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Ras inhibition results in growth arrest and death of androgen-dependent and androgen-independent prostate cancer cells

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Abbreviations:

DMEM, Dulbecco modified Eagle's medium

EGF, epidermal growth factor

ERK, extracellular

signal-regulated

kinase

FGF, fibroblast growth factors

FTS, *S-trans*, *trans*-

farnesylthiosalicylic acid

IGF, insulin-like growth factor

mAb, monoclonal antibody

MAPK, mitogen-activated

protein kinase

PBS, phosphate buffered saline

PI3K, phosphoinositide 3-kinase

PKC, protein kinase C

RTK, receptor tyrosine kinase

ABSTRACT

Prostate cancer is one of the most frequently diagnosed cancers in human males. Progression of these tumors is facilitated by autocrine/paracrine growth factors which activate critical signaling cascades that promote prostate cancer cell growth, survival and migration. Among these, Ras pathways have a major role. Here we examined the effect of the Ras inhibitor *S-trans*, *trans*-farnesylthiosalicylic acid (FTS), on growth and viability of androgen-dependent and androgen-independent prostate cancer cells.

FTS downregulated Ras, inhibited signaling to Akt and reduced the levels of cell-cycle regulatory proteins including cyclin D1, p-RB, E2F-1 and cdc42 in LNCaP and PC3 cells. Consequently the anchorage-dependent and anchorage-independent growth of LNCaP and PC3 cells were inhibited. FTS also induced apoptotic cell death which was inhibited by the broad-spectrum caspases inhibitor, Boc-asp-FMK. Our study demonstrated that androgen-dependent and androgen-independent prostate cancer cells require active Ras for growth and survival. Ras inhibition by FTS results in growth arrest and cell death. FTS may be qualified as a potential agent for the treatment of prostate cancer.

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SDS-PAGE, sodium dodecyl
sulfate polyacrylamide gel
electrophoresis
TGF, transforming growth factors

1. Introduction

Prostatic carcinoma is one of the most common male cancers and the second leading cause of cancer-related death [1]. Prostate cancer cell growth is controlled by several factors including androgen, growth factors and their respective receptors. In early stage of prostate cancer androgens are required for cell proliferation and survival. Therefore, 80% of the patients respond to androgen deprivation therapy with reduced tumor size. However the effectiveness of androgen ablation in the management of advanced prostate cancer is of limited value, with the median length of response being only 18–24 months [2,3]. The transition of the prostate cancer cell to an androgen-independent phenotype is a complex process that involves selection and outgrowth of pre-existing clones of androgen-independent cells as well as adaptive up-regulation of genes that help the cancer cells to survive and grow after androgen ablation [3].

Alterations of growth factors and their receptors may lead to the development of cancer [4]. Increases in autocrine and paracrine growth factor loops are among the most commonly reported changes correlated with progression of prostate cancer. Growth factors and their receptors have been reported to be overexpressed in advanced prostate cancer including epidermal growth factor (EGF), transforming growth factors (TGF)- α and β , fibroblast growth factors (FGF), and insulin-like growth factors (IGF) [5–7]. Activation of the growth factor receptors leads to increased Ras activation in advanced prostate cancer. Ras activation as a result of increased EGF receptor (ErbB-1) signaling was reported in 40% of prostate tumors and occurred with greater frequency in advanced stages [8]. Overexpression of ErbB-2 and ErbB-3 receptors has been implicated in the neoplastic transformation of prostate cancer [9] and expression of ErbB-2/neu in prostate cells induced cell transformation [10]. Although the role of oncogenes and growth factors in prostate carcinoma is still unclear, overexpression of the epidermal growth factor receptor (ErbB-1) and the proto-oncogene ErbB-2 has been reported in prostate tumors, and ErbB-2 has been related to poor prognosis and distant metastasis [11].

Ras signaling is activated in a large fraction of human tumors [4]. Although constitutively active mutants of Ras are commonly encountered in some types of cancer they appear to be relatively rare in prostate cancer, with possible exception in Japanese patients [12–14]. In spite of this prostate cancer cells exhibit elevated levels of activated MAP kinases, which are targets of Ras, in correlation with tumor stage and grade [15]. It has been also demonstrated that Ras activation play a role in altering prostate cancer cells toward decreased hormone dependence and increased malignant phenotype [16]. Expression of constitutively active H-Ras in LNCaP cells was sufficient for progression toward androgen independence

in terms of tumorigenicity and it correlated with activation of MAP kinase signaling.

Because Ras signalling represents a convergence point for numerous diverse extracellular signals, Ras and its effectors may be appropriate targets for therapeutic intervention. Ras is post translationally modified by addition of a farnesyl lipid group that allows Ras attachment to the membrane. Attempts have been made to block Ras or Ras dependent functions in prostate cancer cell lines using farnesyl transferase inhibitors [17–19]. *S-trans*, *trans*-farnesylthiosalicylic acid (FTS) is a synthetic Ras inhibitor which structurally resembles the carboxy-terminal farnesylcysteine group common to all Ras proteins. FTS acts as a functional Ras antagonist in cells affecting Ras-membrane interactions, dislodging the protein from its anchorage domains, facilitating its degradation, and thus reducing cellular Ras content [20,21]. FTS has been shown to inhibit the growth of H-Ras and K-Ras and N-Ras transformed rodent fibroblasts *in vitro* [22,23]. More recent studies have shown that FTS can inhibit the anchorage-dependent growth of LNCaP and CWR-R1 cells [24]. In the present study we examined the impact of FTS on the anchorage-dependent and anchorage-independent growth of the prostate cancer cell lines, PC3 and LNCaP, and determined its effects on the survival of these cells.

2. Materials and methods

2.1. Materials and buffers

FTS was prepared by a general procedure, purified and analyzed as described in detail elsewhere [25]. Pan Ras Ab (Ab-3) was purchased from Calbiochem (La Jolla, CA). Polyclonal rabbit anti Rb (C-15), anti E2F-1 (c-20), and anti cyclin D1 (M-20) antibodies, were purchased from Santa-Cruz Biotechnology (Santa-Cruz, CA). Polyclonal rabbit anti phospho-cdc2 (Thr161) was purchased from cell Signaling. Polyclonal rabbit anti Mapk and anti phospho-Mapk antibodies were purchased from New England Biolabs Inc. Rabbit (polyclonal) anti survivin Ab (FL-142) was from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal Rat anti Tubulin was kindly provided by Prof. Elisha Orr, MabGenics GmbH (Giessen, Germany). Ac-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (Ac-DEVD-7AMC) was purchased from Biomol Research Laboratories (Plymouth Meeting, PA, USA). Boc-asp-FMK was from ICN (Biomedicals Inc.). All other reagents were from Sigma. HNTG buffer contained 20 mM HEPES (pH 7.5), 150 mM NaCl, 0.1% Triton X-100, and 10% glycerol. Solubilization buffer contained 50 mM HEPES (pH 7.5), 150 mM NaCl, 1% Triton X-100, 1 mM EGTA, 1 mM EDTA, 1.5 mM MgCl₂, 10% glycerol, 0.2 mM sodium orthovanadate, 2 mM phenylmethylsulfonyl fluoride (PMSF), 10 μ g/ml aprotinin, and 10 μ g/ml leupeptin.

2.2. Cell lines

The human LNCaP and PC3 cells were grown in RPMI-1640 supplemented with antibiotics and 10% heat-inactivated fetal bovine serum (FBS). Cells were incubated at 37 °C in 5% CO₂ in air, and the medium was changed every 3–4 days. Cells were passaged when 70% confluent using trypsin/Di-sodium ethylenediaminetetra-acetic acid (EDTA) (Biological Industries, Kibbutz Beit Haemek, Israel).

2.3. Cell survival assays

LNCaP and PC3 cells were plated in 96-well plates at a density of 7000 and 10,000 cells/well respectively in medium containing 5% FBS. 24 h later, FTS or 0.1% DMSO (control) were added. Cell survival was determined by using the [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl] tetrazolium bromide (MTT) assay, which determines mitochondrial activity in living cells [26]. MTT 0.1 mg/ml was incubated with the analyzed cells for 2 h at 37 °C. Living cells can transform the tetrazolium ring into dark blue formazan crystals, which can be quantified by reading the optical density at 550–650 nm after lysis of the cells with acidic isopropanol. Staining of nuclei with the fluorescent DNA dye bisbenzimidazole (Hoechst 33258) was used to estimate the number of dying cells. Hoechst staining was performed on live cells by incubation with Hoechst solution (2 µg/ml) for 10 min. Dead cells were scored by nuclear staining and nuclear morphology. Following staining the, cells were photographed. The instrument used was Olympus optical inverted phase-contrast microscope Model IX70 (20× magnification). Quantification of the results was performed by counting 10 random fields. Stained nuclei were considered as dead cells and unstained cells were counted as live cells.

2.4. Cell cycle analysis

For cell cycle analysis, LNCaP and PC3 cells were seeded in six-well plates at a density of 3.5×10^5 cells/well, in medium containing 5% FBS, in the presence of 0.1% DMSO (control) or in the presence of the indicated concentrations of FTS. After 3 days cells were washed once with PBS. Propidium iodide (50 µg/ml) and Triton (0.05%) were added. The stained cells were analyzed in a fluorescence-activated cell sorter (FACS-can; Becton and Dickinson) within 1 h. The percentage of cells at different phases of the cell cycle was determined by using the WinMDI 2.1.4 program.

2.5. Assay for DEVDase activity

Caspase activity was measured in terms of assayed DEVDase activity. PC3 and LNCaP cells were seeded at a density of 2×10^6 cells in 10 cm plates, in medium containing 5% FBS. 24 h later, 50 µM, 75 µM FTS or 0.1% DMSO (control) were added. After 2 days incubation with FTS cells were collected, centrifuged, washed with PBS and resuspended in 100 µl of extraction buffer (50 mM Tris-HCl, pH 7.4, 1 mM EDTA and 10 mM EGTA). Cells were lysed by three rounds of freezing and thawing. The extracts were then centrifuged for 5 min at $14,000 \times g$. The resulting supernatants were normalized for

protein content (approximately 150 µg) and assayed for DEVDase activity using the fluorescent synthetic peptide Ac-DEVD-7AMC (50 µM) in the reaction buffer (50 mM Tris-HCl, pH 7.4, 1 mM DTT and 2 mM MgCl₂). Fluorescence at 360 nm excitation and at 460 nm emission was measured after incubation for 30 min at 37 °C. DEVDase activity induced by FTS is presented as arbitrary units.

2.6. Soft-agar assays

Cells were seeded at a density of 6500 cells/well in 96 well plates in RPMI medium containing 10% FBS. The cells were mixed with 0.05 ml (per each well) of 0.33% noble agar, and the mixture was poured onto a layer of 0.05 ml 1% noble agar in RPMI medium containing 10% FBS. The upper layer of the agar was covered with 0.1 ml of medium. The agar layers contained either 0.1% DMSO (control) or FTS dissolved in 0.1% DMSO. Assays were performed in at least six repeats. The number and sizes of LNCaP and PC3 colonies were estimated on day 14, using a binocular and a light microscope with the image analyzer program Image pro-Plus.

2.7. Lysate preparation and Western immunoblotting

Cells were seeded in six well plates at a density of 3.5×10^5 cells/well, in medium containing 5% FBS, in the presence of 0.1% DMSO (control) or in the presence of the indicated concentrations of FTS. After treatment, cells were solubilized in lysis buffer. Lysates were cleared by centrifugation. For direct electrophoretic analysis, a boiling gel sample buffer was added to cell lysates. For determination of Ras levels cells were homogenized, and fractionated ($100,000 \times g$ pellet and supernatant) as detailed previously [21]. Lysates or cellular fractions were then resolved by SDS-polyacrylamide gel electrophoresis (PAGE) through 7.5% or 10% gels and electrophoretically transferred to nitrocellulose membrane. Membranes were blocked for 1 h in TBST buffer (0.05 M Tris-HCl pH 7.5, 0.15 M NaCl and 0.1% Tween 20) containing 6% milk, blotted with primary antibodies for 2 h, followed by 0.1 µg/ml secondary antibody linked to horseradish peroxidase. Immunoreactive bands were detected with the enhanced chemiluminescence reagent (Amersham Corp.).

3. Results

3.1. FTS reduces levels of Ras and inhibits cell-cycle regulators in PC3 and LNCaP cells

In order to examine the effect of Ras inhibition on the malignant phenotype of androgen-responsive and androgen-insensitive prostate cancer cells we first determined whether the Ras inhibitor, FTS, can affect Ras in such cells. We incubated LNCaP (androgen-responsive) or PC3 (androgen-insensitive) cells with 50 µM FTS or 75 µM FTS for 48 h and determined the levels of Ras in lysates of the cells by Western immunoblotting with pan anti-Ras Ab. We found that PC3 and LNCaP express comparable amounts of Ras as judged by densitometry; the ratio of the Ras bands recorded in lysates of equal number of cells was 1 ± 0.085 ($n = 3$). FTS treatment

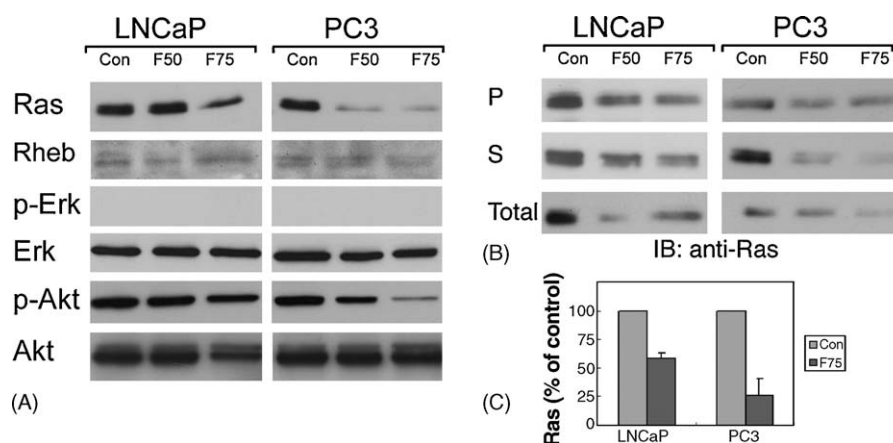


Fig. 1 – FTS induces decrease in the levels of total Ras and phosphorylated PKB. Cells were plated in six-well plates at a density of 3.5×10^5 cells/well in medium containing 5% FBS, in the presence of 0.1% DMSO (control) or in the presence of the indicated concentrations of FTS for 48 h. (A) Total cell lysate was subjected to Western blot analysis. Anti-Ras and anti-Rheb antibodies were used to determine the total Ras and RheB levels in the cells. Anti-phospho-ERK, anti-ERK, anti-phospho-Akt and anti-Akt antibodies were used to determine the activity of ERK and Akt. (B) Levels of expression of Ras in total cell homogenates (expression levels) and in the particulate (P100) and cytosolic fractions (S100) were determined by immunoblotting as detailed in Section 2. Results of a representative experiment are shown. Immunoreactive bands were detected with the enhanced chemiluminescence reagent. Data represent one of three experiments with similar results. (C) Densitometric analysis of the effect of FTS on the cellular levels of Ras. Data were normalized to the levels of Erk and are presented as the means of the ratio between the normalized Ras levels recorded in 75 μM FTS treated cells and in the control DMSO treated cells. Bars: S.D.

caused a significant reduction in the total amount of cellular Ras in both cell lines (Fig. 1A). In addition we found that FTS caused a reduction in the particulate fraction of the cells and in the cytosol (Fig. 1B). Under the same conditions FTS had no effect on the level of the prenylated Rheb GTPase (Fig. 1A). Analysis of the reduction in the amounts of cellular Ras in the two cell lines revealed a clear statistical significance (Fig. 1C). These results are consistent with the known action of FTS as a Ras inhibitor [27]. Moreover, in line with earlier reports that FTS does not reduce the cellular levels of the prenylated γ subunit and of heterotrimeric G proteins [21] and of the prenylated RhoA [43], they demonstrate lack of an effect on the RheB protein in prostate cancer cells. These observations point to the apparent selectivity of FTS towards Ras. Next we examined the level of activation of ERK as readout of the prominent Ras/Raf/MEK/ERK pathway. ERK and activated p-ERK were determined in lysates of control and FTS-treated cells by Western immunoblotting using anti-ERK and anti-p-ERK Abs. Although both cell lines exhibited significant amounts of ERK we did not detect active p-ERK (Fig. 1). These results show that ERK activation does not correlate with the sensitivity to androgen because both the androgen-sensitive LNCaP and the androgen-insensitive PC3 cells exhibited no p-ERK. These experiments suggested that if FTS can affect prostate cancer cell growth through its action on Ras, it would do so by pathways other than the Raf/MEK/ERK. We next examined the effects of FTS on active p-PKB/Akt as a readout of PI3K activation. Akt and activated p-Akt were determined in lysates of control and of FTS-treated cells by Western immunoblotting using anti-Akt and anti-p-Akt Abs. As shown in Fig. 1 both PC3 and LNCaP exhibited significant amounts of

p-Akt and FTS induced a reduction in the amount of the active protein. These results suggest that FTS inhibited the Ras signal to PI3K and Akt. It is important to note that in both cell lines there is no PTEN activity [28] leading to relatively high levels of

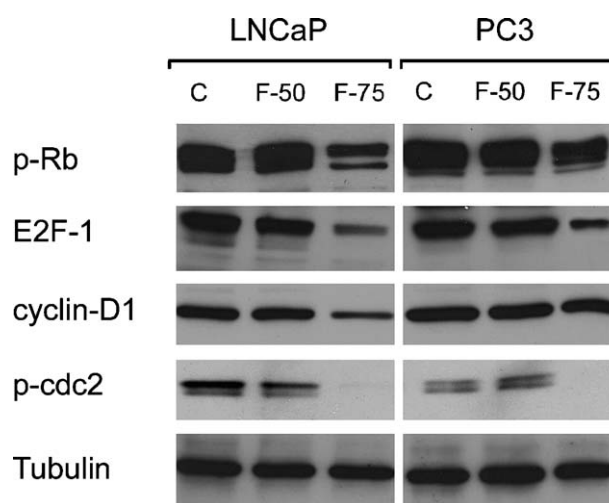


Fig. 2 – FTS treatment reduces the levels of phospho-Rb, E2F-1, cyclin D1 and phospho-cdc2. Cells were treated in the presence of 0.1% DMSO (zero drug control) or in the presence of the indicated concentrations of FTS for 48 h in medium containing 5% FBS. Whole cell lysates were prepared and immunoblotted with anti-phospho-Rb, anti-E2F-1, anti-cyclin D1, anti-phospho-cdc2, or anti β -tubulin antibodies. Data represent one of three similar experiments.

PIP3 and thus of active p-Akt whose activation depends on PIP3. Nonetheless, the production of PIP3 requires of the active PI3K whether or not PTEN is active. Thus inhibition of the Ras-dependent activation of PI3K will eventually result in reduction in PIP3 levels, consistent with our observation that FTS reduced levels of p-Akt.

We next decided to examine whether FTS can affect regulators of the cell cycle which are known to be controlled by various Ras pathways, not only by active ERK. A major role of Ras in G1 progression is to inactivate Rb protein through the activation of G1 Cdk and the subsequent activation of the transcription factor E2F-1 [29–31]. This has been shown to occur through the stimulation of cyclin D1 transcription as well as increased levels of cyclin D1/Cdk4 kinase activity [32,33]. First, we determined the effect of FTS on the levels of cyclin D1. We found that FTS reduced the amounts of cyclin D1 in both of the prostate cell lines (Fig. 2). Consistent with this observation we found that FTS reduced the levels of p-RB protein, of E2F-1 and of p-cdc-2 which is transcriptionally controlled by E2F-1 [29,34]. These results indicate that FTS would affect prostate cancer cell growth by inhibiting cell cycle progression.

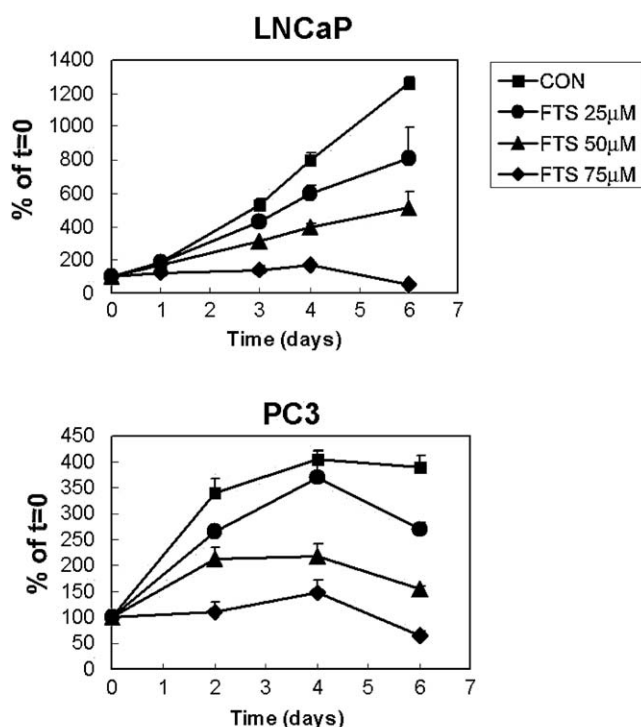


Fig. 3 – FTS inhibits cell growth in a dose-dependent manner. LNCaP and PC3 cells were plated in 96-well plates at a density of 7000 and 10,000 cells/well, respectively, in medium containing 5% FBS in the presence of 0.1% DMSO (control) or in the presence of the indicated concentrations of FTS. The extent of cell growth was determined daily using the colorimetric MTT assay. Results are presented as fold induction over the control untreated cells at day 0, and are mean \pm S.D. of six determinations. The FTS-induced reduction in cell number at all concentrations used was statistically significant as of day 3 (LNCaP) and day 2 (PC3) of the treatment ($p < 10^{-5}$). Each experiment was repeated at least three times with similar results.

3.2. FTS inhibits growth and induces apoptosis in PC3 and LNCaP cells

We next examined the impact of Ras inhibition by FTS on growth of PC3 and LNCaP cells using the MTT method (Fig. 3). We found that FTS caused a time- and dose-dependent reduction in cell number (Fig. 3). The number of cells in the FTS (25–75 μ M) treated cultures increased with time at a significantly lower rate than that in the untreated cells. This was apparent on days 2–4 in PC3 and LNCaP cultures treated with 25 and 50 μ M FTS (Fig. 3). Similar results were obtained in experiments in which serum concentrations were elevated from 5% (Fig. 3) to 10% (not shown) except that growth inhibition under the later conditions developed at a slower rate. These results suggested that FTS inhibited the growth of PC3 and LNCaP cells. However, in both cell lines we found that the drug treatment reduced the number of cells more than what could be expected from growth inhibition only. For example, on day 6 the number of cells in cultures treated with 75 μ M FTS was lower than that recorded on day zero. Therefore we suspected that FTS also induced cell death, particularly because Ras is known to provide survival signals acting as an anti-apoptotic factor [35].

To analyze the possibility that FTS induced death of PC3 and LNCaP cells we determined the fraction of apoptotic cells either by flow cytometry analysis or by staining the cells with the DNA-specific dye Hoechst. The flow cytometry analysis showed that the fraction of sub-G1 (representing dead cells) was increased from 1.4% in the controls to 23% and 12%, respectively in LNCaP and PC3 cells after 72 h incubation with 75 μ M FTS but not with 50 μ M FTS (Fig. 4). These results indicate that the observed reduction in cell number (Fig. 3) was in part due to FTS-mediated cell death (Fig. 4). This conclusion was strengthened by fluorescent dye exclusion experiments (Fig. 5). In these experiments the cells were incubated for 3 days with or without FTS at the indicated concentrations, and then stained for 10 min with Hoechst dye to detect dead cells. Photomicrographs were taken immediately after staining. Stained nuclei were considered as dead cells and unstained nuclei were counted as live cells. Typical images of control and FTS-treated cells demonstrate that the drug induced death of both PC3 and LNCaP cells (Fig. 5A). Quantification of the results was performed by counting 10 random fields (Fig. 5B). As shown, 75 μ M FTS but not 50 μ M FTS induced an increase in the number of dead cells; the number of live cells was decreased at both drug concentrations. The effect on cell death was more profound in LNCaP cells as compared with PC3 cells (Fig. 5) consistent with the results obtained by flow cytometry analysis (Fig. 4). Taken together our results indicate that FTS induces both cell growth inhibition and cell death in the androgen-dependent and androgen-independent prostate cancer cell lines examined.

To examine whether the death induced by FTS was apoptosis, we used the multicaspase inhibitor Boc-Asp-FMK (BAF) and estimated cell viability by the MTT assay (Fig. 6A). We found that the FTS-mediated reduction in viable cells was inhibited by BAF. However, BAF did not block completely the FTS-induced reduction in viable cells (Fig. 6A). This is because the FTS-induced reduction in viable cells is in part due to

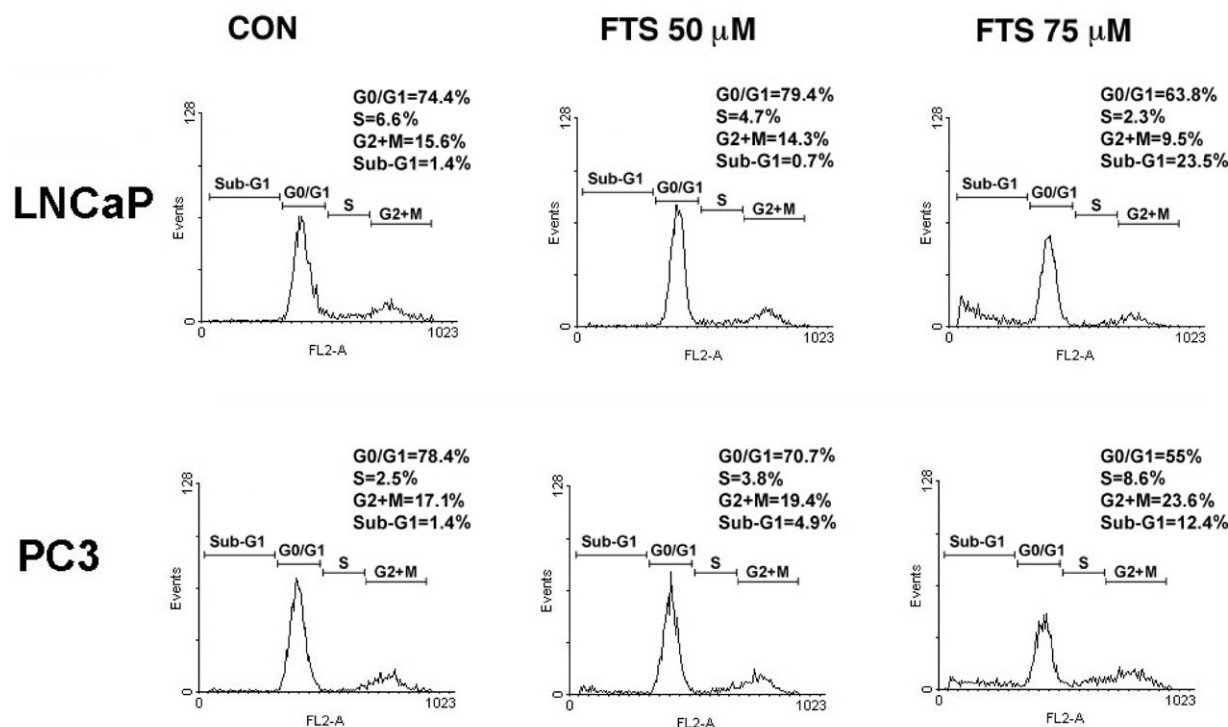


Fig. 4 – FACS analysis of FTS-mediated effects in LNCaP and PC3 cells. LNCaP and PC3 cells were plated in six-well plates at a density of 3.5×10^5 cells/well, in medium containing 5% FBS, in the presence of 0.1% DMSO (control) or in the presence of the indicated concentrations of FTS. Cells were harvested after 3 days and analyzed for DNA content by flow cytometry. The percentages of cells at the G0/G1, S, G2 + M and sub-G1 phases of the cell cycle are indicated. The sub-G1 cell fraction represents cells undergoing apoptosis. The experiments were repeated three times with similar results.

inhibition of cell growth (Fig. 3), which is not affected by caspase inhibitors.

The involvement of caspases in the FTS-mediated death of PC3 and LNCaP cells was further supported by direct measurements of caspase activity in the FTS treated cells. These measurements were performed using the caspase substrate DEVD-7-AMC (DEVDase activity) [36]. We used cell extracts prepared from the FTS-treated and non-treated cells (Fig. 6B). As shown, FTS induced a significant increase in caspase activity in LNCaP and PC3 cells. Extracts were also used for the determination of the levels of survivin, a member of the IAP gene family which was found to be up-regulated in various human cancers [37,38] and to inhibit caspase-3, -7 and -9 [39]. As shown in Fig. 6C FTS caused a dose-dependent reduction in the levels of survivin in LNCaP and PC3 cells, consistent with earlier reports that Ras positively regulates survivin expression [40]. Thus, the FTS induced apoptosis of the prostate cancer cells seems to be associated with a combined mechanism of caspase activation and relief of caspase inhibition by survivin.

3.3. FTS inhibits the anchorage-independent growth of PC3 and LNCaP cells

Next we examined the impact of FTS on the anchorage-independent growth of LNCaP and PC3 cells using soft-agar assays. The cells were plated on soft agar and maintained in culture for 2 weeks, then stained with MTT. The number and

size of colonies were then estimated. Results of a typical experiment are shown in Fig. 7. LNCaP cells formed relatively large colonies in soft agar. The total colony number was not changed by 50 μ M FTS treatment but the size of colonies was significantly decreased. Since at this concentration FTS did not induce LNCaP cell death (Fig. 5) these results indicate that FTS inhibited the anchorage-independent growth of the cells. However, at a higher concentration (100 μ M) FTS induced a decrease in both colony size and colony number reflecting inhibition of anchorage-independent growth and cell death.

PC3 cells formed relatively small colonies. Both the number and the size of PC3 colonies were significantly reduced in the presence of 50 μ M FTS. Since 50 μ M FTS did not induce PC3 cell death (Fig. 5) these results indicate that FTS inhibited the anchorage-independent growth of these cells. Interestingly, comparison of the results obtained at the non-killing concentration of FTS in the two cell lines shows that PC3 cells are more sensitive than LNCaP cells to anchorage-independent growth inhibition. As in the LNCaP cells, colony formation in PC3 cells was completely inhibited in the presence of 100 μ M FTS.

4. Discussion

Prostate cancers typically begin as androgen-sensitive lesions but frequently develop into androgen-insensitive lesions with the progression to advanced stages. Autocrine relationships

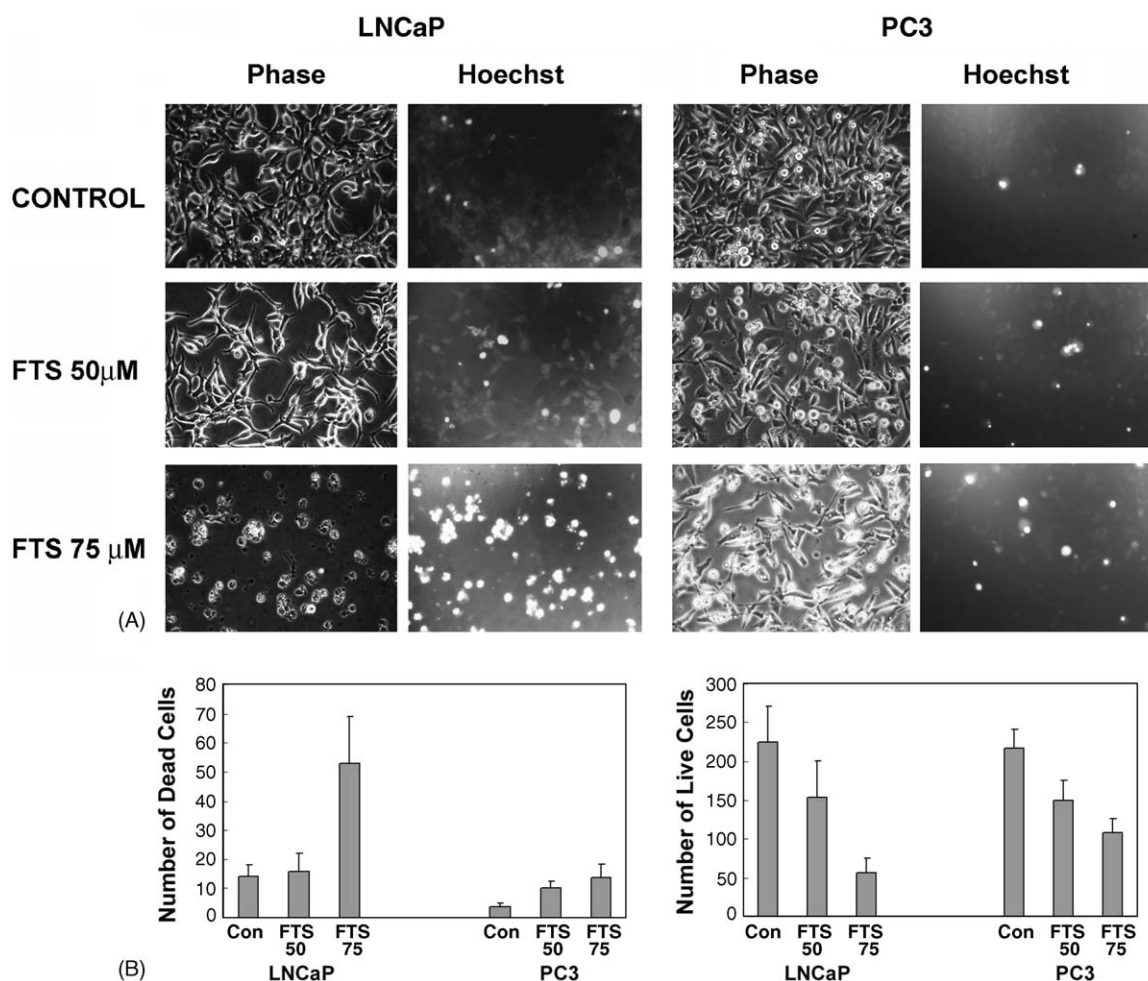


Fig. 5 – FTS-induced cell death of prostate cancer cell lines: LNCaP and PC3 cells were treated as described in Fig. 3. (A) Cells were stained with Hoechst, after 3 days of incubation with FTS. Photomicrographs were taken immediately after Hoechst staining. (B) Quantification of the results presented in B, was performed by counting 10 random fields. Stained nuclei were considered as dead cells. Note that in LNCaP cells, more dead cells were observed after treatment with 75 μ M FTS, than in PC3 cells. The total cell number is represented as the mean \pm S.D. of 10 determinations.

between EGFR, TGF- α and EGF in prostate cancer cell lines, primary tumors and androgen-independent metastatic tumors, have been suggested [7,41,42]. Other growth factors such as fibroblast growth factors (FGF), and insulin-like growth factors (IGF) and their receptors were also implicated in prostate cancer progression [5,6]. Since these factors activate signaling that converges at the levels of Ras it was reasonable to assume that inhibition of Ras will affect the uncontrolled growth of prostate cancer cells. In addition since growth factors provide survival signals which also involve Ras activation it was reasonable that inhibition of Ras may cause prostate cancer cell death. Our results show that the Ras inhibitor FTS induced both growth arrest and apoptosis of the androgen-dependent LNCaP cells and of the androgen-independent PC3 cells. These results are in accord with the observed growth inhibition of LNCaP and CWR-R1 prostate cancer cells by FTS which were clearly associated with the reduction in cell cycle regulators [24]. However our studies demonstrate for the first time that FTS induces apoptosis in prostate cancer cells and inhibits their anchorage-independent growth.

Activation of Ras signaling pathways is essential to exit a quiescent state and progress through G1 phase of the cell cycle. Ras regulates several cell cycle proteins; it inactivates Rb protein through the activation of G1 Cdks. This has been shown to occur through the stimulation of cyclin D1 transcription as well as by increasing cyclin D1/Cdk4 kinase activity [34]. Consistent with this role of Ras in the regulation of cell cycle progression we demonstrate that FTS treatment reduces the levels of phosphorylated-Rb, E2F, cyclin D1 and phosphorylated cdc2 proteins in prostate cancer cells (Fig. 2). Similar results were obtained earlier in other human cancer cell lines, including melanoma [43] neuroblastoma [44] and glioblastoma [45] pointing to a universal mechanism of growth inhibition by FTS. The observation that this mechanism of growth inhibition operates in prostate cancer cells independent of sensitivity to androgen suggests that: (i) FTS may be used in combination with androgen ablation in androgen-dependent prostate cancer (ii) FTS may be used as a single drug treatment for prostate cancers that grow and spread independently of androgen. These notions are strengthened by the

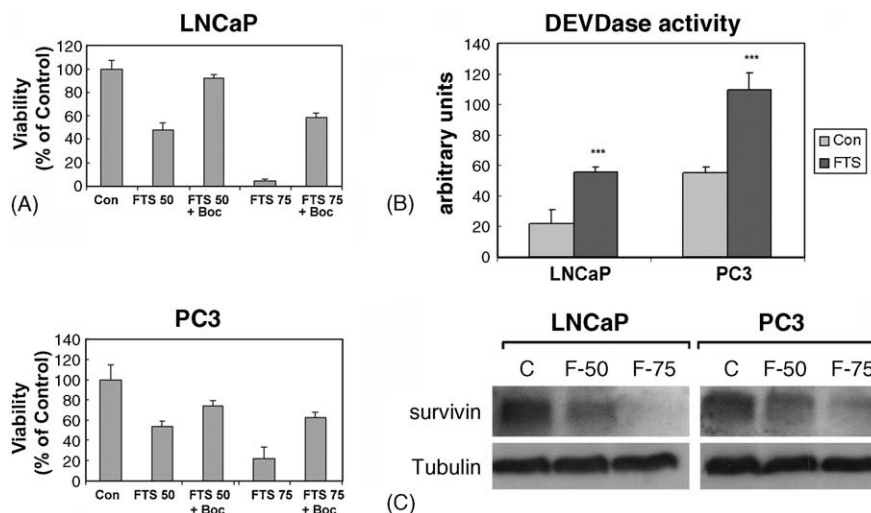


Fig. 6 – The involvement of Caspases in FTS-induced cell death. (A) Cells were plated in 96-well plates at a density of 8000 cells/well in medium containing 5% FBS in the presence of 0.1% DMSO (control) or in the presence of the indicated concentrations of FTS, with or without 100 μ M Boc-asp-FMK (caspase inhibitor). The MTT assay was performed 3 days later. Results are presented as fold induction over the control untreated cells, and are mean \pm S.D. of six determinations. Each experiment was repeated at least three times with similar results. (B) DEVDase activity was determined in lysates of 2 days FTS treated cells, as well as of control 0.1% DMSO treated cells, as described in Section 2. The results shown are of a representative experiment (one of three experiments with similar results). The data shown are mean \pm S.D. ($p < 4 \times 10^{-4}$) (C) Cells were treated as described in Fig. 2. Total cell lysates was subjected to Western blot analysis. Anti-survivin antibodies were used to determine the levels of survivin in the cells. Anti- β -tubulin antibodies were used as a control. Immunoreactive bands were detected with the enhanced chemiluminescence reagent (note that the levels of survivin was reduced to 60% and 5% in LNCaP cells treated with 50 or 75 μ M FTS, respectively, and to 65% and 35% in PC3 cells treated with 50 or 75 μ M FTS, respectively). Data represent one of three similar experiments.

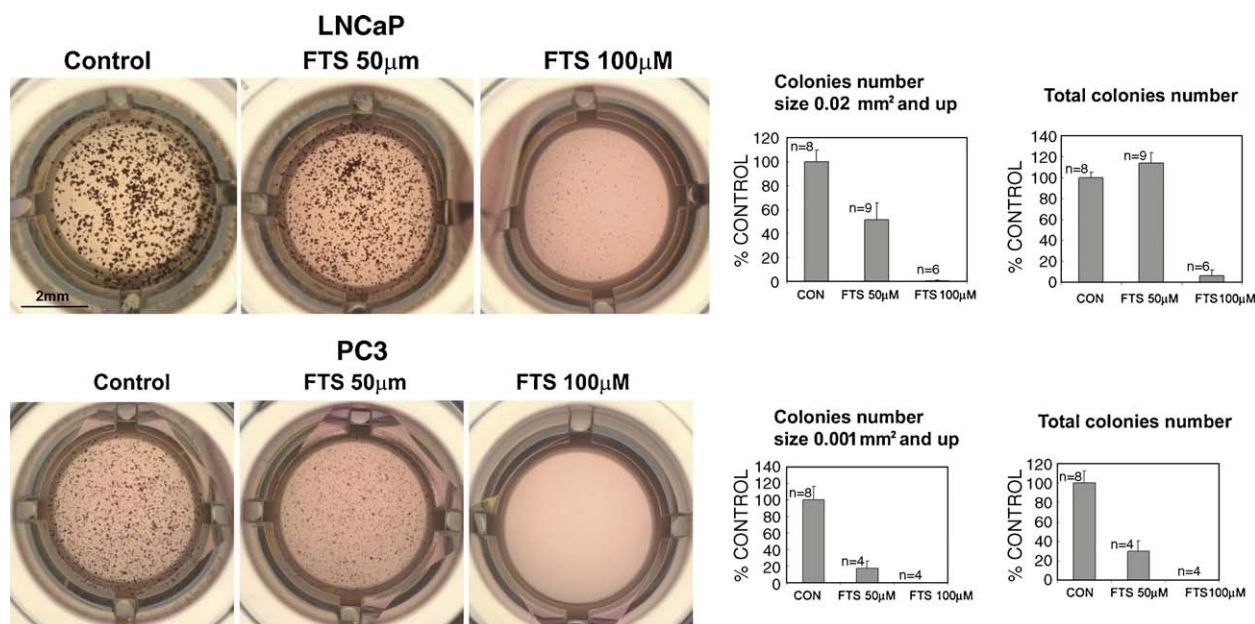


Fig. 7 – FTS inhibits anchorage-independent growth of human prostate cancer cells. Cells were seeded in soft agar (6500 cells/well in 96 well plates) in medium containing 10% FBS, 0.3% agar, in the presence of 0.1% DMSO (zero drug control) or in the presence of the indicated concentrations of FTS. The extent colony formation was determined 2 weeks later. Cells were dyed with MTT and the wells were photographed and colonies were counted. Results are presented as percentage of the control untreated cells, and are the mean \pm S.E.M. of the indicated number of determinations (n). Each experiment was repeated at least three times with similar results.

observation that the FTS-induced apoptosis is not associated with androgen-dependency (Figs. 4 and 5). Nonetheless, it is interesting to point out that the two lines exhibited different sensitivities to FTS. The effect was apparently stronger in the androgen-dependent LNCaP cells than in PC3 cells.

The FTS-induced death of prostate cancer cells appears to be a caspase-dependent apoptotic cell death since it was inhibited by the caspase inhibitor Boc-asp-FMK, and since FTS induced an increase in caspase activity. Early experiments in Ras-transformed rat intestinal epithelial (RIE) cells showed that FTS cancels resistance to anoikis, apparently by the inhibition of Ras and the consequent caspase-dependent cell death [35]. However, in Ras-transformed RIE cells as well as in a number of human tumor cell lines grown in the presence survival signals (e.g. in the presence of serum) FTS does not induce cell death [35]. For example, pancreatic cancer cells and colon carcinoma cells [46] grown in the presence of serum do not die when they are treated with FTS alone. Prostate cancer cells appear, however, to be highly sensitive to FTS as they apoptose even in the presence of survival signals. A similar phenomenon has been recently described in human glioblastomas [45]. Like in prostate cancer cells, the FTS-induced cell death is associated with activation of intrinsic apoptotic pathways (including cytochrome c release, and activation of caspases) and downregulation of survivin in glioblastoma (unpublished observations). It appears that the relief of survivin inhibition of intrinsic apoptotic pathways [47] is sufficient for the facilitation of tumor cell death under certain circumstances or genetic backgrounds, as in glioblastoma and in prostate cancer cells. However, it is most likely that this effect is operable because it is accompanied by activation of death pathways or by preventing survival signals. It is most likely that in prostate cancer cells and in glioblastoma [45] FTS inhibits Ras-dependent autocrine survival signals [43] allowing activation of caspases (Fig. 6). Since the activation of caspases is accompanied by downregulation of survivin, which otherwise would have blocked the active enzymes, the cells undergo caspase-dependent cell death. Interestingly, the incidence of PTEN mutations is very high in both prostate cancer and glioblastoma [48], indicting that both tumor types are most likely “addicted” to high PIP3-dependent survival signals. Perhaps this explains why prostate cancer cells and glioblastoma apoptose in the presence of FTS while some other tumors do not.

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