect chromosomes from degradation and fusion (1-4). It has been recognized that telomeres shorten with cell senescence, and therefore telomere length is an indication of cell longevity (1). Telomerase is a ribonucleoprotein that is responsible for keeping the lengths of telomeres intact in rapidly proliferating cells. Telomerase activity is undetectable or very low in most normal tissues, but is higher in proliferating tissues, including malignant tumors, strongly suggesting a key role in carcinogenesis (4, 5).

Studies on telomerase activity in human prostate tissues and cell lines showed that no normal tissue and few benign prostatic hyperplasia samples exhibited telomerase activity, whereas 84% of the malignant tissues were highly active (6, 7). The activation of telomerase during cell immortalization and the restricted pattern of telomerase activity in normal cells have raised the possibility that telomerase inhibitors might be useful agents that selectively kill malignant cells, while sparing normal cells. When the telomerase RNA subunit was disrupted by antisense RNA expression, antisense-expressing cell clones exhibited cell growth arrest and cell death (8, 9).

We have recently shown that diethylstilbestrol (DES), being an estrogen, inhibited telomerase activity in prostate cancer cells, even in the presence of androgens (10).

Indole-3-carbinol (I3C), a common phytochemical in cruciferous vegetables, demonstrated its ability to function as an anticarcinogenic agent on different levels (11-16), such as induction of p53-independent apoptosis (11); G-1 cell cycle arrest and inhibition of expression of cyclin-dependent kinase 6 (CDK6) protein kinase and prostate specific antigen (PSA) (12, 13); inhibition of phosphorylation of retinoblastoma (Rb) protein (13) and AKT kinase (14); and inhibition of nuclear factor (NF)-κB pathway (13, 16).

In the present study, we examined the effects of I3C on the telomerase of prostatic cell lines LNCaP and PC3, which represent different stages of prostate cancer development. Due to our recent findings (10) showing an inhibitory effect of DES on telomerase, the combined effect of I3C with DES on telomerase activity and mRNA expression and cell viability was also examined.

Materials and Methods

Cell culture and treatments. Human prostate cancer LNCaP and PC3 cells were maintained as previously described (10). Briefly, LNCaP and PC3 cells were treated with 0, 100, or 250 μM I3C (Sigma, St. Louis, MO, USA) with and without 0, 10, 25, or 50 μM diethylstilbestrol (DES).
DES (Sigma). Telomerase gene expression (hTERT) was examined after 24 h and telomerase activity after 48 h. At the end of the incubation periods, the cells were trypsinized, washed, counted, tested for viability, and frozen at –70˚C until further analysis.

Quantitative telomerase activity. The telomerase activity assay was performed using TRAPaze Telomerase Detection Kit (Quantitative Telomerase Detection Kit; US Biomax, Inc., Rockville, MD, USA), based on the Telomeric Repeat Amplification Protocol (TRAP) method (1). Cell extracts were prepared from the frozen, harvested cells according to kit instructions as previously described (10).

hTERT real-time quantitative RT-PCR. Total RNA was isolated from the frozen cell pellet specimen using the SV Total RNA Isolation System (Promega, Madison, WI, USA) including a DNase digestion step. RNA quantity and quality were spectrophotometrically determined.

The mRNA expression of hTERT and the endogenous housekeeping gene encoding for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were quantified using real-time PCR analysis by Taqman Technology (Applied Biosystems, Foster City, CA, USA). The GAPDH analysis served both as a control for RT-PCR performance and as a reference for relative quantification. The primers, the probes and the conditions of real-time PCR were used as described previously (10).

Two separate calibration curves were constructed for hTERT and for GAPDH using serial dilutions of untreated cell line RNA. The results were expressed as hTERT/GAPDH ratios.

Statistical analysis. The results were expressed as mean±standard error (SE). Student’s t-test was used for data analysis. A p-value of 0.05 or less was considered significant.

Results

In the present study, the effects of I3C on telomerase activity and expression in the prostatic cell lines PC3 and LNCaP were investigated. The effect of combined treatment (I3C and DES) was also examined. The I3C concentration ranged from 0 to 250 μM and the DES ranged from 0 to 50 μM.

I3C reduced telomerase mRNA and activity in both prostate cancer cell lines. Figure 1 demonstrates the effect of I3C on telomerase mRNA expression and activity in the LNCaP cell line, which represents androgen-dependent, prostate cancer cells. I3C significantly reduced the telomerase mRNA expression in a dose-dependent manner (100 μM vs. control (no treatment), p=0.046; 250 μM vs. control, p=0.028; 250 μM I3C vs. 100 μM I3C p=0.001). I3C at 250 μM also had an inhibitory effect on telomerase activity (p=0.028).

Figure 2 demonstrates the effect of I3C on telomerase mRNA expression and activity in the PC3 cell line. I3C at 250 μM had a significant inhibitory effect on both telomerase mRNA expression (p=0.033) and activity (p=0.001). I3C at 100 μM had no significant effect on telomerase activity and gene expression.

Treatment with I3C and DES together reduced both telomerase activity and hTERT expression in the LNCaP line. The effects of I3C (100 μM or 250 μM) in the presence of DES (10, 25, or 50 μM) is shown in Figures 3 and 4. The addition of 50 μM DES to 100 μM I3C significantly reduced telomerase expression compared to I3C alone (p=0.031, Figure 3). As to telomerase activity, the combined effect of 100 μM I3C and 50 μM of DES had a significant cumulative inhibitory effect on telomerase activity compared to I3C (p=0.002) or DES (p=0.015) (Figure 4). Since I3C at 250 μM in any combination with DES was cytotoxic, no activity or expression results were obtained. The inhibitory effect of DES alone on telomerase mRNA expression and activity in both cell lines was in agreement with previous results (10).
Combined I3C and DES reduced telomerase activity and gene expression in the PC3 line. The effect of I3C (100 μM or 250 μM) in the presence of DES (10, 25, or 50 μM) in PC3 is shown in Figures 5 and 6. The addition of 10-50 μM of DES to 100 μM of I3C had no significant effect on telomerase expression compared to I3C or DES alone (Figure 5). However, the addition of 250 μM I3C to 10-50 μM DES significantly reduced telomerase mRNA expression (250 μM I3C+10 μM DES vs. 10 μM DES, \( p=0.028 \); 250 μM I3C+25 μM DES vs. 25 μM DES, \( p=0.001 \); 250 μM I3C+50 μM DES vs. 50 μM DES, \( p=0.03 \), Figure 5).

The combined treatment of 25 or 50 μM of DES and 250 μM of I3C significantly reduced telomerase mRNA expression compared to 250 μM I3C alone (\( p=0.043 \); \( p=0.05 \), respectively, Figure 5). As to the combined effects on telomerase activity, 250 μM I3C combined with 25 μM DES significantly reduced telomerase activity compared to DES alone (\( p=0.04 \)). It should be emphasized that the combination of 250 μM I3C with 50 μM DES almost totally inhibited telomerase activity (vs. 250 μM I3C alone, \( p=0.003 \); vs. 50 μM DES alone, \( p=0.05 \)).

**Discussion**

Telomerase, which is responsible for telomere lengthening, plays a pivotal role in tumorigenesis (3, 5, 17). In the present study, we examined the effects of I3C alone and in combination with DES on telomerase activity and gene expression in both androgen-dependent (LNCaP) and androgen-independent (PC3) prostate cancer cell lines.

The idea for this study was derived from research that demonstrated the ability of I3C to mediate G-1 cell cycle arrest in prostate cancer cells (12, 13, 15, 16). Li et al. (18)
and Sarkar and Li (16) used microarray gene expression profiling to show that I3C regulated many genes that are important for control of the cell cycle. The effect of I3C on prostate cancer cells was supported by the following outcomes: induction of p53-independent apoptosis (11); G-1 cell cycle arrest and inhibition of expression of CDK6 protein kinase and PSA (12, 13); inhibition of phosphorylation of Rb protein (13) or AKT kinase (14), and inhibition of NF-κB pathway (18). Linking these data with the pivotal effect telomerase has on the control of cell proliferation, we focused on examining a possible role of I3C as a modulator of telomerase activity in LNCaP and PC3 prostate cancer cells.

In contrast to DES, which inhibited both telomerase activity and gene expression in LNCaP cells, and activity only in PC3 cells (10), I3C inhibited activity and gene expression in both cell lines (Figures 1 and 2), although to a lesser extent in the PC3 line. We believe that this might be due to each cell line representing different stages of prostate cancer (LNCaP cells, the early stage and PC3, the advanced stage). The fact that DES down-regulated telomerase activity in the PC3 line, with no effect on hTERT mRNA expression could be due to a direct effect of the drug on the enzyme itself, such as dephosphorylation (19). Western blot techniques which might have confirmed this, were not used in the present study because of our experience and that of others that showed cross-reactivity of the monoclonal antibodies against hTERT (10, 20).

Although telomerase gene expression of PC3 (the androgen-independent cell line) was not affected by DES (10), its combination with I3C (250 μM) showed a significantly reduced hTERT mRNA expression. As to LNCaP, the combination of I3C and DES demonstrated a cumulative inhibitory effect on both telomerase activity and gene expression. These results provide clues regarding the possible involvement of androgen receptors in controlling telomerase gene expression.

We also examined the effect of the combined treatment of I3C and DES on cell viability. Surprisingly, the combination of 250 μM I3C with 50 μM DES dramatically decreased the viability of the PC3 cells to 29.7%±3.1% (vs. control (0), p<0.0001; vs. 50 μM DES, p=0.0001). In the LNCaP cell line, the combination of I3C at 250 μM with DES at 50 μM drastically decreased the number of cells (results not illustrated). These findings are supported by the studies of Souli et al. (21) and Weng et al. (22) that showed significant tumor suppression in xenograft tumors in mice treated with I3C or its derivatives.

Conclusion

To the best of our knowledge, this study was the first attempt to investigate the effect of I3C, both in the presence and absence of DES, on the telomerase activity and mRNA expression of prostate cancer cells. Without having any idea yet, about the mechanisms behind the ability of DES and I3C to inhibit telomerase activity and expression, we demonstrated that the combination of these compounds significantly down-regulated telomerase and halted cell proliferation. Since this phenomenon occurs in both low-grade (LNCaP) and high-grade (PC3) prostate cancer cells, it raises the possibility of using I3C with low concentrations of DES for treating prostate cancer.

References