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Tea Polyphenols Decrease Serum Levels of Prostate-Specific Antigen, Hepatocyte Growth Factor, and Vascular Endothelial Growth Factor in Prostate Cancer Patients and Inhibit Production of Hepatocyte Growth Factor and Vascular Endothelial Growth Factor *In vitro*

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Abstract

The purpose of this study was to determine the effects of short-term supplementation with the active compounds in green tea on serum biomarkers in patients with prostate cancer.

Twenty-six men with positive prostate biopsies and scheduled for radical prostatectomy were given daily doses of Polyphenon E, which contained 800 mg of (–)-epigallocatechin-3-gallate (EGCG) and lesser amounts of (–)-epicatechin, (–)-epigallocatechin, and (–)-epicatechin-3-gallate (a total of 1.3 g of tea polyphenols), until time of radical prostatectomy. Serum was collected before initiation of the drug study and on the day of prostatectomy. Serum biomarkers hepatocyte growth factor (HGF), vascular endothelial growth factor (VEGF), insulin-like growth factor (IGF)-I, IGF binding protein-3 (IGFBP-3), and prostate-specific antigen (PSA) were analyzed by ELISA. Toxicity was monitored primarily through liver function enzymes. Changes in serum components were analyzed statistically using the Wilcoxon signed rank test. Cancer-associated fibroblasts were treated with EGCG, and HGF and VEGF protein and mRNA levels were measured.

HGF, VEGF, PSA, IGF-I, IGFBP-3, and the IGF-I/IGFBP-3 ratio decreased significantly during the study. All of the liver function tests also decreased, five of them significantly: total protein, albumin, aspartate aminotransferase, alkaline phosphatase, and amylase. The decrease in HGF and VEGF was confirmed in prostate cancer–associated fibroblasts *in vitro*.

Our results show a significant reduction in serum levels of PSA, HGF, and VEGF in men with prostate cancer after brief treatment with EGCG (Polyphenon E), with no elevation of liver enzymes. These findings support a potential role for Polyphenon E in the treatment or prevention of prostate cancer.

According to the American Cancer Society, prostate cancer is the most diagnosed malignancy in men and the second leading cause of cancer mortality in men in the United States, with 27,000 dying each year. Rates of recurrence for early-stage disease are relatively high, and mortality rates for late-stage disease have not improved significantly over the past 10 years (1). Increasingly, many researchers have focused their efforts on the potential use of natural products and have reevaluated earlier epidemiologic data and initiated laboratory research to determine their potential efficacy as cancer therapy. Green tea polyphenols have been regarded as showing potential in this area (2). A promising study done in Italy recently showed that consumption of green tea polyphenols significantly delayed the progression of high-grade prostate intraepithelial neoplasia to prostate cancer (3). This trend has been found to continue into the second year despite the lack of consumption of green tea polyphenols (4).

The major bioactive polyphenol present in green tea is (–)-epigallocatechin-3-gallate (EGCG). Additional catechins found in green tea include (–)-epicatechin (EC), (–)-epigallocatechin (EGC), and (–)-epicatechin-3-gallate (ECG). Epidemiologic studies have revealed a reduction in colon cancer incidence in individuals that consumed tea (5), an inverse correlation between urinary tea polyphenols and gastric cancer (6), and an improved prognosis of stage I and II breast cancer patients in those patients that drank five or more cups

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of green tea (7). More recently, a meta-analysis suggested a modest effect on lung cancer risk (8). Tea consumption has also been shown to be associated with a lower prostate cancer risk (9, 10). Furthermore, it has been suggested that the lower prostate cancer risk in Asian men may be associated with their green tea consumption (11). However, the epidemiologic data are still inconclusive, with some studies showing possible benefits and others finding no effect on risk ratios for cancer (9, 12).

We have previously shown that EGCG and ECG can inhibit the HGF/c-Met signaling pathway in both breast (13) and prostate carcinoma cells.[6] The HGF/c-Met pathway is deregulated in numerous types of malignancies, including breast, prostate, and gastric cancers (14). In prostate cancer, the transmembrane receptor c-Met is often overexpressed in primary tumors and metastases. High levels of c-Met are directly correlated with Gleason score and associated with poorly differentiated tumors (15, 16). Additionally, high serum levels of the c-Met ligand, HGF, have been found to be associated with metastatic disease and decreased overall survival (17, 18). Often the overproduction of HGF occurs in cancer-associated fibroblasts located in the stroma surrounding the tumor (16, 19). Deregulation of the HGF/c-Met pathway leads to increased proliferation, motility, and invasion (14).

Several other serum biomarkers associated with poor prognosis in prostate cancer patients include insulin-like growth factor (IGF)-I, IGF binding protein-3 (IGFBP-3), and VEGF. High serum levels of IGF-I have been shown to be directly associated with prostate cancer risk through its proliferative and antiapoptotic effects (20). IGFBP-3 normally functions to inhibit IGF-I signaling, and several epidemiologic studies have found an inverse association between IGFBP-3 and prostate cancer risk, although some controversy exists with regard to IGFBP-3 and risk correlatives (20, 21). The ratio of IGF-I/IGFBP-3 has therefore been suggested to be a potential prostate cancer biomarker (21). VEGF plays an important role in angiogenesis, a process necessary for tumor growth and metastasis (22). The density of new vessel growth is associated with clinically aggressive prostate cancer and disease progression (23). It has been reported that higher levels of VEGF are present in the serum and plasma of men with prostate cancer compared with healthy controls, and increased VEGF levels are associated with metastatic disease and biochemical progression (24, 25). A recent meta-analysis has shown that VEGF levels in serum or plasma are ~2.18 and 1.85 times greater (weighted average of all cancer studies analyzed), respectively, in prostate cancer patients versus healthy controls (26). Finally, HGF and VEGF levels have been found in some studies to increase early during tumorigenesis (27–31), suggesting that the use of natural compounds that can block the HGF/c-Met, VEGF, and/or IGF-I signaling axis may show promise both as chemopreventive agents and as anticancer agents slowing the progression of established tumors.

Given our *in vitro* results showing that EGCG inhibits HGF-stimulated c-Met signaling and downstream effects in breast and prostate cancer cells, we initiated a phase II clinical trial in prostate carcinoma patients to determine if oral administration of Polyphenon E (containing EGCG and, at lesser amounts, ECG, EGC, and EC) could decrease biomarker levels, including HGF, prostate-specific antigen (PSA), VEGF, and IGF-I, in patient serum and tissue. The results for the serum portion of our study are reported here.

Materials and Methods

Study drugs

Polyphenon E, 200 mg EGCG per capsule, is an investigational agent manufactured and originally supplied by Mitsui-Norin Co., Ltd. and now by Polyphenon E. International, Inc. The active pharmaceutical ingredient of Polyphenon E is a purified tea fraction containing 80% to 98% total catechins by weight; the main component is EGCG, which comprises 50% to 75% of the material. Other catechins are present at levels of 12% or below, including EC, EGC, ECG, and gallocatechin gallate. Polyphenon E also contains caffeine (<2% in one lot measured) and small quantities of unidentified products. The safety of Polyphenon E was previously established in a phase I study (32). Patients were asked to take four capsules with a meal (total of 1.3 g Polyphenon E) for a total of 800 mg EGCG daily. The time of drug administration varied between patients, subject to their surgical schedules, but an average of 6 wk was anticipated.

Participants

Men of ages 18 to 75 y, with a recent diagnosis of stage I, II, or III prostate cancer, who were scheduled for radical prostatectomy were recruited for the study. Exclusion criteria included a known malignancy at any site other than prostate; participation in another research study within the last 3 mo; having consumed five or more cups of green tea within 1 wk before biopsy; or having any of the liver enzymes alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, albumin, or total bilirubin greater than the upper limit of normal (ULN).

Study design

The study design was an open-label, single-arm two-stage phase II clinical trial to evaluate the effects of Polyphenon E administered during the interval between prostate biopsy and radical prostatectomy. Safety was monitored by serum chemistries; interviews for toxicity were conducted by telephone contact at week 3, a hepatic panel, and a toxicity interview in person at the time of prostatectomy. During the course of the study, the Food and Drug Administration added the requirement that a hepatic panel, including amylase and lipase, be done after 4 wk on the study and every 4 wk thereafter. For grade 1 toxicity, elevation of alanine aminotransferase (>ULN–2.5 × ULN), the study drug was to be withheld until recovery to normal. For grade 2 (2.5 × ULN–5.0 × ULN), grade 3 (5.0 × ULN–20.0 × ULN), or grade 4 (>20.0 × ULN) alanine aminotransferase elevation, the study drug was to be discontinued, and repeat hepatic panels were obtained weekly until enzymes resolved to normal. Vomiting of the study drug on 2 consecutive days was cause for withdrawal. The primary study objective was to determine the effects of Polyphenon E on tissue markers (e.g., tumor cell c-Met expression and phosphorylation levels). Secondary end points, the focus of this article, included the effects on serum biomarkers VEGF, IGF-I, IGFBP-3, and PSA. Protocol adherence was measured by medication diaries and pill counts. Compliance was defined as having taken 75% or more of the daily medications. The number of subjects was planned to be between 14 and 25. The first phase consisted of a scheduled preliminary analysis done after 14 evaluable subjects had completed the protocol. Several positive responses to treatment were apparent, so the second stage was initiated, with the addition of 11 more subjects for a total of at least 25 evaluable subjects. This design was expected to yield 95% probability of detecting at least one positive response, if

[6] Duhon et al., manuscript in review.

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the probability of a positive response was ≥20% (27). A positive response was defined as a ≥50% change in tissue biomarkers. The planned interim analysis found by immunohistochemical measurement that p-Met and p-AKT tissue levels were <50% of levels in matched controls. The tissue data will be presented in another article (33).

Biomarker assays

Venous blood samples were collected before study and before surgery in plastic vacutainers and kept on ice until processing. The samples were spun at 2,000 rpm at 4°C for 15 min in a swing bucket centrifuge, and the serum layer was removed via plastic transfer pipettes into microcentrifuge tubes. Serum was immediately stored at −80°C and kept frozen until analysis. Debate exists on the most accurate analysis of VEGF levels, serum versus plasma, because VEGF has been shown to be released from platelets on serum preparation (26). Serum was chosen in this study for several reasons, including the observation that platelets may be involved in tumor metastasis and reflect the biology of cancer, and therefore platelet-derived VEGF may reflect the biology of cancer (34). Additionally, in this study, each patient served as their own control and comparisons were not made across treatment groups. Routine clinical chemistry tests and the hepatic panel were done in the Louisiana State University Health Sciences Center (LSUHSC) clinical laboratory, and assays for PSA, IGF-I, and IGFBP-3 were done by a commercial laboratory (DSL Laboratories, Webster, TX) using the highly sensitive immunoradiometric assay. Nonrelated serum samples were used as controls and the coefficients of variation for all assays were <7.0% within each assay and <12.0% between each run. All quality control methods and controls were within specifications for each assay. All pretreatment and posttreatment samples were run in the same batch analysis and all samples were blinded. ELISA assays (R&D Systems) to detect HGF and VEGF were done in a blinded fashion in the investigator's laboratory. Duplicate aliquots of each sample were run and averaged, and concentrations determined based on standard curves. Pretreatment and posttreatment samples from the same patients were assayed in the same batch for ELISA. The intra-assay coefficient of variation for HGF and VEGF was <7.0% and the interassay coefficient of variation for HGF and VEGF was <9.0%.

Western blot analysis and *in vitro* ELISA

The HPS-19B primary prostatic fibroblast cell line was kindly provided by the David Rowley laboratory and maintained in DMEM (Cellgro), 5% Nu-Serum (BD Biosciences), 5% fetal bovine serum (Cellgro), 5 μg/mL insulin (Sigma), 0.5 μg/mL testosterone (Sigma), and penicillin/streptomycin (Cellgro). WPMY-1 cells, a SV40 large-T immortalized stromal cell line, were obtained from American Type Culture Collection and maintained in DMEM (Cellgro), 5% fetal bovine serum, and penicillin/streptomycin. HPS-19B and WPMY-1 cells were treated in serum-free medium for 48 or 24 h, respectively, with or without 5 or 10 μmol/L EGCG. Conditioned media and protein lysates were harvested by adding 125 μL of boiling Laemmli sample buffer (125 mmol/L Tris, 4% SDS, 0.01% bromophenol blue, 30% Sucrose, 5% β-mercaptoethanol) to the cells. Ten microliters of the lysates were run on a 10% acrylamide gel, transferred onto polyvinylidene difluoride, blocked, and probed with antibodies to the proteins listed below. Antibodies used included phospho-extracellular signal–regulated kinase (ERK), phospho-signal transducer and activator of transcription 3 (STAT3), phospho-c-jun NH_2-terminal kinase (Cell Signaling Technology), total ERK (Santa Cruz Biotechnology), and tubulin (Lab Vision). ELISAs were done with undiluted samples according to the manufacturer's recommended protocol.

Reverse transcription-PCR analysis

RNA was isolated from WPMY-1 cells treated in serum-free medium overnight with 5 and 10 μmol/L EGCG with the 5 Prime RNA isolation kit (5 PRIME, Inc.) according to the manufacturer's recommended protocol. Reverse transcription was done using the Superscript RT kit from Invitrogen. Briefly, 2 μg of RNA were reverse transcribed using oligo dT primers. Remaining RNA was removed by the addition of RNase H. Real-time reverse transcription-PCR (RT-PCR) was done on the Bio-Rad iCycler, using gene-specific proprietary primers purchased from Qiagen with SYBR Green. All results were normalized to glyceraldehyde-3-phosphate dehydrogenase. The experiment was done three times and the RT-PCR reaction was done in duplicate. Statistical analysis was done by Student's *t* test.

Luciferase assay

The 181-HGF luciferase construct was made by amplifying the promoter region of the *HGF* gene, using the forward primer 5′-TAGAGCTCTGTTTGGTGCTGTTGAAGG-3′ and the reverse primer 5′-ATAAGCTTGCCTGGGTGAAAGAATCCTG-3′, and subcloned into the *Sac*1-*Hin*dIII site of the pGL3-Enhancer vector (Promega). The

Table 1. Serum markers levels before and after Polyphenon E treatment

Serum biomarker	*n**	Before	After	After–before		*P*†
		Mean (SD)	Mean (SD)	Mean‡ (SD)	Percent change	
HGF (pg/mL)	25	1,009.2 (521.2)	782.9 (373.3)	−226.3 (289.4)	−18.9	<0.001
VEGF (pg/mL)	25	395.2 (287.2)	345.5 (264.4)	−49.7 (104.2)	−9.9	0.032
PSA (ng/mL)	25	11.0 (6.2)	9.3 (5.1)	−1.12 (1.94)	−10.4	0.012
IGF-I (ng/mL)	24	238.0 (107.0)	210.0 (119.9)	−28.0 (57.2)	−11.0	0.024
IGFBP-3 (ng/mL)	24	4,064 (917)	3,773 (1,109)	−291 (606)	−7.9	0.009
IGF-I/IGFBP-3	24	0.0595 (0.024)	0.0522 (0.026)	−0.0029 (0.0123)	−3.4	0.028

*Assay problems on one or more samples reduced the sample size from 26.
†Wilcoxon signed rank test comparing before and after values.
‡Mean of differences does not always equal difference of the means.

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luciferase assay was done by transfecting 2 μg of 181-HGF and 0.1 μg Renilla luciferase per well of a 24-well plate into 75% confluent WPMY-1 cells with ExGen 500 (Fermentas, Inc.) according to the manufacturer's recommended protocol. Fresh medium was added 8 h later. The following day, the cells were treated with or without 5 or 10 μmol/L EGCG in serum-free medium for 6 or 24 h. Lysates were harvested and read on a luminometer with the Dual-luciferase reporter assay kit (Promega) according to the manufacturer's recommended protocol.

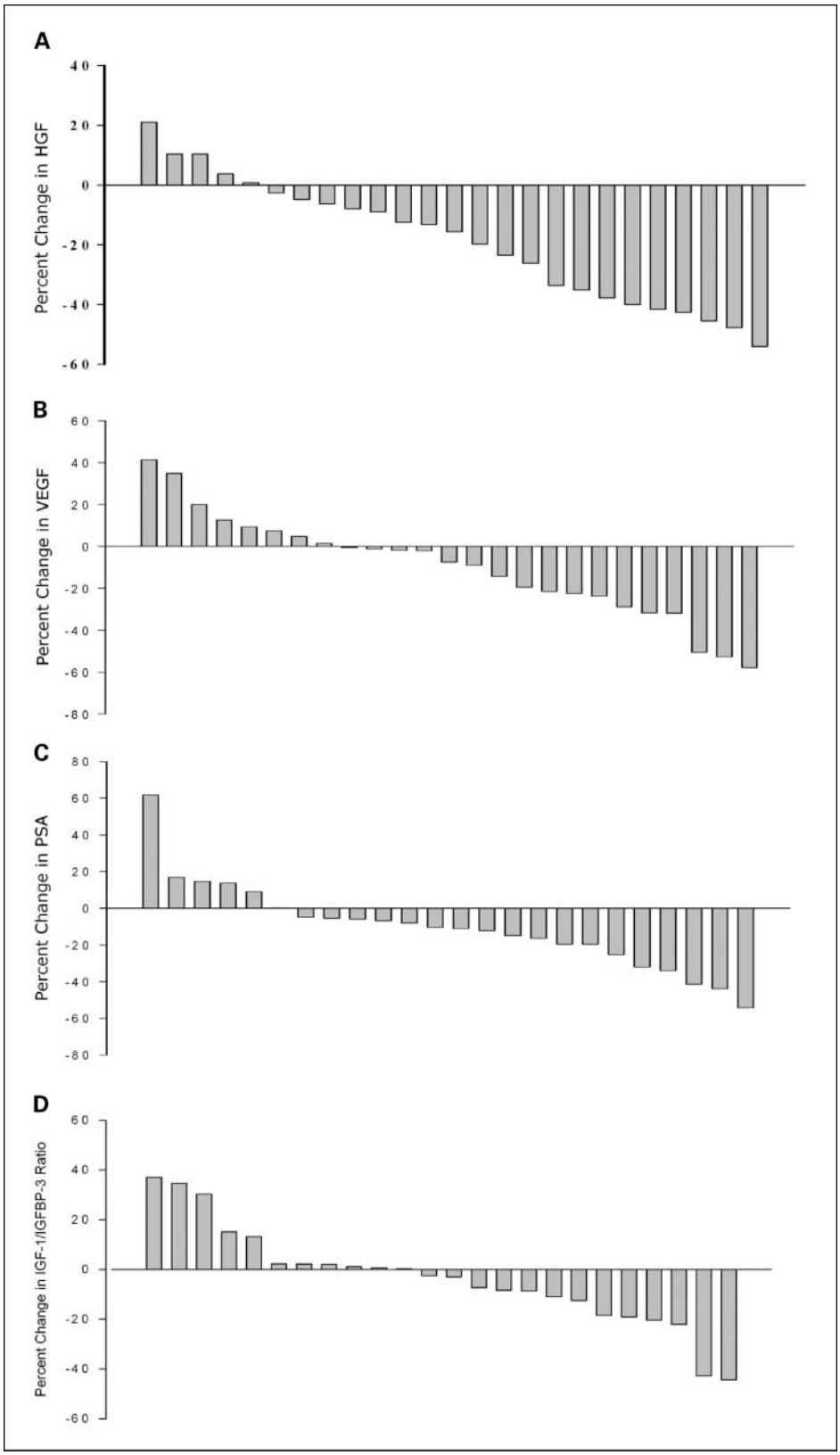

Fig. 1. Polyphenon E decreases levels of HGF, VEGF, PSA, and the IGF-I/IGFBP-3 ratio in men with prostate cancer. Men self-administered 800 mg EGCG daily over a median period of 34.5 d. Serum was taken before treatment and after treatment and analyzed for serum HGF and VEGF levels by ELISA or for serum PSA, IGF-I, and IGFBP-3 by a commercial laboratory. The percent change was determined by comparing posttreatment levels with pretreatment levels. Data were sorted numerically and percent change was plotted. Each column represents an individual patient.

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Table 2. Liver function tests before and after Polyphenon E treatment

Serum biomarker	*n*	Before	After	After–before*		*P*†
		Mean (SD)	Mean (SD)	Mean (SD)	Percent change	
Total protein (g/dL)	26	7.48 (0.51)	6.66 (0.96)	−0.82 (0.98)	−10.7	<0.001
Albumin (g/dL)	26	4.17 (0.29)	3.68 (.078)	−0.51 (0.81)	−11.9	0.002
Bilirubin (mg/dL)	26	0.64 (0.29)	0.62 (0.27)	−0.13 (0.26)	5.9	0.74
Conjugated bilirubin (mL/dL)	10‡	0.20 (0.11)	0.21 (0.12)	0.0 (0.01)	8.9	1.00
Alanine aminotransferase (units/L)	26	27.0 (16.0)	26.2 (14.3)	−1.88 (7.60)	−0.7	0.19
Aspartate aminotransferase (units/L)	26	28.8 (26.1)	25.5 (20.5)	−4.13 (8.75)	−7.6	0.021
Alkaline phosphatase (units/L)	26	77.1 (20.2)	67.5 (18.8)	−10.25 (9.89)	−13.0	<0.001
γ-Glutamyl transpeptidase (units/L)	11‡	56.6 (26.6)	58.8 (43.9)	−4.18 (8.28)	−6.2	0.17
Amylase (units/L)	11‡	105.5 (68.0)	71.4 (45.5)	−21.2 (30.4)	−14.0	0.028
Lipase (units/L)	10‡	30.8 (16.5)	24.2 (13.6)	−8.5 (13.4)	−16.8	0.097

*Mean of differences does not always equal differences of the means.
†Wilcoxon signed rank test comparing paired before and after values.
‡These tests were added after the study had started; data were available for only 11 patients.

Ethical and regulatory considerations

The research was approved by the LSUHSC Institutional Review Board and all Health Insurance Portability and Accountability Act guidelines were followed. An approved Investigational New Drug was obtained before study initiation. All subjects signed informed consent documents.

Statistical methods

Due to the non-Gaussian distribution of the data and the relatively small sample size, nonparametric statistical methods were used. Before and after values for each end point were compared using the Wilcoxon signed rank test; Spearman's nonparametric correlation coefficients were used to compare the degrees of change between end points. Multivariate linear regression was done to test the effect of age, time on drug, and race on the degree of change in the end points. Univariate tests included Mann-Whitney test for racial differences in pretreatment measures and degree of change. Analysis was done using SPSS 16.0 for Windows (SPSS, Inc.).

Results

Polyphenon E administration lowers serum levels of HGF, VEGF, IGF-BP3, IGF-I, and PSA in men with prostate cancer

The phase II clinical trial to assess the effects of Polyphenon E on serum levels of cancer biomarkers enrolled 33 men. However, two were later discovered to be ineligible; one withdrew early; one refused to take any of the study medication; two did not go to surgery for medical reasons; and no presurgical serum was available for one man. Of the 26 participants, ages ranged from 41 to 68 years with a mean of 58.5 years; 62% of the enrolled patients were African American. The time on the study ranged from 12 to 214 days with a median of 34.5 days. Four patients were on the study between 56 and 73 days; one unusual case was on treatment 214 days. Nine patients were on the study between 33 and 53 days, and 12 were on the study between 12 and 32 days. No obvious differences in magnitude of response by time on drug were noted. However, a trend existed in that the percentage of patients with decreasing PSA increased with more time on drug; 85% of the patients above the median time on drug had decreasing PSA levels, versus 64% for those below the median time on drug. However, due to the small sample size, this trend was not significant ($P = 0.35$). The patient who was on the study for 214 days had numerous delays in his prostatectomy date and his physician chose to keep him on the study because no adverse effects were observed. Only one patient reported mild nausea from taking the study drug, before being instructed to take the medicine with a meal rather than before a meal.

Laboratory analysis of the serum markers, HGF, VEGF, PSA, IGF-I, and IGFBP-3, was done as described in Materials and Methods, and changes in serum biomarkers are shown in Table 1. A significant decrease was observed in serum levels of HGF, VEGF, PSA, IGF-I, and IGFBP-3 (all $P < 0.03$, Wilcoxon signed rank test). The IGF-I/IGFBP-3 ratio also changed significantly. Figure 1 shows these changes for HGF, VEGF, PSA, and the IGF-I/IGFBP-3 ratio across the spectrum of patients. Ten of 25 patients had a ≥25% decrease in their HGF levels, whereas 6 of 25 patients had a ≥25% decrease in their VEGF levels. Age, race, and time on drug did not have a significant effect on the changes in serum biomarkers. In general, patients who changed in one marker also changed in one or more other markers in the same direction. For example, of 18 patients whose PSA levels decreased, 14 of them also experienced a decrease in HGF, 12 in VEGF, 12 in IGF-I, and 17 in IGFBP-3. However, although this suggests a trend, the correlation in the magnitude of the change was not significant, most likely due to the small number of patients enrolled for this trial. Additionally, 10 of 26 patients had a ≥10% decrease in both VEGF and HGF (Spearman's ρ correlation coefficient = 0.711, $P < 0.001$ two-tailed).

Polyphenon E shows no adverse effects on liver function

Isolated case studies have suggested that high doses of EGCG may have adverse effects on liver function. Therefore,

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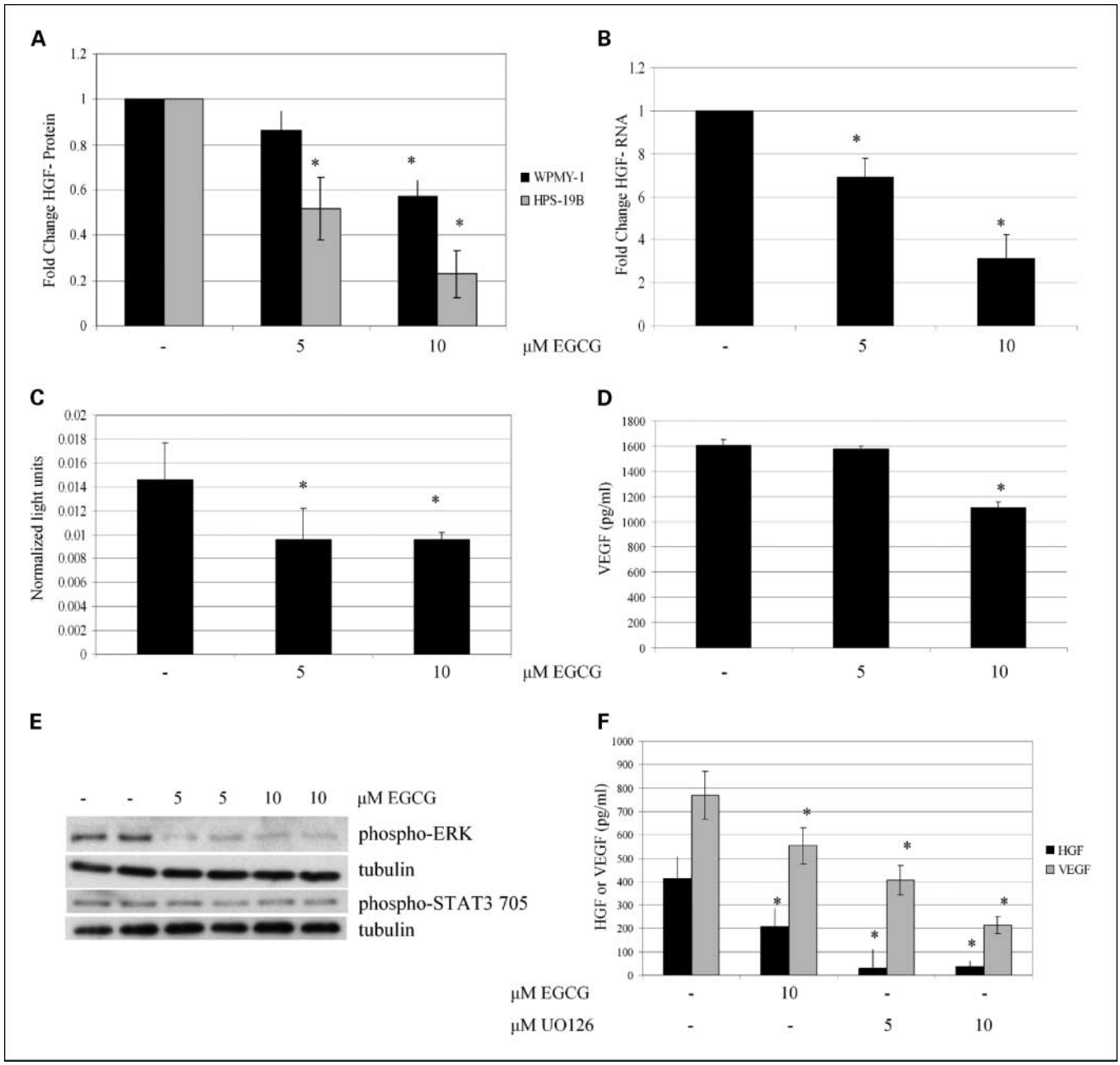

Fig. 2. EGCG decreases levels of HGF and VEGF in cancer-associated fibroblasts. *A*, WPMY-1 and HPS-19B cancer-associated fibroblasts were treated for 24 or 48 h, respectively, with 5 or 10 μmol/L EGCG. Conditioned medium was harvested and assessed by ELISA for secreted HGF. Fold change was determined relative to control. *B*, RNA was isolated from control and 5 or 10 μmol/L EGCG–treated WPMY-1 fibroblasts. Real-time RT-PCR analysis was done for HGF. All values were normalized against glyceraldehyde-3-phosphate dehydrogenase and fold change was determined relative to control. Results are averages of three independent experiments. *C*, HGF luciferase assays were done by transfecting the 181-bp HGF promoter construct and Renilla-luciferase into WPMY-1 fibroblasts. The following day, the cells were treated with or without 5 or 10 μmol/L EGCG for 24 h in serum-free medium. Lysates were read on a luminometer and normalized to Renilla luciferase. *D*, 24-h conditioned media from WPMY-1 fibroblasts treated with or without 5 or 10 μmol/L EGCG were analyzed for VEGF by ELISA. *E*, WPMY-1 fibroblasts were treated with or without 5 or 10 μmol/L EGCG in serum-free medium for 6 h. Lysates were harvested and run by Western blot analysis for phospho-p42/44 ERK, phospho-STAT3, and tubulin. *F*, WPMY-1 cells were treated with or without 10 μmol/L EGCG or 5 or 10 μmol/L UO126 for 24 h in serum-free medium. Conditioned medium was assessed for secreted HGF and VEGF levels by ELISA. *, statistically significant differences compared with control samples. All statistical analyses were done by Student's *t* test.

we assessed potential effects on liver enzymatic function by analyzing total protein, albumin, bilirubin, conjugated bilirubin, alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, γ-glutamyl transpeptidase, amylase, and lipase. Changes in hepatic enzymes are shown in Table 2. Interestingly, all enzymes but conjugated bilirubin decreased during the study, five of them significantly: total protein, albumin, aspartate aminotransferase, alkaline phosphatase, and amylase ($P < 0.05$, Wilcoxon signed rank test). All values were within normal limits throughout the study. Except for total protein and albumin, all changes were in a favorable direction, suggesting that in our study, Polyphenon E administration does not have adverse effects on liver function. Additionally, we observed no significant changes in body mass index ($P = 0.80$).

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EGCG inhibits production of HGF and VEGF in prostate cancer–associated fibroblasts

High levels of HGF are associated with metastatic disease and decreased survival in prostate cancer (16–18). Our results suggesting that Polyphenon E could decrease levels of HGF in prostate cancer patients prompted us to assess the effect of EGCG on HGF expression *in vitro*. HGF is normally secreted from the surrounding stromal fibroblasts, where it activates c-Met in tumor cells. We treated the primary cancer-associated prostate fibroblast cell line HPS-19B and the immortalized prostate fibroblast line WPMY-1 under serum-free conditions with 5 and 10 μmol/L EGCG for 48 and 24 hours, respectively. Conditioned medium was harvested and ELISA was done. Results in Fig. 2A show that EGCG inhibits HGF secretion in prostate fibroblast cells, thereby supporting the results that we have observed in our phase II study. Intracellular and extracellular ELISAs were done to determine if EGCG treatment affects total levels of HGF. Results show that EGCG decreased both the intracellular and extracellular levels of HGF as early as 6 hours, suggesting that EGCG is not just affecting secretion (data not shown).

Real-time RT-PCR analysis was done with WPMY-1 fibroblasts treated for 24 hours with and without 5 and 10 μmol/L EGCG in serum-free medium. Results from three independent experiments show that EGCG significantly decreased mRNA levels of HGF in a dose-dependent manner, suggesting that EGCG is affecting either RNA transcription or degradation (Fig. 2B). To determine if EGCG blocks HGF transcription, luciferase assays were done with the 181-bp upstream region of the HGF gene fused to the luciferase gene. EGCG treatment significantly decreased luciferase activity at 24 hours by ~35% (Fig. 2C).

EGCG has been shown to inhibit VEGF production in numerous model systems both *in vitro* and *in vivo* (35–39). The EGCG-mediated decrease in VEGF is suggested to be at the level of transcription through inhibition of STAT3 and/or activator protein-1 (35, 36, 38). We also assessed if EGCG treatment could decrease VEGF levels in prostate-associated fibroblasts. WPMY-1 cells were treated for 24 hours and conditioned medium was assessed for secreted VEGF by ELISA. Similar to HGF, EGCG significantly decreased VEGF levels (Fig. 2D).

Few studies have assessed the signaling pathways affecting transcription of the human HGF gene. STAT3 has been shown to regulate HGF levels through Src, and the mitogen-activated protein kinase cascade has been implicated in the transcriptional regulation of the human HGF gene (40–42). To assess which signaling pathways were targeted by EGCG thereby decreasing HGF levels in human fibroblasts, WPMY-1 (Fig. 2E) and HPS-19B (data not shown) cells were treated with and without 5 or 10 μmol/L EGCG for 6 hours, and protein lysates run by Western blot analysis. Figure 2E shows that EGCG had no affect on the phosphorylated, active forms of STAT3 or c-Jun NH_2-terminal kinase (data not shown) in WPMY-1 cells. EGCG treatment did inhibit phosphorylation of ERK p42/p44 (Fig. 2E). To confirm that ERK p42/44 plays a role in HGF production, WPMY-1 fibroblasts were treated with or without 10 μmol/L EGCG or 5 or 10 μmol/L of the p42/44 ERK inhibitor UO126 for 24 hours. ELISA analysis of WPMY-1 conditioned medium shows that ERK inhibition decreases secreted levels of HGF and VEGF, similar to that with EGCG treatment. These observations suggest that EGCG may block HGF production through inhibition of ERK signaling (Fig. 2F).

Discussion

We report here that men diagnosed with prostate cancer who take 1.3 g daily of green tea catechins (800 mg EGCG) show a significant reduction in serum HGF, VEGF, IGF-I, IGFBP-3, and PSA with a median dosing period of 34.5 days. Liver function tests revealed no abnormalities, suggesting that this dose of tea polyphenols is safe when administered for a few months. The data are consistent with a growing body of work suggesting a protective effect of catechins against liver toxicity in cell culture and mice (43–46). In addition, *in vitro* experiments show that EGCG blocks the production of VEGF and HGF in at least two different prostate cancer–associated fibroblast cell lines. For HGF, inhibition by EGCG seems to be at the level of transcription, most likely via inhibition of the p42/44 mitogen-activated protein kinase pathway. These data suggest that green tea polyphenols could be useful as adjuvant therapy in men with prostate cancer by lowering the levels of cytokines that contribute to prostate cancer progression.

Over the past few years, the trend in clinical research trials has been toward development of biomarker end points to determine the efficacy of a new drug (47). This is based, in part, on that fact that the typical accepted end point of overall survival requires large and costly trials, thus slowing the development of potentially useful therapeutic agents. Changes that are observed in the surrogate end point should reflect changes in a more relevant clinical parameter such as overall survival.

HGF shows a variety of activities *in vitro* and in animal models that are consistent with its ability to promote prostate cancer progression, including stimulation of cell motility and invasion, increased survival of tumor cells, and angiogenesis (17, 48, 49). In addition, it has been reported that levels of HGF in the serum correlate with stage of prostate cancer and, in some cancers, may actually predict overall survival (17, 18, 50). Furthermore, levels of c-Met, the receptor for HGF, predict overall survival in many cancers; thus, inhibiting the HGF/c-Met axis is predicted to increase life expectancy (15, 16, 51). Therefore, levels of HGF could have predictive value with regard to metastatic disease, and agents such as Polyphenon E that can lower serum levels of HGF might be therapeutically useful at slowing the progression of tumors dependent on HGF activity. Although the reductions in serum levels of HGF that we observed were not robust, it is quite possible that even relatively small reductions could have a large biological effect in terms of prostate cancer progression.

Lowering biological levels of VEGF in patients with advanced cancer is predicted to increase their overall survival time. Therefore, it is reasonable to propose that reductions in the levels of this cytokine could slow the progression of prostate cancer. Thus, the reduction in serum levels of VEGF in men consuming Polyphenon E seems promising, and additional longer-term studies will be needed to determine if lowering VEGF and HGF serum levels actually translates into a more favorable clinical outcome.

Polyphenon E also lowered serum levels of PSA, although the change was modest. This result, although promising,

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needs to be interpreted with caution. Changes in a number of physiologic parameters can alter the serum levels of PSA, and many of these are not related to cancer progression (52). Furthermore, levels of PSA may not accurately reflect tumor size or changes in cancer cell number.

We also observed a significant reduction in the levels of IGF-I and IGFBP-3 in men taking Polyphenon E. Levels of IGF and IGFBP-3 have been studied for a number of years and the results are still controversial about their roles in prostate cancer development or progression (53–55). A recent study concluded that there was no association between levels of these two proteins and prostate cancer, although interestingly, the authors noted that prediagnostic levels of IGFBP-3 correlated with prostate cancer risk, but only for Black men (56).

Our results are also consistent with the published trial from Bettuzzi and colleagues (3). In their trial, men with high-grade prostate intraepithelial neoplasia were given either extracts of green tea or a placebo for 1 year (3). One third of the men in the control group developed prostate cancer within the year, whereas only 3% of the men in the treated group developed cancer. This trend has continued well into the second year (4). In addition, although not significantly different, the trend for the treated group was lower levels of PSA as compared with the placebo group. Other biomarkers in the serum were not analyzed in this study. The HGF/c-Met axis is proposed to already be activated in men with high-grade prostate intraepithelial neoplasia (57), and thus the reduction in progression to cancer with green tea polyphenols might, in part, be due to the inhibition of this signaling pathway.

Few published trials have assessed the effects of green tea catechins on prostate cancer biomarkers and progression. A study from Jatoi et al. (58) used a dosing schedule of 6 g of tea per day in water in six divided doses. Only 1 of 42 patients showed a >50% reduction in PSA, and many of the patients complained of tea toxicity. They concluded that green tea seemed to be of limited value in the treatment of androgen-independent prostate cancer and, at the dosages used, seemed to have side effects. Our study using higher doses resulted in few side effects, presumably because Polyphenon E is formulated to have low caffeine levels. In addition, none of our patients had advanced prostate cancer, and thus the fact that green tea at lower doses than what our patients received had no effect on advance prostate cancer is not surprising. A second study done in Canada accrued patients with androgen-independent prostate cancer, and these patients were given 250 mg of green tea extracts twice a day (59). The primary end point of the trial was PSA or measurable disease progression. They concluded that green tea had minimal clinical activity against hormone-refractory prostate cancer. The authors hypothesized that serum levels of EGCG probably did not exceed 0.2 μmol/L and this could be one of the reasons tea polyphenols were ineffective. Our trial is based on a previous phase I dosing trial that showed that concentrations exceeding 1 μmol/L could be achieved with oral administration of Polyphenon E, with maximal achievable plasma levels at ~4 hours after ingestion (60). Therefore, the positive effects green tea had on our biomarkers could be related to the higher serum levels of EGCG.

Over the last 10 years, there has been an increasing awareness that the tumor stroma plays a very important role in cancer progression (61). A number of cells associated with the stroma, including macrophages, endothelial cells, and fibroblasts, provide factors that facilitate proliferation, survival, and invasion of tumor cells. The Rowley laboratory has studied the importance of reactive stroma in prostate cancer progression and has characterized cancer-associated fibroblasts that seem to play a critical role in tumor-stroma interaction (62). A better understanding of the functions of stroma cells in cancer progression may lead to therapeutic approaches to target tumor and stromal cells.

HGF is primarily expressed and secreted by stromal fibroblasts, although autocrine production can occur in late-stage prostate cancer (63). Therefore, a reduction in serum HGF in patients treated with Polyphenon E prompted us to consider the effect of tea polyphenols on cancer-associated fibroblasts. Our results clearly show that EGCG, the main catechin in Polyphenon E, rapidly blocked the production of HGF at physiologically relevant concentrations. The block seems to be at the level of transcription and may involve inactivation of the p42/44 mitogen-activated protein kinase pathway. HGF transcription has been shown to be regulated by STAT and Src (40, 41), but EGCG had no apparent effect on these pathways in the fibroblasts we examined. In addition, we observed that EGCG blocked the production of VEGF in cancer-associated fibroblasts; this cytokine plays a critical role in the angiogenic process. Our data in prostate fibroblasts agree with several publications showing that EGCG can reduce VEGF levels at the transcriptional level in cell culture as well as mouse models (35–39).

These combined results have potential significance because they show that green tea polyphenols can target both c-Met in tumor cells and the production of HGF and VEGF in stromal fibroblasts. We propose that this will be a more potent approach therapeutically than targeting only one cell type. Current studies using immunohistochemical approaches are evaluating the reactive stroma resected in tissue samples from patients that consumed Polyphenon E in our trial.

The Bettuzzi et al. study (3) suggests that tea polyphenols will be most effective when used to delay progression of dysplasias to cancer, or to slow progression of small tumors *in situ* or growing at metastatic sites. Tea polyphenols may be even more effective in combination with other natural products or with targeted agents. For instance, a recent phase II clinical trial showed that consumption of pomegranate extract significantly slowed the increase rate of PSA in prostate cancer patients (64). The main polyphenol in the serum after ingestion of pomegranate extract is ellagic acid, and we have determined that ellagic acid is 10 times more effective than EGCG in blocking activation of c-Met by a distinct mechanism.[7] It would be interesting to analyze the effectiveness of the two groups of polyphenols in preclinical and clinical trials. Others have recently determined that combinations of certain polyphenols such as EGCG with Tarceva, an epidermal growth factor receptor inhibitor, are more effective in preclinical cancer models than either agent alone (65). Tarceva is currently used therapeutically to treat lung cancer and head and neck cancer, and acquired resistance or chronic resistance to this targeted agent has limited the success of Tarceva. Based on these studies, we have initiated a phase I/II trial examining the effectiveness of Polyphenon E plus Tarceva in the treatment of lung cancer patients.

[7] Duhon et al., in preparation.

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In conclusion, our results show a significant reduction in serum levels of PSA, HGF, IGF-I, IGFBP-3, and VEGF in men with prostate cancer after brief treatment with EGCG (Polyphenon E), with no elevation of liver enzymes. The decrease in serum HGF and VEGF by EGCG was supported by *in vitro* studies revealing that EGCG blocks production of HGF and VEGF in cancer-associated fibroblasts. These data support a potential role for Polyphenon E in the treatment or prevention of prostate cancer and suggest that these findings should be verified by larger, placebo-controlled clinical trials. The effects of different doses, long-term administration, and combination with other drugs remain to be seen.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Cancer Prevention Research

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Jerry McLarty, Rebecca L.H. Bigelow, Mylinh Smith, et al.

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