Tocopherol Transfer Protein Sensitizes Prostate Cancer Cells to Vitamin E*

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Prostate cancer is a major cause of mortality in men in developed countries. It has been reported that the naturally occurring antioxidant α-tocopherol (vitamin E) attenuates prostate cancer cell proliferation in cultured cells and mouse models. We hypothesized that overexpression of the tocopherol transfer protein (TTP), a vitamin E-binding protein that regulates tocopherol status, will sensitize prostate cancer cells to the anti-proliferative actions of the vitamin. To test this notion, we manipulated the expression levels of TTP in cultured prostate cells (LNCaP, PC3, DU145, and RWPE-1) using overexpression and knockdown approaches. Treatment of cells with tocopherol caused a time- and dose-dependent inhibition of cell proliferation. Overexpression of TTP dramatically sensitized the cells to the apoptotic effects of α-tocopherol, whereas reduction (“knockdown”) of TTP expression resulted in resistance to the vitamin. TTP levels also augmented the inhibitory effects of vitamin E on proliferation in semi-solid medium. The sensitizing effects of TTP were paralleled by changes in the intracellular accumulation of a fluorescent analog of vitamin E and by a reduction in intracellular levels of reactive oxygen species and were not observed when a naturally occurring, ligand binding-defective mutant of TTP was used. We conclude that TTP sensitizes prostate cancer cells to the anti-proliferative effects of vitamin E and that this activity stems from the ability of protein to increase the intracellular accumulation of the antioxidant. These observations support the notion that individual changes in the expression level or activity of TTP may determine the responsiveness of prostate cancer patients to intervention strategies that utilize vitamin E.

Prostate cancer is a major public health problem in developed countries, and it constitutes the second leading cause of cancer deaths among males in the United States. Like all malignant diseases, prostate cancer is a culmination of multiple genetic and epigenetic insults, which affect key regulatory features of cell proliferation. Although family history and ethnicity are key risk factors in the susceptibility to prostate cancer, no single gene has been identified as a high penetrance heritable prostate cancer trait to date. Current treatments of prostate cancer rely primarily on surgical and hormonal intervention strategies, coupled with sensitive diagnostic tools aimed at early detection. The incidence, prevalence, and mortality associated with prostate cancer, together with its slow progression from intraepithelial neoplasia (found in men <30 years of age) to clinical disease (found mostly in men >50 years of age), make chemoprevention an attractive therapeutic approach for this disease.

Accumulation of reactive oxygen species (ROS) and oxidative damage are thought to play important roles during oncogene-induced transformation (1–3) and specifically in the initiation and progression of prostate cancer (4–6). For example, androgens are shown to increase intracellular ROS levels (7–9) and affect the transition to androgen independence (10, 11). Similarly, reduced expression of antioxidant enzymes and elevated ROS levels are observed in human prostate cancer samples (12). These observations give rise to the hypothesis that elevated oxidative stress is an important contributing factor in prostate tumorigenesis and that antioxidants may have anticarcinogenic functions (13).

Vitamin E (denoting α-tocopherol here) is an essential dietary lipid that functions as the principal lipid-soluble antioxidant in most animal and plant species. Supplementation with vitamin E is thought to be an effective chemopreventive strategy for a number of oxidative stress-related pathologies, such as ataxia (14, 15), amyotrophic lateral sclerosis (16), Alzheimer disease (17, 18), and Parkinson disease (19). Importantly, dietary supplementation with vitamin E was shown to inhibit carcinogenesis in a number of induced cancers (20). In a transgenic model of hepatocellular carcinoma, dietary supplementation with vitamin E brought about a remarkable decrease in DNA damage and in tumor incidence (3). In the prostate, vitamin E was shown to induce cell death and antagonize androgen actions in cultured cells (21–24) and to delay tumorigenesis in

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3 The abbreviations used are: ROS, reactive oxygen species; TTP, tocopherol transfer protein; NBD, 12-(N-methyl-N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)); PARP, poly(ADP-ribose) polymerase.
genetic models of the disease in rats (25) and mice (26). Despite the profound anti-carcinogenic effects of vitamin E in the model systems described above, studies on the efficacy of vitamin E in human prostate cancers have been inconclusive. Two large scale intervention studies documented a protective role for vitamin E in reducing prostate cancer incidence (27, 28). On the other hand, recent results from the Selenium and Vitamin E Chemoprevention Trial (SELECT), arguably the most comprehensive randomized, placebo-controlled prostate cancer intervention trial to date, indicated that supplementation of healthy men with vitamin E was not associated with a reduced risk for prostate cancer (29). Thus, available data obtained in defined laboratory models have not been supported by trials in human populations.

A possible explanation for this apparent paradox is that individual genetic traits modify clinical responses to vitamin E. Because the α-tocopherol transfer protein (TTP) is the major regulator of vitamin E status in humans, we set out to examine whether expression of this protein affects the sensitivity of prostate cancer cells to anti-proliferative activities of vitamin E. We show here that TTP markedly enhances the sensitivity of prostate cancer cells to tocopherol-induced inhibition of cell proliferation. The data further indicate that these effects involve the well established antioxidant activity of vitamin E.

EXPERIMENTAL PROCEDURES

Cells—LNCaP, DU145, and PC3 cells were maintained in RPMI 1640 media supplemented with 2 mm l-glutamine (Invitrogen), 10% fetal bovine serum (Hyclone). RWPE-1 cells were maintained in keratinocyte serum-free media supplemented with bovine pituitary extract and epidermal growth factor (Invitrogen). HepG2-Tet-On-TTP cells were grown in DMEM containing 10% fetal bovine serum, 200 μg/ml gentamicin (G418, Invitrogen), and 100 μg/ml hygromycin B, and TTP expression was induced with 1 μg/ml doxycycline as described earlier (30). To generate cell lines that stably overexpress TTP, we used two approaches. 1) The TTP open reading frame fused with 5′-hemagglutinin (HA) tag was cloned into the pDNA3.1-Hygro (+) vector (Invitrogen) and the construct transfected into LNCaP cells using FuGENE 6 (Roche Applied Science). 2) The HA-TTP cDNA was cloned into the lentiviral expression vector pLEX (OpenBiosystems) and lentivirus particles assembled in HEK293T. Lentiviral particles were then transfected into LNCaP cells using FuGENE 6 (Roche Applied Biosystems) and lentivirus particles assembled in HEK293T. Lentiviral particles were then used for Polybrene-mediated transduction using standard protocols. Reduced expression of endogenous TTP was achieved by using two independent assays.

DNA Fragmentation—Cells were treated with tocopherol (or ethanol, 1% v/v) for 2 or 4 days prior to collection and fixing with 0.25% formaldehyde (37 °C for 10 min). Fixed cells were resuspended in 10% PBS in methanol and incubated at −20 °C overnight. After two washes in PBS, cells were treated with 20 μg/ml RNase at 37 °C for 30 min, chilled on ice for 10 min, and incubated with 50 mg/ml propidium iodide, 0.1% Nonidet P-40, 0.1% azide overnight. Cells were filtered through strainer tubes (BD Biosciences) prior to flow cytometry on a Beckman Coulter Epics MCL-XL cell analyzer at the Case Comprehensive Cancer Center Cytometry Core.

Cleavage of Poly(ADP-ribose) Polymerase (PARP)—The caspase-3-dependent cleavage of PARP was measured by immunoblotting with specific antibodies (Cell Signaling). Following the indicated treatments, cells were scraped, washed with PBS, and lysed in 20 mM HEPES, pH 7.4, 1 mM EDTA, pH 8.0, 150 mM NaCl, 1% Igepal, 20 mM sodium fluoride, 20 mM β-glycerol phosphate, 1 mM sodium vanadate, and 200 μM PMSF (Buffer 1). Lysates were freeze-thawed twice, and cell debris was removed by centrifugation prior to SDS-PAGE and immunoblotting.

Anchorage Dependence of Proliferation—TTP-expressing or control LNCaP cells were suspended in RPMI 1640 medium containing 10% FBS and 0.3% agar and plated over a layer of the same medium containing 0.5% agar (33, 34). Cells were fed with media containing 0.3% agar every 2 days for 12 days, after which time colonies were quantified by manual counting.

Fluorescence Microscopy—Cells were cultured on poly-d-lysine-coated coverslips, fixed with 3.7% paraformaldehyde for 10 min, and permeabilized with 0.1% Triton X-100 for 5 min. Paraformaldehyde was quenched with 75 mM ammonium chloride, 20 mM glycerine in PBS for 10 min. Washed slides were blocked in PBS containing 0.2% BSA, 0.004% Triton X-100, and 1% goat serum (Hyclone) for 25 min at room temperature and mounted in SlowFade (Invitrogen). Prior to fixation, NBD-tocopherol (5 μM as serum complex) was loaded into cells overnight, and visualized as described earlier (35). Mitochondria were stained by incubating the cells with 0.5 μM Mitotracker CMX ROS (Invitrogen) for 15 min at 37 °C prior to fixation. Confocal images were collected on a Zeiss 510 Meta confocal microscope at the Case Western Reserve University Neuroscience Imaging Center. For quantitative measurements of NBD-tocopherol accumulation (Figs. 4B, 5, B and C), cells were loaded with the fluorescent analog for 24 h, washed, and fixed, and fluorescent images were collected on a Leica DMI 4000B inverted microscope. Fluorescence intensity in each field was quantified using Scion Image software and normalized to cell number (determined by manual counting in duplicate images obtained with differential interference contrast).
Measurements of Intracellular ROS—Cells were cultured in serum-free RPMI 1640 medium for 2 days, washed twice with phenol red-free Hanks’ buffered salt solution (Hyclone), and incubated for 2 h with dihydrofluorescein diacetate (10 μg/ml, Invitrogen) and 2’,7’-dichlorofluorescein fluorescence (excitation, 485 nm; emission, 535 nm) measured in a plate reader as described earlier (36). Duplicate plates were stained with the DNA stain Hoechst 33258 (Invitrogen, 5 μg/ml), and fluorescence (excitation, 365 nm; emission, 460 nm) was measured on a plate reader. Shown are 2’,7’-dichlorofluorescein fluorescence values after normalization to Hoechst fluorescence.

Miscellaneous—Vitamin E secretion was measured with [14C]tocopherol (0.18 μCi/ml, GE Healthcare) as described previously (30). The ligand binding affinity of TTP was determined by fluorimetric titrations with NBD-tocopherol, as described previously (37, 38). Recombinant proteins were expressed and purified, as described previously (39, 40), following induction at 37 °C for 3 h with 0.4 mM isopropyl 1-thio-β-D-galactopyranoside for TTP or overnight at 25 °C with 0.25 mM isopropyl 1-thio-β-D-galactopyranoside for TTP(L183P). Immunodetection was done using antibodies against α-tubulin (Sigma), against the HA tag of the ectopic TTP (clone HA.11, Covance), or against endogenous TTP (clone 12-D7 from H. Arai or 81096 from R. Farese, University of California, San Francisco). Statistical analyses were performed using an unpaired Student’s t test with GraphPad Quickcalcs freeware.

RESULTS

Multiple studies demonstrated that vitamin E inhibits the proliferation of prostate cancer cells in culture (22–24) as well as in mouse models of the disease (25, 26). In virtually all vertebrates, the levels of tocopherol are primarily regulated by the action of TTP, a tocopherol-binding protein that has been well characterized in its primary site of expression, the liver (cf. Refs. 41, 42).

Although it is not currently known how vitamin E is processed in extra-hepatic sites, expression of the TTP transcript has been demonstrated also in other tissues, including the prostate (43).
We hypothesized that the biological activity of vitamin E is determined by its intracellular concentration, which in turn is determined by the expression level of TTP. To test this hypothesis, we generated stable TTP-expressing LNCaP cells derived from a human androgen-dependent prostate cancer line (44) in which vitamin E is growth-inhibitory (23, 43). As shown in Fig. 1A, treatment of control LNCaP cells with tocopherol resulted in a significant reduction in cell viability, down to <50% of the initial cell number after 11 days. Treatment of TTP-expressing LNCaP cells with vitamin E completely inhibited cell proliferation, indicating that expression of TTP greatly enhances the growth-inhibitory effect of vitamin E. Interestingly, overexpression of TTP in and of itself (in the absence of exogenously added tocopherol) also attenuated cell proliferation, albeit to a lesser degree (30% decrease in cell proliferation after 11 days). To determine the dose dependence of this effect, we cultured control and TTP-expressing LNCaP cells in the presence of different concentrations of tocopherol, and we measured their viability after 10 days. We chose tocopherol concentrations that span the range found in plasma of severely deficient (low micro-molar) and normal (20–40 μM), and supplemented (50–80 μM) humans (45). As shown in Fig. 1B, vitamin E inhibited cell proliferation in a dose-dependent fashion within a physiologically relevant concentration range (25% growth inhibition at 70 μM tocopherol). Overexpression of TTP enhanced the anti-proliferative actions of the vitamin (60% growth inhibition at 70 μM). Next, we examined whether expression of TTP and treatment with tocopherol attenuate the transformed phenotype of LNCaP cells. Because tumorigenic potential in vivo is closely correlated with anchorage-independent proliferation in vitro (46), we measured the ability of LNCaP or LNCaP-TTP cells to proliferate in semi-solid medium. As shown in Fig. 1C, >80% of control LNCaP cells formed colonies when suspended in 0.3% agar. Expression of TTP and treatment with tocopherol diminished proliferation in soft agar by 50 and 75%, respectively. Treatment of LNCaP-TTP cells with tocopherol all but abolished colony formation in soft agar. These results demonstrate that tocopherol inhibits the proliferation of prostate cancer cells and that expression of TTP greatly enhances this inhibitory effect.

To gain insight into the molecular mechanisms that underlie the anti-proliferative actions of vitamin E and TTP, we examined how expression of the protein and treatment with tocopherol affect the distribution of cells across different phases of the cell cycle. LNCaP or LNCaP-TTP cells were treated with vitamin E (or vehicle) for 2 days, and fixed cells were subjected to flow cytometry after staining with propidium iodide. The data (Fig. 1F) show that expression of TTP as well as treatment with tocopherol significantly increased the fraction of cells in the sub-G₁ phase, reflecting enhanced apoptosis. When tocopherol was added to TTP-expressing cells, the effect was greatly enhanced (~8-fold). This effect was not due to clonal variability, because multiple independent TTP-expressing clones exhibited significant increase in sub-G₁ content (Fig. 1E). Notably, treatment of LNCaP-TTP cells for 4 days with tocopherol (Fig. 1E) resulted in a dramatic increase in the fraction of cells undergoing apoptosis (~20-fold over vehicle controls) as compared with that observed over 2 days of vitamin treatment (compare Fig. 1, D and F). Finally, the TTP-induced increase in apoptosis was reversed when TTP expression was knocked down using TTP-specific shRNA (Fig. 1F). These data indicate that overexpression of TTP greatly sensitizes LNCaP cells to the apoptotic effects of vitamin E. In support of this notion, TTP expression and vitamin E treatment increased the cleavage of poly(ADP-ribose) polymerase (PARP), an established indicator of programmed cell death (47). As shown in Fig. 1, G and H, the cleaved form of PARP is absent in untreated control LNCaP cells. Treatment with vitamin E caused a marked time-dependent (Fig. 1G) and dose-dependent (Fig. 1H) increase in PARP cleavage. Importantly, the effect of tocopherol on PARP cleavage was dramatically increased in LNCaP cells that overexpress TTP. Notably, TTP elicited a measurable increase in PARP cleavage also in the absence of exogenously added tocopherol (see “Discussion”). Taken together, these results support and extend previous reports that tocopherol induces apoptotic cell death in prostate cancer cells (23, 24). Furthermore, these results show that expression of TTP greatly potentiates vitamin E-induced apoptosis.

To examine the generality of this phenomenon, we tested how the expression of TTP affects cell proliferation in other prostate cell lines. Specifically, we used overexpression and knockdown approaches to manipulate TTP levels in the aggressive, metastasis-derived DU145 and PC3 cell lines (48, 49) and in the less transformed, immortalized prostatic epithelia cells RWPE-1 (50). Similar to our observations in LNCaP cells, overexpression of TTP in PC3 and RWPE-1 cells drastically increased the fraction of cells that undergo apoptosis in response to vitamin E treatment (Fig. 2, A and B). Conversely, repression of endogenous TTP expression in PC3 and DU145 cells using lentiviral transduction of shRNA rendered the cells markedly resistant to tocopherol (Fig. 2, C and D). Collectively, these data demonstrate that the sensitivity of various prostate...
FIGURE 2. Apoptotic actions of TTP and tocopherol in different prostate cancer cell lines. Vitamin E-induced apoptosis was measured by cell cycle analysis as described in Fig. 1D. TTP was stably overexpressed in PC3 (A) or RWPE-1 cells (B) using lentiviral transduction. Reduction of endogenous TTP expression in PC3 cells (C) or DU145 cells (D) was achieved using the indicated shRNA constructs. Asterisks indicate significant differences at p values <0.05.
cancer cell lines to vitamin E is greatly influenced by the expression level of TTP.

An unexpected finding from these studies is that TTP in and of itself (i.e. in the absence of exogenously added tocopherol) has pronounced effects on cell proliferation, transformation, and apoptosis (Fig. 1). Such behavior could indicate that the apoptotic effects of TTP arise from novel, ligand-independent activities of the protein. To address this possibility, we characterized the biological effects of TTP(L183P), a naturally occurring mutation that causes familial vitamin E deficiency in humans (51). First, we measured the effect of the mutation on ligand binding using fluorimetric titrations with NBD-tocopherol, a fluorescent analog of vitamin E that binds to TTP with high affinity and that we have characterized extensively in vitro (37–39) and in vivo (30, 35). We found that the L183P mutation reduces the affinity of TTP to vitamin E by ~100-fold (K_d = 1.47 ± 0.22 μM for TTP(L183P) versus 16 ± 5.5 nm for the wild-type protein; Fig. 3A). We note that micromolar range affinity for tocopherol is exhibited only by proteins that do not bind vitamin E specifically, such as Sec14p or the supernatant protein factor (52). Thus, we conclude that TTP(L183P) is severely impaired in ligand binding. When expressed in LNCaP cells, TTP(L183P) was completely ineffective in sensitizing cells to the apoptotic actions of α-tocopherol, assayed by cell cycle...
analysis (Fig. 3B), proliferation assays (data not shown), or cleavage of PARP (Fig. 3C). These observations indicate that the anti-proliferative actions of TTP are dependent on the ability of the protein to bind vitamin E. The anti-proliferative activities of TTP observed in the absence of added vitamin E (Fig. 1) likely represent the presence of serum-derived tocopherol in the culture media (determined to be 2–3 μM by gas chromatography/mass spectrometry, data not shown), rather than from novel, ligand-independent activities of the protein.

To address the molecular mechanisms that underlie the apoptotic actions of TTP, we examined the effect of the protein on the intracellular levels of vitamin E. It has been established that in hepatocytes, TTP stimulates the export of tocopherol to circulating lipoproteins (30, 53), yet its function in other cell types is presently unknown. Thus, we compared the ability of the protein to facilitate the secretion of tocopherol from LNCaP cells and HepG2 hepatocytes (30). TTP-expressing HepG2 hepatocytes and LNCaP prostate cancer cells were loaded with [14C]tocopherol and washed, and release of the radiolabeled vitamin to the culture media was measured. As reported previously (30, 53), expression of TTP in hepatocytes caused a pronounced increase in the efflux of labeled tocopherol to the media (~3-fold; Fig. 4A). However, tocopherol efflux from LNCaP cells was not affected by the expression of TTP (Fig. 4A). We conclude that the mechanisms by which TTP sensitizes LNCaP cells to the anti-proliferative actions of vitamin E do not involve changes in tocopherol egress. We next considered the possibility that the levels of TTP limit the intracellular concentration of the vitamin and in turn its biological activity. To quantitate intracellular tocopherol levels, we measured the accumulation of NBD-tocopherol. LNCaP cells were incubated with NBD-tocopherol for 24 h and
washed, and intracellular NBD-tocopherol was visualized with the aid of fluorescence microscopy. As shown in Fig. 4B, cells that overexpress TTP accumulated ~2-fold more NBD-tocopherol than control LNCaP cells. Importantly, overexpression of the ligand binding-defective TTP(L183P) had no effect on the intracellular levels of NBD-tocopherol (Fig. 4B). These findings suggest that TTP-induced sensitization of LNCaP cells to tocopherol stems from enhanced accumulation of the vitamin. This notion is strengthened by the observations that in different prostate cell lines, the extent of NBD-tocopherol accumulation mirrors the endogenous expression levels of TTP (Fig. 5, A–C), such that a plot of accumulated NBD-tocopherol as a function of TTP levels reveals a linear relationship (Fig. 5D). Moreover, the basal level of apoptosis among the different cell lines is a linear function of the endogenous expression levels of TTP among these cell lines (Fig. 5, E and F).

Because tocopherol is a critical antioxidant, we examined how TTP expression and/or vitamin E treatment affects the intracellular levels of ROS. Using dichlorofluorescein diacetate, a cell-permeable dye that fluoresces upon oxidation by free radicals (54), we found that the basal level of ROS in LNCaP cells significantly decreased upon treatment with tocopherol as well as by expression of TTP (Fig. 4C). The most pronounced reduction in ROS levels was observed when TTP-expressing cells were cultured in the presence of tocopherol, where ~50% of the endogenous ROS was quenched. These results suggest that the anti-carcinogenic activities of TTP and vitamin E stem from their combined action in scavenging intracellular radicals.

To obtain further mechanistic insights into the apoptotic effects of vitamin E and TTP, we determined the subcellular localization pattern of NBD-tocopherol using confocal fluorescence microscopy. As shown in Fig. 6A, NBD-tocopherol exhibits a punctate distribution pattern in LNCaP cells, displaying significant overlap with the distribution pattern of the mitochondrial marker MitoTracker. Because in hepatocytes NBD-tocopherol is primarily localized to endosomes and lysosomes (30), these findings indicate that the intracellular fate of the vitamin is cell type-specific. Accumulation of dietary vitamin E in mitochondria was previously reported in some tissues (55, 56). We did not observe significant co-localization of NBD-tocopherol with markers of the endoplasmic reticulum, lysosomes, endosomes or peroxisomes (data not shown). We did not observe any significant change in the mitochondrial localization of NBD-tocopherol when TTP was overexpressed. Next, we determined the intracellular distribution of the TTP protein in LNCaP cells. As shown in Fig. 6B, TTP, like its ligand, was primarily localized to mitochondria. This distribution pattern was specific, as it was not observed in the absence of anti-TTP antibodies (Fig. 6B), yet it was observed with antibodies directed against either TTP (Fig. 6B) or against the HA tag to which the protein was fused (data not shown). The localization of TTP did not change upon treatment of the cells with vitamin E (100 μM α-tocopherol; data not shown). From these data, we conclude that TTP and vitamin E localize to the mitochondria in LNCaP cells. Taken together, our observations suggest that the pro-apoptotic activities of TTP may stem from the ability of the protein to increase the level of α-tocopherol in mitochondria, thereby perturbing redox balance in this organelle.

**DISCUSSION**

The slow progression of prostate cancer from intraepithelial neoplasia to clinical disease makes primary chemoprevention an attractive therapeutic route for this disease. Unique promise in this regard is held by the dietary antioxidant α-tocopherol (vitamin E), which was shown to cause growth arrest and apoptosis of prostate cancer cells in vitro (21–24) as well as in mouse models of the disease (25, 26). Despite these promising observations, studies in human populations have been disappointing. Notably, the Selenium and Vitamin E Cancer Prevention Trial (SELECT) was recently terminated after early indications that supplementation of healthy men with vitamin E did not reduce prostate cancer risk (29). The apparent paradox between the unmistakable anti-carcinogenic effects of vitamin E in preclinical models of prostate cancer and the absence of any impact in human clinical trials prompted us to consider the notion that responsiveness to tocopherol reflects individual, genetically determined trait(s) that are significantly heterogeneous among healthy human subjects. If this is the case, averaging response data from large numbers of participants in a clinical trial could minimize the impact of individual variations, such that no apparent response is observed in the pooled data. In support of this notion, Kelly et al. (57) showed that levels of plasma tocopherol, both basal and in response to supplementation, are remarkably stable and are subject to large inter-individual variations. Thus, it appears that vitamin E status should be added to a growing list of metabolic phenotypes that are consistently different between individuals and can be used to compose an individual ‘metabolic fingerprint’ (58).

The major biochemical determinant that specifically regulates vitamin E status in humans is the hepatic TTP. The protein is predominantly expressed in parenchymal cells of the liver, where it catalyzes the secretion of dietary α-tocopherol into circulating lipoproteins that deliver the vitamin to peripheral tissues (41, 42, 59). The critical role of TTP in regulating vitamin E status is evident from the fact that naturally occurring mutations in the TTPA gene lead to vitamin E deficiency, accompanied by neurodegeneration and ataxia (14, 60). Mice in which the ttpA gene has been disrupted exhibit similar pathological phenotypes (61–64). Importantly, the relative plasma levels of vitamin E in the TTP<sup>−/−</sup>, TTP<sup>+/−</sup>, and wild-type mouse models mirror the expression levels of TTP in these animals (62, 63). We therefore hypothesized that changes in the
Indicated cell lines were treated with tocopherol for 48 h, and apoptosis was measured as in Fig. 1. Accumulation of NBD-tocopherol in the indicated cell lines was measured as in Fig. 4. Data were averaged from experimental replicates. 

**FIGURE 5.** TTP and Prostate Cancer

**FIGURE 6.** NBD-tocopherol and TTP localize to mitochondria in LNCaP cells. A, LNCaP cells expressing TTP or control vector were loaded with NBD-tocopherol and imaged by confocal fluorescence microscopy as described under “Experimental Procedures.” Images are representative of two independent experiments. B, intracellular localization of TTP in LNCaP-TTP cells was determined with immunofluorescence confocal microscopy using anti-TTP (top) or control (bottom) antibodies, as described under “Experimental Procedures.” Images are representative of two independent experiments. Scale bar, 10 μm.

expression levels of TTP affect the intracellular concentration and, in turn, the biological activity of vitamin E. We were especially interested in examining this question in the context of prostate cells, as cancer of the prostate is uniquely responsive to vitamin E, and expression of TTP mRNA in cells from this tissue has been documented (43).

We show that, as reported previously (23, 24, 43), tocopherol inhibits the proliferation of LNCaP cells (Fig. 1). Importantly, the expression level of TTP is highly correlated with cell sensitivity to the anti-proliferative actions of the vitamin in four different prostate cells as follows: LNCaP, PC3, DU145, and RWPE-1. Specifically, TTP sensitized the cells to tocopherol-induced apoptosis, as seen in proliferation and transformation assays, cell cycle analyses, and visualization of PARP cleavage (Figs. 1–3). Multiple lines of evidence suggest that the mechanisms that underlie these effects involve the antioxidant activity of vitamin E. First, LNCaP-TTP cells accumulate significantly more NBD-tocopherol as compared with control cells (Fig. 4B). Second, LNCaP-TTP cells display a significant decrease in the levels of intracellular reactive oxygen species (Fig. 4C). Finally, the naturally occurring L183P mutation abolishes binding of tocopherol by TTP in vitro and concomitantly eliminates the sensitizing actions of the protein when overexpressed in prostate cancer cells (Fig. 3). These results indicate that when TTP is expressed at high levels, prostate cancer cells accumulate vitamin E, which reduces intracellular levels of ROS, thereby inducing apoptosis. Indeed, a growing body of evidence points to oxygen-based free radicals as intracellular messengers that transduce proliferative signals and are required for malignant transformation (1, 2, 65) especially in prostate cancer cells (6, 13). It is therefore possible (and likely) that individual polymorphic variations in TTP determine the impact of vitamin E on relevant disease states in an individual. This notion is validated by the findings of Wright et al. (66), who reported that T/A substitution at position −980 of the TTP promoter is associated with modest changes in basal plasma vitamin E levels. Evaluation of additional polymorphic changes in TTPA awaits further genetic and biochemical analyses.

The molecular mechanism(s) by which TTP sensitizes prostate cancer cells to vitamin E-induced apoptosis remain incompletely understood. It was previously reported that, in hepatocytes, tocopherol is primarily localized to the endocytic pathway (30, 67, 68). It is therefore surprising that in LNCaP cells, TTP and vitamin E accumulate in mitochondria (Fig. 6). These results raise the possibility that processing and perhaps actions of vitamin E are regulated in a tissue-specific manner. It is interesting to note in regard to this localization that it was recently reported that some vitamin E analogs specifically target the mitochondrial cell death pathway (69).

Finally, the data provided here are of clinical relevance on two levels. First, it is possible that manipulation of prostate-specific TTP levels could be used to enhance the anti-tumorigenic effects of vitamin E supplementation. In addition, individual genotyping of the TTPA gene could serve as a marker for evaluating potential benefits of vitamin E supplementation of individual patients.
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