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Resveratrol Worsens Survival in SCID Mice With Prostate Cancer Xenografts in a Cell-Line Specific Manner, Through Paradoxical Effects on Oncogenic Pathways

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Abstract

BACKGROUND—Resveratrol increases lifespan and decreases the risk of many cancers. We hypothesized resveratrol will slow the growth of human prostate cancer xenografts.

METHODS—SCID mice were fed Western diet (40% fat, 44% carbohydrate, 16% protein by kcal). One week later, human prostate cancer cells, either LAPC-4 (151 mice) or LNCaP (94 mice) were injected subcutaneously. Three weeks after injection, LAPC-4 mice were randomized to Western diet (control group), Western diet plus resveratrol 50 mg/kg/day, or Western diet plus resveratrol 100 mg/kg/day. The LNCaP mice were randomized to Western diet or Western diet plus resveratrol 50 mg/kg/day. Mice were sacrificed when tumors reached 1,000 mm³. Survival differences among groups were assessed using Cox proportional hazards. Serum insulin and IGF axis were assessed using ELISAs. Gene expression was analyzed using Affymetrix gene arrays.

RESULTS—Compared to control in the LAPC-4 study, resveratrol was associated with decreased survival (50 mg/kg/day—HR 1.53, $P = 0.04$; 100 mg/kg/day—HR 1.22, $P = 0.32$). In the LNCaP study, resveratrol did not change survival (HR 0.77, $P = 0.22$). In combined analysis of both resveratrol 50 mg/kg/day groups, IGF-1 was decreased ($P = 0.05$) and IGFBP-2 was increased ($P = 0.01$). Resveratrol induced different patterns of gene expression changes in each xenograft model, with upregulation of oncogenic pathways E2F3 and beta-catenin in LAPC-4 tumors.

CONCLUSION—Resveratrol was associated with significantly worse survival with LAPC-4 tumors, but unchanged survival with LNCaP. Based on these preliminary data that resveratrol may be harmful, caution should be advised in using resveratrol for patients until further studies can be conducted.

Keywords

resveratrol; prostate cancer; xenograft; IGF-1; E2F3; beta-catenin

INTRODUCTION

Prostate cancer is the most common cancer in men in America, with an estimated 217,730 new cases and 32,050 deaths in 2010 [1]. The majority of men with prostate cancer take supplements or some form of natural therapy either alone or in combination with conventional medical treatments [2]. One of these “natural” therapies which has gained in popularity in the past few years, is resveratrol, a phytoalexin naturally found in red wine. Resveratrol, with its anti-oxidant effect, was originally described as a possible explanation for the “French Paradox” in which Frenchmen consuming high amounts of saturated fat seemed to be protected from coronary heart disease by the red wine they drank [3].

Both in vitro and in vivo studies have shown beneficial effects of resveratrol on aging, immune regulation, inflammation, vascular endothelial dysfunction, diabetes, metabolic syndrome, and many cancers [4]. Resveratrol has shown a beneficial effect in a limited number of in vivo studies for the treatment of prostate cancer [5,6]. All human clinical trials reported to date using pure resveratrol (not in wine or grapes) have investigated bioavailability or pharmacokinetics and not cancer prevention or treatment [7].

Obesity increases overall mortality and cancer-specific mortality in some populations [8], while caloric restriction increases lifespan and inhibits cancer growth [9,10]. In prostate cancer specifically, weight loss reduces the risk of non-metastatic high-grade disease [11]. While these and other benefits of caloric restriction make it an attractive treatment strategy in humans, long-term adherence is poor. A more achievable method to attain these same benefits is supplementation with resveratrol, since resveratrol has been shown to counteract the detrimental effects of a Western diet (high fat, high carbohydrate) in non-cancer animal models [12]. Caloric restriction and resveratrol induce common molecular mechanisms important in prostate cancer, including reduction of serum insulin and IGF-1, and increased SIRT1 [7,9,13–17]. These observations suggested that resveratrol would exert the same effect on prostate cancer growth that caloric restriction exerts. Specifically, we hypothesized that resveratrol would inhibit the growth of prostate cancer, and we sought to test this using LAPC-4 and LNCaP xenografts.

MATERIALS AND METHODS

Cell Culture

The human CaP cell line, LAPC-4, was obtained from Dr. William Aronson (UCLA, Los Angeles, CA). LAPC-4 was maintained in Isocove’s Modified Dulbecco’s Medium (Gibco, Invitrogen Corp., Carlsbad, CA) with 10% fetal bovine serum, glutamine, 1 nm R1881 (Perkin Elmer, Waltham, MA) and antibiotics (50 IU/ml penicillin; 50 mg/ml streptomycin). The other human CaP cell line, LNCaP, was obtained from American Type Culture Collection. LNCaP cells were grown in modified RPMI 1640 with 10% fetal bovine serum. Cells were incubated at 37°C in 5% CO₂ and harvested by trypsinization in log phase growth at 70–80% confluence on the same day as tumor injection.

Experimental Compound and Diet Composition

Resveratrol, extracted from the bushy knotweed at >99% purity as determined by both thin layer and gas chromatography was obtained from Sigma–Aldrich (St. Louis, MO). All diets were prepared by Test Diet (Richmond, IN). Western diet (WD; 40% fat, 44% carbohydrate, 16% protein by kcal) was made as previously described [18,19]. The investigational diets were prepared by geometrically mixing powdered resveratrol into sucrose with a mortar and pestle to assure even distribution, then blending this mixture with the remaining dietary components. Four different investigational diets were designed to test a dose response to resveratrol. Based on the average daily feed consumption per mouse during previous studies in our laboratory using the identical WD [18,19], we calculated the concentration of resveratrol in feed that would give each mouse approximately 10, 20, 50, or 100 mg of resveratrol per kilogram of mouse weight per day. Mice were fed the pelleted WD or experimental diet ad libitum orally.

Animal Studies

After obtaining approval from the Duke University Institutional Animal Care and Use Committee, an exploratory study was performed, followed by two separate primary studies. A total of 397 male Severe Combined Immunodeficient (SCID) mice, strain CB17SC, aged 7 weeks were obtained from Taconic (Hudson, NY). Mice were housed 5/cage and fed an ad libitum WD without resveratrol until randomization. Two weeks thereafter, cells in 0.1 ml Matrigel (BD Biosciences, San Jose, CA) were injected subcutaneously into the flank of each mouse. The exploratory study was performed injecting 4×10^5 LAPC-4 cells in 146 mice using resveratrol doses similar to those reported previously (10 mg/kg/day and 20 mg/kg/day resveratrol) [12]. When the highest dose in this exploratory study demonstrated a trend toward increased survival (HR 0.81, $P = 0.30$, 95% CI 0.54–1.21, Supplementary Fig. 1), we surmised this dose was too low, especially considering the known poor bioavailability of resveratrol [20]. We then designed two primary studies using higher resveratrol doses. One study used 4×10^5 LAPC-4 cells while the other study used 1×10^5 LNCaP cells injected per mouse. Tumors were measured twice weekly. Three weeks after injection, mice were randomized by a computer algorithm to make tumor size and body weight the same in each experimental group and allowed to eat only the experimental diet. Mice were sacrificed when tumors reached 1,000 mm³ by caliper measurement.

In the exploratory study, the experimental groups were fed WD with no resveratrol (control, $n = 49$), 10 mg/kg/day resveratrol (RV10, $n = 49$), or 20 mg/kg/day resveratrol (RV20, $n = 48$). In the primary LAPC-4 study, they were fed no resveratrol (control, $n = 50$), 50 mg/kg/day resveratrol (RV50, $n = 50$), or 100 mg/kg/day resveratrol (RV100, $n = 51$). In the LNCaP study, the groups were fed no resveratrol (control, $n = 49$) or 50 mg/kg/day resveratrol (RV50, $n = 50$). In this final group, five mice died from equipment malfunction (cage flooding) and were excluded from analysis (final $n = 45$). A RV100 group was not included in the LNCaP study because the prior LAPC-4 study demonstrated that RV50 had a stronger effect on modulating tumor growth than RV100, and the RV50 was thought to be easier to achieve in future human studies, should they have been justified by these preclinical findings.

Serum and Tissue Analysis

At sacrifice, fasting blood (minimum 4 hr of fasting) was obtained via cardiac puncture. Fasting blood glucose at sacrifice was determined using a standard handheld glucometer. After centrifugation, serum was stored at -80°C until analyzed. Serum from the five median surviving mice per group was assayed for murine IGF-1 and the IGF-binding proteins (IGFBPs)-1, -2, and -3 using mouse-specific in-house enzyme-linked immunoassays (ELISA) as described previously [21,22]. The IGF-I assay has a sensitivity of 0.1 ng/ml and

no cross reactivity with IGF-II. The intra- and inter-assay coefficients of variations were <10% in the 1–10 ng/ml range. The mouse IGFBP-3 assay has a sensitivity of 0.2 ng/ml. The intra- and inter-assay coefficient of variations were <6% and <8%, respectively, in the range from 1 to 6 ng/ml.

Liver, kidneys, and testicles were weighed as a crude measurement of toxicity. Frozen tumors were sectioned and stained for CD31 with immunofluorescence using standard protocols [23,24]. The primary antibody was rat anti-mouse CD31 (BD Biosciences). The secondary antibody was donkey-anti-rat IgG, Alexa 488 (Invitrogen, Carlsbad, CA). Slides were immediately photographed and images analyzed using ImageJ computer software (NIH, Bethesda, MD [25]). The number of fluorescent pixels in the area of interest was divided by the total number of pixels in that area to calculate the relative amount of CD31, and therefore percent vascular volume.

Gene Arrays

Total RNA was extracted from the snap frozen tumors of mice in the control and RV50 groups of both the LAPC4 and LNCaP studies using the mir-Vana miRNA Isolation Kit (Ambion, Austin, TX). The tissue was ground in a denaturing lysis buffer and extracted with acid-phenol:chloroform. RNA was purified on glass-fiber filters. The quality of the resulting RNA was checked by 260/280 nm absorbance ratio and by electrophoresis on an Agilent bioanalyzer. The RNA integrity number was >7.0 for all samples. Total RNA was processed and prepared for the GeneChip using the manufacturer's protocols (Affymetrix, Santa Clara, CA). In brief, the first strand of cDNA was synthesized from 2 µg of total RNA using a T7-Oligo(dT) Primer and SuperScript II reverse transcriptase. T4 DNA polymerase was used to make the second strand of cDNA. Biotinylated cRNA was generated with the IVT Labeling Enzyme Mix. After cleaning, the cRNA was fragmented for 35 min at 94°C and applied to Affymetrix U133A GeneChip arrays. After processing, arrays were scanned with the GeneChip® Scanner 3000. Affymetrix U133A GeneChip arrays contain > 22,000 probe sets and 14,500 human genes.

Gene Expression Analysis

Array quality control data were examined, and arrays with a housekeeping GAPDH 3'/5' ratio >3 were excluded. For arrays passing quality control, RMA (Robust Multichip Average) expression values [26,27] were computed from Affymetrix microarray data using the Bioconductor [28] Affy software package.

GSEA v2.07 (<http://www.broad.mit.edu/gsea>) was performed based on prespecified treatment phenotype and previously published methods [29]. LAPC4 and LNCaP data were analyzed both separately and together. As the LAPC4 and LNCaP xenografts were processed for microarray analysis at different times, we used the normalizing algorithm ComBat [30] to adjust for batch effects prior to combining data. Predefined gene sets from MSigDB v. 3.0 were preprocessed to exclude sets with <10 and >500 genes and 1,000 iterations were performed per analysis with a signal to noise metric used to rank genes based upon their differential expression across groups. Gene sets with a nominal *P*-value <0.05 were considered statistically significant.

Pathway analysis was applied using six oncogenic pathway expression signatures (Ras, Src, β-catenin, Myc, E2F3, and PI3 kinase) [31] as well as an androgen receptor (AR) activity signature [32]. Only the 22,215 gene probes present on the Affymetrix U133A array were used in the application of these signatures. The 10% of probes with the lowest standard deviation in the training set of each signature were excluded, and binary regression (BinReg) analysis was applied to the remaining 90% of probes. The parameters for this analysis are as

follows: (1) samples were quantile-normalized based on the collection of probe-specific medians in the training data, (2) probes were shift/scale normalized such that the mean and variance of each probe were the same in the training and validation sets to correct for batch effects, (3) singular value decomposition factors were built upon the training data only, (4) 1,000 burn-ins and 5,000 iterations were used for the application of the Markov chain Monte Carlo algorithms, and (5) 95% confidence interval. The number of genes and metagenes used for building the binary regression models, based on above-cited work, are included as Supplementary Table II.

Statistical Analysis

Body weights, organ weights, IGF axis hormones, CD31, glucose, and insulin were compared across groups using the Kruskal–Wallis test. Two-way comparisons were performed using the rank-sum test. Time from randomization to sacrifice was compared among groups using the log-rank test. The hazard ratios for death versus the control group were assessed using a Cox proportional hazards model. For endpoints measured in only 5 or 10 mice because of expense (such as gene arrays) the subset was chosen from mice with survival closest to the median survival of the group in order to make them as representative as possible. All statistical analyses were performed using Stata 10.0 (StataCorp. College Station, TX). $P < 0.05$ was considered statistically significant and all tests were two-sided.

RESULTS

Metabolic Endpoints

All mice ate their assigned diets without apparent toxicity. Median body weights were similar across groups in the LNCaP study (Supplementary Table I). In the LAPC-4 study, the RV50 group was significantly heavier than the other groups by approximately 1 g (approximately 4% of body weight; $P = 0.02$). All organ weights in all groups were similar in both studies with the singular exception of heavier kidneys in the RV50 group of the LAPC-4 study (Table I). Blood glucose was also similar among all groups in all studies (all $P > 0.7$).

Survival

In the LAPC-4 study, RV50 significantly decreased survival (HR 1.53, $P = 0.04$) while RV100 did not significantly change survival (HR 1.22, $P = 0.32$, Fig. 1 and Table I). In the LNCaP study, there were no significant differences between the control group and RV50 (HR 0.77, $P = 0.22$, Fig. 2 and Table I).

IGF Axis

Insulin was significantly lower in the resveratrol treated groups of the LAPC-4 study, but was unrelated to resveratrol in the LNCaP study (Table II). IGF axis hormones were analyzed for each study separately then reanalyzed after combining the data from both studies (Table II). Resveratrol significantly decreased IGF-1 in the LAPC-4 study ($P = 0.01$) and the combined analysis ($P = 0.05$). Resveratrol significantly increased IGFBP-2 in the LNCaP study ($P = 0.04$) and in the combined analysis ($P = 0.01$) while a slight trend in the same direction was seen in the LAPC-4 study. Resveratrol significantly decreased the IGF-1/IGFBP-3 ratio (a measure of free IGF-1) in the LAPC-4 study ($P = 0.01$), but not in the LNCaP study or in the combined analyses.

Percent Vascular Volume

The percent vascular volume, measured by CD31 stained pixels per area, was similar among all treatment groups in the LAPC-4 study (all $P > 0.5$, data not shown). CD31 stains were

not performed on the LNCaP study specimens since there was no significant survival difference in the treatment groups compared to control.

Gene Arrays

GSEA revealed a diverse set of gene sets significantly enriched with resveratrol treatment. Importantly, resveratrol induced different patterns of gene expression in each xenograft model. Comparison of treated and untreated LAPC-4 xenografts revealed 26 gene sets, including E2F1 and E2F3 regulated gene sets, were enriched in the treatment group, while 26 gene sets were enriched in untreated xenografts. Analysis of expression patterns in treated LNCaP xenografts found enrichment of 49 gene sets including the TGFbeta pathway. When expression data from both studies were combined, 37 gene sets were enriched in xenografts treated with resveratrol, whereas 26 gene sets were enriched in the untreated mouse tumors.

Our pathway analysis revealed a significant difference in E2F3 pathway expression in LAPC-4 xenografts after treatment with resveratrol (Fig. 3, Mann-Whitney *U*-test $P=0.0003$). There was no difference in E2F3 pathway activation in LNCaP xenografts, or when the data were combined. Furthermore, we observed a borderline significant increase in beta-catenin pathway activity with resveratrol treatment in LAPC-4 xenografts (Fig. 4, Mann-Whitney *U*-test $P=0.0549$), which was also not observed in LNCaP or in combined data. LNCaP xenografts exhibited no significant differences with respect to the pathway expression signatures we interrogated, and no individual oncogenic pathway trended toward any increased activity with resveratrol treatment.

DISCUSSION

Resveratrol has been touted as a virtual panacea for multiple medical conditions through many peer-reviewed articles. In this study, we sound a note of caution that the effects of resveratrol may be cell line or mutation dependent. In SCID mice, resveratrol promoted the growth of prostate cancer LAPC-4 xenografts while having no significant effect on LNCaP xenografts. These two cell lines are known to have different mutation profiles. LAPC-4 cells have wild-type androgen receptor [33], p53 mutation [33,34], and wild type PTEN genes [35]. LNCaP cells have an androgen receptor mutation that can be activated by steroids other than testosterone [33], p53 wild type [33,34,36,37], and PTEN mutant [35]. Perhaps the disparate survival data of these two xenografts is partially explained by their disparate mutation profiles. If this is true, some men with prostate cancer may not be affected by resveratrol whereas others may be harmed since human prostate cancer displays a wide variety of mutations [38].

This study was originally intended to provide the translational science basis for beginning clinical trials using resveratrol to slow prostate cancer progression in humans. Based on prior studies as discussed in Section Introduction, we expected to see a benefit of resveratrol and were surprised by our findings that it may be harmful in some cases. Given our findings, we believe that proceeding to phase 1 trials of resveratrol in human prostate cancer could be potentially dangerous to some patients. While additional molecular testing to elucidate resveratrol's mechanism of action in prostate cancer would have been performed if the primary endpoint had been favorable, we chose not to pursue further testing since it would be of mechanistic interest only if resveratrol is never used to prevent or treat prostate cancer.

Gene expression analysis with microarrays was utilized to help elucidate the mechanisms for the differential effects of resveratrol on the different xenografts. GSEA identified upregulation of E2F1 target genes with resveratrol treatment in LAPC-4 cells. A speculative mechanism relating this to the worse survival is the following: E2F1 deregulation typically

triggers a variety of cellular responses including p53-mediated apoptosis. LAPC-4 cells are p53 mutant, and therefore have an ablated apoptotic response to E2F1 pathway upregulation. Resveratrol treatment then favors E2F1 mediated proliferation and increased xenograft growth. Furthermore, in the LNCaP p53 wild type this could explain the slightly improved survival—albeit not significant. Another speculative mechanism is as follows: resveratrol is known to indirectly activate Sirt1, a class III histone deacetylase. SIRT1 activation by resveratrol deacetylates FOXO1, typically inhibiting apoptosis in PTEN wt prostate cancer models [39]. Since LAPC-4 is PTEN wt, apoptosis is inhibited, and thus you have greater tumor burden. Since LNCaP is PTEN mutant, SIRT1 activation by resveratrol leads to apoptosis.

Our two independent expression analyses also discovered upregulation of genes associated with E2F3 activation in our LAPC-4 xenografts. E2F3, another member of the E2F family, is decidedly a pro-proliferative signaling molecule, which could help explain why resveratrol stimulated LAPC-4 xenograft growth. Increased expression of E2F3 in human tumors was found to be an independent predictor of poor outcome in prostate cancer [40], a fact that makes our findings particularly ominous. Beta-catenin, which leads to transcription of several cancer-related genes [41], was also upregulated in our LAPC-4 xenografts. These mechanisms taken together may overpower the antiproliferative effects of resveratrol in cells bearing mutations in apoptotic pathways. This oncogenic effect of resveratrol has been shown in a study of human breast cancer cells [42].

Resveratrol in our study lowered IGF-1 and raised IGFBP-2, effects which are associated with decreased prostate cancer risk [43–45]. These effects on the IGF axis in our study did not translate into slowed prostate cancer growth. This finding leads us to hypothesize that the direct stimulatory effects of resveratrol on oncogenic gene pathways may be greater than the protective effect on the IGF1 axis.

A prior study investigated the effects of resveratrol in vivo using TRAMP (transgenic adenocarcinoma mouse prostate) mice. In this study, resveratrol decreased the incidence of poorly differentiated prostatic adenocarcinoma. The mechanisms of this action included decreased IGF-1, phospho-ERKs 1 and 2, and increased estrogen receptor-beta [5]. In the present study, we confirmed the IGF-1 down-regulation. Our model has important differences from the TRAMP model. First, ours is a treatment model while TRAMP is a prevention model using mice which are genetically programmed to always develop prostate cancer. Second, TRAMP prostate cancer is of mouse origin and has neuroendocrine differentiation that may only be relevant to high-grade cancers, while LAPC-4 and LNCaP are of human origin and potentially represent an earlier stage prostate cancer. Third, our study shows an effect on survival, not just tumor grade.

Our results should be interpreted with caution given the limitations of our study. First, SCID mice have altered macrophage/neutrophil function and no T-and B-cells [46]. Since the immune system is involved in fighting prostate cancer, xenografts in SCID mice may behave somewhat differently than cancer in the human prostate. Second, our pathway signature results should be interpreted with caution as they were generated from human mammary epithelial cells. However, these signatures do represent a broad program of transcriptional response to specific oncogenic stimuli, and have been applied with success to prostate cancer expression data [32]. Additionally, the androgen receptor activity signature was generated from LNCaP expression data. Third, resveratrol has poor oral bioavailability and extensive first pass metabolism [47], which may blunt its pharmacologic effect. If the resveratrol could be administered by a method that achieved a higher concentration in the blood and slower metabolism, the biologic effects seen in this study may be magnified, though this requires specific testing in future studies. Finally, resveratrol has been shown to

prolong life in mice fed a WD but not in mice fed a standard diet [12]. Consuming a healthy diet may also blunt the effects of resveratrol on cancer.

CONCLUSIONS

In these studies using nearly 250 mice, resveratrol was associated with significantly worse survival with LAPC-4 tumors, but unchanged survival with LNCaP tumors. Resveratrol caused IGF axis alterations normally associated with slowed tumor growth, but the LAPC-4 tumors grew faster. Gene array data showed E2F3 and E2F1 upregulation in LAPC-4 but not LNCaP tumors, suggesting that the direct stimulatory action of resveratrol on oncogenic pathways may overcome its protective effects in some types of prostate cancer. Based on these preliminary data that resveratrol may be harmful, caution should be advised in using resveratrol for patients until further studies can be conducted.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

IGF1	insulin-like growth factor 1
IGFBP	IGF binding protein

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Fig. 1.
Mouse survival in the LAPC-4 study.

Fig. 2.
Mouse survival in the LNCaP study.

Fig. 3.
E2F3 pathway activity.

Fig. 4.
β-catenin pathway activity.

TABLE I

Survival (vs. Control; Cox Proportional Hazards)

Study group	Hazard ratio (95% CI)	P-value
LAPC-4 study		
RV50	1.53 (1.03–2.27)	0.04
RV100	1.22 (0.83–1.81)	0.32
LNCaP study		
RV50	0.77 (0.51–1.17)	0.22

TABLE II

Serum IGF Axis Hormone Levels (Median Values; *P*-value From Kruskal–Wallis Test in LAPC-4 Study, Rank Sum Test for LNCaP Study)

Study group	IGF-1 (ng/ml)	IGFBP-1 (ng/ml)	IGFBP-2 (ng/ml)	IGFBP-3 (ng/ml)	IGF1/IGFBP3 ratio	Blood glucose (mg/dl)	Insulin (ng/ml)
LAPC-4 study							
No RV (n = 10)	205	4.0	258	2,053	0.10	219	2.4
RV50 (n = 10)	162	3.2	319	1,928	0.08	206	1.1
RV100 (n = 9)	179	3.6	272	2,139	0.08	203	0.4
<i>P</i> -value	0.01	0.82	0.14	0.39	0.01	0.87	0.03
LNCaP study							
No RV (n = 8)	128	9.3	206	1,747	0.07	216	6.0
RV50 (n = 9)	142	6.2	286	1,763	0.08	222	3.9
<i>P</i> -value	1.0	0.36	0.04	0.63	0.70	0.72	0.31
Combined							
No RV (n = 18)	179	5.8	208	2,035	0.09	217	4.6
RV50 (n = 19)	148	4.9	307	1,884	0.08	215	2.3
<i>P</i> -value	0.05	0.46	0.01	0.10	0.14	0.91	0.13