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Disparate results between proliferation rates of surgically excised prostate tumors and an in vitro bioassay using sera from a positive randomized controlled trial

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Abstract

In vitro bioassay has been used extensively to test the effects of culturing cancer cells in sera from humans participating in dietary interventions, i.e, studies of modified intake of nutrients for the purpose of reducing cancer risk or progression. It has been hypothesized that cell proliferation rates determined by the in vitro bioassay indicate whether modification of dietary intake could decrease cancer cell growth in vivo. It has been suggested, however, that the in vitro bioassay may not correlate with tumor cell proliferation rates in prostate cancer. We investigated the concordance of cell proliferation rates from surgically excised prostate tumor tissue with the in vitro bioassay using sera from matched patients. We used samples from an earlier randomized clinical trial that showed that supplementation with flaxseed significantly inhibited prostate cancer cell proliferation rates in vivo as indicated by Ki67 staining in tumor specimens. Proliferation rates of LNCaP, DU145 and PC3 cell lines cultured in 10% human sera from participants in the flaxseed trial were determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Spearman's Rho correlation coefficients (ρ) indicated no association between Ki67 staining in prostate tumors and the in vitro bioassay for the three cell lines. These

disparate findings suggest that the in vitro bioassay may not provide an accurate assessment of the environment in vivo.

Keywords

cancer; diet; exercise; neoplasia; proliferation; prostate

The in vitro bioassay was described by Tymchuk et al. (2001) and has been used to test the effects of human sera on cultured cancer cells. Typically, 10% human sera from pre- and post-intervention subjects are used in place of standard fetal bovine serum (FBS) to grow cells in culture. The results have been used as indicators of whether the intervention could potentially modify cancer cell growth in vivo. Most studies that have used the in vitro bioassay have shown that cellular proliferation is significantly reduced when cells are grown in sera from the post-intervention period compared to the pre-intervention period (Tymchuk et al. 2001, Ornish et al. 2005, Pantuck et al. 2006, Aronson et al. 2010, Soliman et al. 2011, Barnard et al. 2008).

Although the in vitro bioassay has been used often during the past decade, particularly with respect to prostate cancer, until recently there has been little information about the concordance between the in vitro bioassay results and cell growth in vivo. In response to a letter to the editor (Azrad and Demark-Wahnefried 2012), Galet and Aronson (2012) provided preliminary data that showed disparity between the in vitro bioassay results and prostate cancer cell growth in vivo using sera and prostate tissue, respectively, from the same subjects; however, this analysis was limited to 48 subjects (Galet and Aronson 2012). The disparity between the two analyses was striking because the same research team reported significant reductions in growth of 22RV1 cells in vitro using pre- and post-intervention sera, and reductions in prostate tumor cell proliferation in the same subjects following a low fat diet supplemented with 5 g/day of fish oil (Aronson et al. 2011). Because of the inconsistent reports, more information is required to determine whether the in vitro bioassay can serve as an accurate surrogate for the in vivo environment.

Previously, we conducted a phase II randomized controlled trial to test the comparative effects of flaxseed supplementation with and without dietary fat reduction (<20% of total energy) using a presurgical model in men with operable prostate cancer. We found that men assigned to the flax-seed supplemented groups during the month prior to surgery had significantly lower tumor proliferation rates, as determined by Ki67, compared to men who did not receive flaxseed supplementation ($p = 0.0013$). Because we had stored sera from both pre- and post-flaxseed supplementation and data on tumor proliferation from 137 subjects, we explored the concordance between proliferation rates of the assay and those in the tumor (Demark-Wahnefried et al. 2008).

Flaxseed provides high concentrations of the plant lignans, secoisolariciresinol and matairesinol, which are converted to the enterolignans, enterolactone and enterodiol, by intestinal microflora. It has been suggested that enterolignans may be anti-carcinogenic owing to their impact on the hormonal milieu and other mechanisms (Chen et al. 2007, 2009). From our earlier study, we determined that physiological concentrations of

enterolignans were correlated significantly with Ki-67 in prostate tumor tissue, a sensitive marker of cellular proliferation rate (Azrad et al. 2013). These results suggest that the anti-proliferative effects of flaxseed were due to enterolignans; however, we were unable to find evidence of the molecular mechanism by which enterolignans could suppress tumor proliferation. Therefore, we planned to use the in vitro assay as a model system to elucidate potential mechanisms by which flaxseed alter tumor growth. The first step, however, was to assess how well the proliferation indices obtained directly from surgical tumor specimens were correlated with those obtained from the in vitro bioassay by using three well characterized cell lines, LNCaP, DU145 and PC3, and using sera obtained immediately prior to surgery from the men from whom the tumor specimens were obtained.

Material and methods

Subjects

All subjects were enrolled in our randomized controlled trial that was designed to test the impact of flaxseed supplementation and/or a low fat diet on tumor proliferation rates in men awaiting prostatectomy for localized prostate cancer (Clinical Trial Number: NCT00049309) (Demark-Wahnefried et al. 2008). From the subjects enrolled in the original clinical trial, included subjects who had both Ki67 staining performed on their surgically-excised prostate tumor and sufficient amounts of stored serum to perform the in vitro bioassay.

Biological samples

Fasting blood samples from the post-dietary intervention period were collected in the morning prior to prostatectomy using 10 ml silicone coated serum separation tubes (Becton Dickinson, Franklin Lakes, NJ). Blood was allowed to clot for approximately 30 min at room temperature, then centrifuged at $1530 \times g$ for 10 min. Serum samples were aliquotted into sterile cryovials and stored at -80°C .

Following prostatectomy, the primary pathologist for the study (RV) reviewed the surgical pathology reports and hematoxylin and eosin (H & E) stained slides for each case. A formalin fixed, paraffin embedded tissue block was chosen for each case based on whether the corresponding H & E section contained adequate tumor tissue that was of a histologic grade and was representative of the case as described in the surgical pathology report. Sections were cut from the selected tissue block and used for immunohistochemistry as described below.

Microscope slides were labeled with a study identification number to protect patient identity. Tissue sections were placed on slides and deparaffinized in an oven at 60°C for 2 h, then cooled to room temperature and immersed in xylene three times for 5 min each. Sections were rehydrated through graded alcohols (100, 95 and 70%) for 5 min each. Antigen retrieval was performed in a pressure cooker with citrate buffer, pH 6 (Dako, Glostrup, Denmark), for 10 min. Sections were allowed to cool and endogenous peroxidase was blocked using 3% H_2O_2 and nonspecific antigen binding was blocked using 3% goat serum (Thermo Scientific, Pittsburgh, PA). To assess tumor proliferation, sections were stained

with the antibody, MIB-1 (Biocare Medical, Concord, CA), diluted 1:200 for Ki-67 hybridoma clone. The use of Ki-67 as a marker of tumor proliferation has been validated in dietary intervention trials and endorsed by the Prostate Cancer Chemoprevention Trial Consensus Panel (Kucuk 2002). The streptavidin-biotin-peroxidase-diaminobenzidine tetrachloride method (Biocare Medical) was used to detect MIB-1 staining and sections were counterstained with hematoxylin. Tonsil tissue with lymphoid hyperplasia served as a positive control. A “delete” section served as a negative reagent control; a tissue section for each case was processed identically to the procedure outlined above, except primary antibody was not added. This allowed us to assess nonspecific staining. No staining was observed on any delete slides. Sections stained for Ki67 were reviewed independently by two pathologists for our study (RV and JFM), both of whom were blinded to the identity of the sections. A random starting point in the tumor was selected at low magnification and sequentially encountered tumor cell nuclei were evaluated at high magnification for Ki-67 positivity. The result was reported as the number of positive nuclei divided by the total number of nuclei evaluated $\times 100$.

Cell lines and in vitro bioassay

LNCaP, DU145 and PC3 cell lines (ATCC, Manassas, VA) were cultured in RPMI 1640 medium (Cellgro™; Corning, Manassas, VA) with 10% FBS (HyClone™; Thermo Scientific, Waltham, MA) in T-75 flasks. Owing to potential variation in hormone and growth factors in commercially available FBS, care was taken to use the same lot for all cell culture experiments. Cells were maintained at 37°C and 5% CO₂ in a humidified incubator and passaged at 80% confluence. Cells were removed from flasks after dissociation with trypsin-EDTA (0.25% trypsin/0.53 mM EDTA in Hanks' balanced salt solution without calcium or magnesium) (ATCC). The in vitro bioassay was performed as described earlier (Ornish et al. 2005, Pantuck et al. 2006, Aronson et al. 2010, Soliman et al. 2011). Cells suspended in RPMI 1640 with 10% FBS, were seeded in poly-D-lysine coated 96-well plates (Becton Dickinson) at a density of 5,000 cells/well for 24 h. Subsequently, culture medium was removed, cells were washed with serum-free medium, then RPMI with 10% FBS (positive control) or 10% human serum were added to the wells and cultures were allowed to grow for 48 h. Consistent with previous studies, neither FBS nor human sera were heat inactivated (Ornish et al. 2005, Pantuck et al. 2006, Aronson et al. 2010, Soliman et al. 2011). Cell proliferation was determined using the CellTiter 96® MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay (Promega, Madison, WI). Results of the bioassay are expressed as percentage of positive control. Each 96-well plate contained LNCaP, DU145 or PC3 cells grown in RPMI with 10% FBS, which served as a positive control. The results of the MTT assay for cells grown in sera from patients were divided by the results of the MTT assay for the positive control, then multiplied by 100. The percentage of positive control was used for statistical analyses.

Statistics

Spearman rank correlation coefficients were determined to correlate the in vitro bioassay and Ki67 staining in surgical tumor tissue. Analyses were conducted using JMP 8.0 (SAS Institute, Inc., Cary, NC) and a *p* value 0.05 was considered statistically significant.

Results

The men who participated in our study were representative of the male population in the USA with respect to race, although more (~ 80%) were overweight or obese. Mean age, prostate specific antigen (PSA) levels, surgical Gleason scores, grades and stages of tumors are given in Table 1.

Correlation coefficients between cellular proliferation rates obtained from Ki67 staining of surgical tumor specimens and cells grown in sera from the same subjects are given in Table 2. No significant correlation was found between the in vitro bioassay results and Ki67 staining from the prostatectomy tumor tissue.

Because 15% of the participants were smokers at the time, we tested whether the disparity between the in vitro bioassay and Ki67 staining was influenced by cigarette smoking. Ki67 staining and cell proliferation rates for smokers and nonsmokers were similar. Spearman correlation tests that were stratified by smoking status also showed disparate results between the in vitro bioassay and Ki67 staining, which suggested that cigarette smoking did not influence our results.

The majority of the study participants were overweight and body weight can influence serum levels of several hormones and growth factors (Ornish 2005). Therefore, we assessed whether the weight status of the participants could have influenced the disparate findings concerning Ki67 staining, but we found no association between body mass index (BMI) and the in vitro bioassay (data not shown). We concluded that neither cigarette smoking nor weight status accounted for the disparity between the in vitro bioassay and Ki67 staining.

We also stratified by tumor stage to test whether there were differences in the relation between the in vitro bioassay and Ki67 staining in patients with early disease and less aggressive cancer versus patients with later stage and more aggressive cancer (Table 3). No significant correlation was observed between the in vitro bioassay and Ki67 staining among the three cell lines for either stage 1 + 2 (less aggressive cancer) or stage 3 (more aggressive cancer). Similarly, no associations were observed when stage 1 was compared to 2 or when stage 2 was compared to 3 in separate analyses (data not shown).

Discussion

We conducted a large study to compare the results of Ki67 staining from surgically excised prostate tumors with results obtained from the in vitro bioassay that used serum from patients matched with Ki67 staining data. The results of the in vitro bioassay did not correlate with Ki67 staining in surgical tumor specimens from matched subjects. Our findings corroborate earlier reports and provide further evidence that the in vitro bioassay may not be an accurate indicator of the environment in vivo (Azrad and Demark-Wahnefried 2012, Galet and Aronson 2012).

Tymchuck et al. 2001 first used the in vitro bioassay and found that LNCaP cells stimulated with sera from 13 men after diet and exercise intervention for 11 days showed significantly decreased (approximately 30%) cell proliferation. These investigators acknowledged that

whether the same reduction in tumor growth occurred in vivo had not been determined. We found that the results from the in vitro bioassay were dissimilar to tumor growth in vivo. Our disparate results are consistent with those reported by Galet and Aronson 2012 who also found no correlation between tumor proliferation rates from prostatectomy tissues collected in a randomized control trial and cell proliferation rates in the in vitro bioassay using matched sera. The report by Galet and Aronson (2012) was limited by its small sample size (n = 48) and the use of a single cell line, 22RV1. We expanded on the report by Galet and Aronson (2012) by using a larger sample and three well characterized cell lines, LNCaP, DU145, PC-3. We tested the in vitro bioassay using these cell lines, because LNCaP cells are androgen-sensitive, whereas DU145 and PC-3 are androgen-independent and are considered models for aggressive disease.

We showed earlier that enterolignans can inhibit cell proliferation in PC-3 cells by IGF1 receptor signaling and can induce caspase-dependent apoptosis in LNCaP cells (Chen et al. 2007, 2009). Therefore, we hypothesized that using sera from men with significantly elevated levels of enterolignans as a result of flaxseed supplementation potentially could alter cell proliferation in androgen-dependent or -independent cell lines; however, we found no correlation of results from the in vitro bioassay with cell proliferation rates from prostate tumors for any of the three cell lines.

The reasons for the disparity between the in vitro bioassay and in vivo cell proliferation are unclear; however, we offer the following possibilities. Cancer cells grown in culture tend to differ from malignant tissue in terms of genetics, epigenetics and gene expression (van Staveren et al. 2009, Smiraglia et al. 2001, Gazdar et al. 2010). Also, cells grown in a single monolayer have different morphology and respond differently to stimuli compared to cells growing in vivo. Although a dietary intervention may have a positive impact on tumor growth in vivo, the physiological differences between cells grown in vitro versus cells grown in vivo may be limitations that may be obscured using the in vitro bioassay. In addition, prostate cancer is a multi-focal malignancy in which tumor foci may differ throughout the gland with regard to aggressiveness and other characteristics (Tanagho and McAninch 1992). Further, it is possible that the lack of correlation may be due to the greater proliferation rates of cancer cells grown in culture compared to cancer cells within the tumor environment in vivo; however, even under these circumstances, we would expect to see evidence of a correlation between Ki67 and the in vitro bioassay. Therefore, the use of the in vitro bioassay to mimic the effects of diet and lifestyle interventions on tumor growth in vivo is questionable.

Our investigation had limitations. Like previous reports of use of the in vitro bioassay (Tymchuk et al. 2001, Ornish et al. 2005, Pantuck et al. 2006, Aronson et al. 2010, Soliman et al. 2011, Barnard et al. 2008), we used commercially available FBS for which the androgen concentration was not known. Although we used the same lot for all experiments, it is possible that the concentration of androgens in the FBS of cultures prior to the addition of patient sera could have either enhanced or masked an effect on cell proliferation. While this possibility is reduced by performing all analyses on the same 96-well plate and by expressing the growth of cells grown in patient sera as the percentage of cells grown in 10%

FBS, our uncertainty about whether the FBS could have contained uncharacteristically high or low androgen concentration is a potential limitation.

Although the in vitro bioassay is attractive because it provides an opportunity to test the effects of an intervention on changes in serum, which presumably impact cell proliferation at the tissue level, the lack of association between the in vitro bioassay and studies that measure proliferation of surgically-excised tumors directly (Demark-Wahnefried et al. 2008) suggest that the in vitro bioassay may not be an ideal model for assessing the impact of lifestyle interventions. Therefore, reports of investigations that use the in vitro bioassay should be interpreted cautiously, because they may not reflect accurately the environment of prostate cancer in vivo.

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Table 1

Baseline characteristics of study subjects

Age, (mean $\pm$ SD)	59.2 $\pm$ 7.3
Race/ethnicity, % (n)	
Caucasian	70 (112)
African-American	26 (42)
Other	4 (7)
Current cigarette smoking, % (n)	15 (24)
BMI, (mean $\pm$ SD)	28.8 $\pm$ 4.1
Serum PSA, (mean $\pm$ SD)	5.7 $\pm$ 3.8
Gleason score, % (n)	
6	66 (106)
7	34 (55)
Stage, % (n)	
I	16 (25)
II	68 (111)
III	16 (25)

Table 2

Spearman correlations between in vitro bioassay and Ki67 staining in malignant prostate tissue

Cell line	ρ	p
LNCaP	-0.077	0.380
DU145	0.019	0.830
PC3	-0.062	0.475

(n = 137)

Results from MTT assay using LNCaP, DU145 and PC3 cell lines were compared to Ki67 staining from surgically excised prostate tumors.

Table 3

Spearman correlations between in vitro bioassay and Ki67 staining in malignant prostate tissue stratified by stage

	ρ	p
Stages 1 and 2		
LNCaP	-0.071	0.467
DU145	0.039	0.686
PC3	-0.045	0.448
Stage 3		
LNCap	-0.120	0.577
DU145	-0.106	0.622
PC3	-0.163	0.448

Results from MTT assay using LNCaP, DU145 and PC3 cell lines were compared to Ki67 staining from surgically excised prostate tumors.