

# Antioxidants Block Prostate Cancer in *Lady* Transgenic Mice

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## ABSTRACT

The development of chemopreventive agents against prostate cancer would benefit from conclusive evidence of their efficacy in animal models that emulate human disease. To date there has been little *in vivo* evidence supporting their preventive capabilities. The 12T-10 *Lady* transgenic model spontaneously develops localized prostatic adenocarcinoma and neuroendocrine cancer followed by metastases, recapitulating the natural history of human prostate cancer in many respects. Using male *Lady* version of the transgenic adenocarcinoma of the mouse prostate mice, we show that administration of antioxidants (vitamin E, selenium, and lycopene) in the diet dramatically inhibits prostate cancer development and increases the disease free survival. Treatment of animals with the antioxidants resulted in a 4-fold reduction in the incidence of prostate cancer compared with the untreated animals. Prostate cancer developed in 73.68% (14 of 19) and 100% (19 of 19) of the animals from the standard and high fat diet, respectively. In contrast, only 10.53% (2 of 19) and 15.79% (3 of 19;  $P < 0.0001$ ) of the animals in the standard and high fat diets supplemented with antioxidants developed tumors. The micronutrients were well tolerated with no evidence of antioxidant-related toxicity. Histopathological analysis confirmed absence of cancer in the additive treated groups. Immunohistochemistry demonstrated a strong correlation between disease-free state and increased levels of the prognostic marker p27<sup>Kip1</sup> and a marked decrease in proliferating cell nuclear antigen expression. These observations provide support for the chemopreventive effect of these micronutrients and some clues as to their mechanism of action.

## INTRODUCTION

Prostate cancer is the second most common cause of cancer deaths among males in the Western world. Progress toward understanding the biology of prostate cancer and the development of new therapies for this disease has been hampered, by the lack of *in vivo* model systems that adequately recapitulates the spectrum of benign, latent, aggressive, and metastatic forms of the human disease. Epidemiological studies have demonstrated a close link between dietary fat consumption, antioxidant supplementation and prostate cancer (1, 2). These observations have been supported by some *in vivo* models of prostate cancer (3).

Dietary antioxidants such as selenium, lycopene, and  $\alpha$ - and  $\gamma$ -tocopherol have been studied as candidate chemopreventive agents (4–7). *In vitro* and *in vivo* studies carried out in our laboratory and by other investigators suggest that these compounds are potential agents for prostate cancer prevention (8–10). This interest is reflected in the decision by the NIH to sponsor the Selenium and Vitamin E Chemoprevention Trial. Vitamin E is a major intracellular antioxidant in cell membrane. It inhibits lipid peroxidation and has a wide range of anticancer properties (11–15). These include both protection against carcinogenesis and inhibition of tumor progression. Fleshner *et al.*,

(10) has shown that supplemental vitamin E can inhibit high-fat-promoted tumor progression in nude mice bearing human LNCaP prostate cancer xenografts. In addition, we have demonstrated that vitamin E inhibits the growth of prostate cancer cells *in vitro* mediated by alteration in cell cycle regulatory molecules, particularly p27 (9).

Selenium prevents cancer in numerous tumor model systems. Studies carried out in our laboratory have shown that selenium induces cell cycle arrest of the androgen-dependent LNCaP human prostate cancer cells but not PC3 (androgen-independent) cells via up-regulation of p27. Clark *et al.*, (16, 17) demonstrated a marked decrease in the incidence of prostate cancer among men supplemented with selenium in a randomized trial. Lycopene is a carotenoid, naturally occurring in tomatoes and other fruits. It is also a potent antioxidant and the most significant free radical scavenger among the carotenoid family (18, 19). A nested case-control study within the context of the Health Professional Follow-Up study revealed that individuals consuming >10 servings per week of tomato-based products had less odds of developing advanced/aggressive prostate cancer (20). One of the lycopene intervention studies suggests that this compound may possess significant anticancer properties (6).

There is much interest in using antioxidant combinations for prevention. Combinations of antioxidants have been shown to act with additive or synergistic effects in certain model systems (21, 22). Recent *in vitro* data from our laboratory suggests that one of the pathways for cancer prevention may be via the p27 tumor suppressor gene (8, 9). A member of the *Cip/Kip* family of cyclin-dependent kinase inhibitors, p27, regulates cell cycle progression from G<sub>1</sub> to S-phase by binding and inhibiting the cyclin E/cyclin-dependent kinase 2 complex (23). Loss of p27 protein has been shown to be associated with development and progression of numerous tumor types, including breast (24), colorectal (25, 26), lung (27), and prostate cancers (28).

The *Lady* transgenic model (29), a less aggressive version of the original transgenic adenocarcinoma of the mouse prostate, which spontaneously develops metastatic CaP, mimics progressive forms of human disease. Using male *Lady* transgenic mice, we show that administration of antioxidants (vitamin E, selenium, and lycopene) in the diet at a human achievable dose significantly inhibits prostate cancer development and increases the disease-free survival of these mice.

## MATERIALS AND METHODS

**Transgenic Mice.** The female *Lady* (12T-10) mice developed on a pure background were obtained from Dr. Robert J. Matusik (Vanderbilt Prostate Center, Vanderbilt University Medical Center, Nashville, TN; ref. 29, 30). Breeders were fed standard pellet mouse feed. After weaning of the animals at 3 to 4 weeks of age, the gender of the offspring was determined, males were separated, and a tail biopsy was collected from each mouse. Tail DNA was used for determination of transgene incorporation by PCR. Transgenic animals were divided into four main groups: group 1, animals on a standard diet; group 2, animals on a standard diet with a combination of antioxidants (vitamin E, selenium, and lycopene); group 3, animals on a 40% calorie diet; and group 4, animals on a diet containing 40% calorie with antioxidants. The antioxidants/supplements added were in proportion to the human equivalent of (per day) 800 IU vitamin E ( $\alpha$ -tocopherol succinate), 200  $\mu$ g of Selenium (seleno-DL-methionine) and 50 mg of lycopene. Transgenic males, included in experi-

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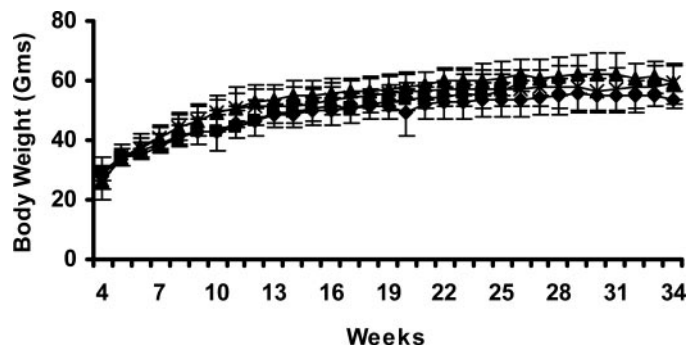


Fig. 1. Growth curve of *Lady* transgenic animals treated with antioxidants. Starting at 4 to 5 weeks until they were 28 to 32 weeks of age, transgenic males were fed diets with a combination of vitamin E, selenium, and lycopene. Results shown are the mean body weights in each experimental group  $\pm$  SD, as a function of age (weeks). The values indicate that antioxidants administered in the diet do not affect body weight. A defined as antioxidants (vitamin E, selenium, and lycopene).

ments at 4 to 5 weeks of age, were fed formulated diets (Purina Mills Test Diet, Richmond, Indiana), until they were 28 to 32 weeks of age. The diets were non-irradiated, stored at 4°C at all times, stable for 6 months (assayed by the company), and free of phytoestrogens. Throughout the study, animals were weighed every other week. Animal care and treatments were conducted in accordance with established guidelines and protocols approved by the Sunnybrook and Women's College Health Sciences Centre, Toronto, Canada and in accordance with the Canadian Council on Animal Care.

**Preparation and Analysis of Blood and Tissues.** Blood sample was obtained by direct heart puncture. Serum was separated and aliquots stored at -80°C. All animals were examined at necropsy for gross organ abnormalities, kidney, liver, spleen, bladder, seminal vesicle, and prostate. Tissues collected at necropsy were routinely fixed in 10% (v/v) buffered formalin. Five microm-

eter sections were cut from paraffin-embedded tissues, mounted on slides, routinely stained with H&E, and processed for histopathological evaluation.

**Immunohistochemistry.** Expression of p27<sup>Kip1</sup> and proliferating cell nuclear antigen (PCNA) proteins were determined immunohistochemically. Paraffin sections of tissue blocks were deparaffinized with xylene, rehydrated and boiled for 10 min in citrate buffer (pH 7.0). Sections were blocked with 0.3% hydrogen peroxide in methanol followed by normal serum and then incubated overnight at 4°C with the primary antibody [anti-p27<sup>Kip1</sup> rabbit polyclonal antibody (Santa Cruz Biotechnology) diluted 1:100 (200  $\mu$ g/ml) in PBS; PCNA rabbit polyclonal antibody (Santa Cruz Biotechnology) diluted 1:50 (200  $\mu$ g/ml) in PBS; anti-SV40 Tag (AB-2) monoclonal antibody (Oncogene Research) diluted 1:100 (200  $\mu$ g/ml) in PBS]. Slides were then reacted with biotin-labeled antirabbit IgG/antimouse IgG and incubated with preformed avidin-biotin peroxidase complex (Vector Laboratories). Metal-enhanced diaminobenzidine substrate (Vector Laboratories) was added. Sections were counterstained with hematoxylin, dehydrated, and mounted.

**Scoring of p27.** Prostate tissue sections from different animals were randomly scored. The prostate tissue cytoplasmic p27 expression was scored based on the intensity of staining (absence of staining, <1+, 1+, 2+, and 3+). This degree of staining was scored by two independent investigators. Cells with weak or strong staining (1+ to 3+) were considered as positive, and cells with <1+ and without cytoplasmic staining were considered negative for p27 expression. Fisher exact test was used for statistical analysis.

**Serum Testosterone.** The Roche Diagnostics Elecsys 2010 Immunoassay System with an automated, random access, multichannel analyzer was used. Samples were incubated with a testosterone-specific biotinylated antibody and a testosterone derivative labeled with a ruthenium complex. After addition of streptavidin-coated microparticles, the complex was bound to the solid phase via interaction of biotin and streptavidin. The reaction mixture was then aspirated into the measuring cell where the microparticles are magnetically captured onto the surface of the electrode. Unbound substances are then removed with ProCell. Application of voltage to the electrode then introduces

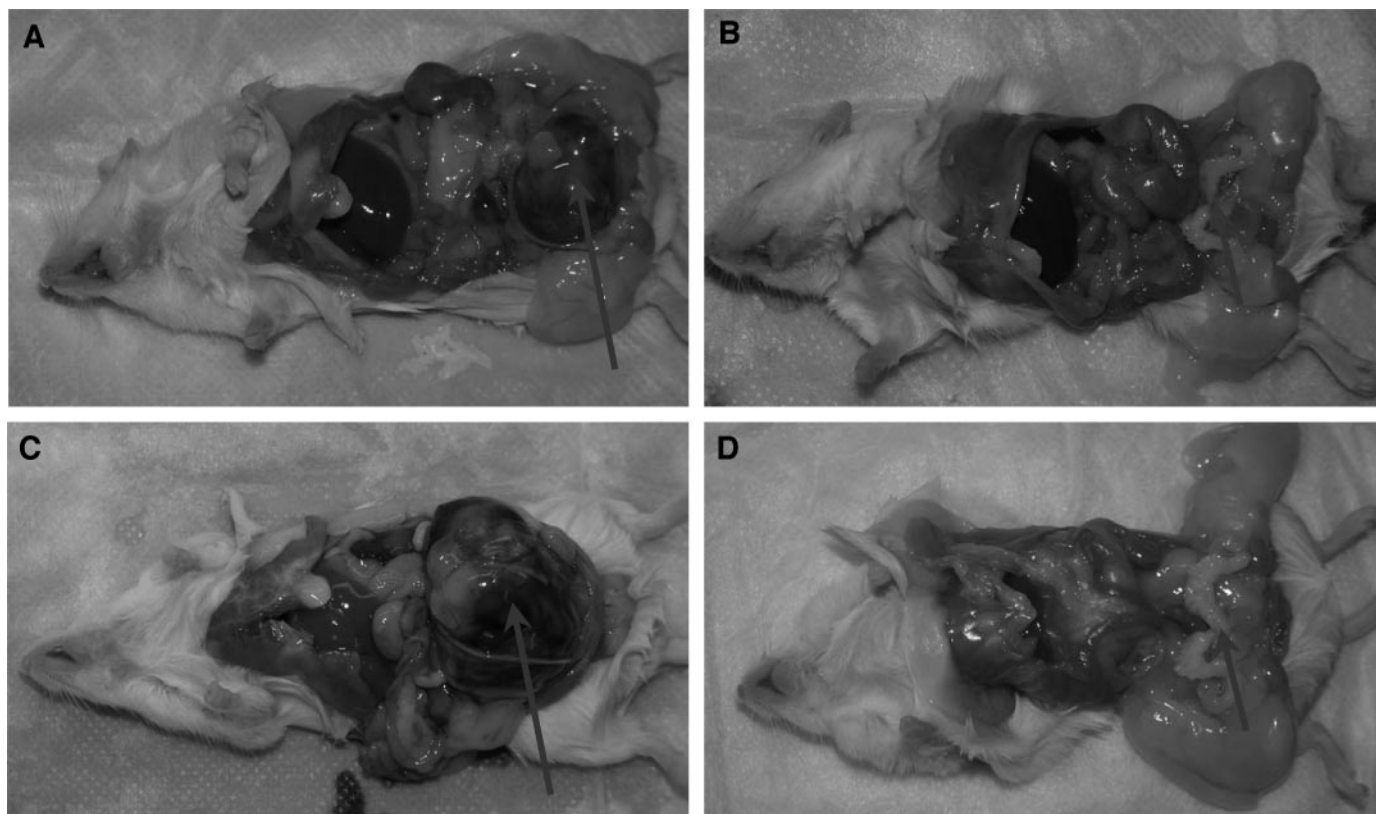


Fig. 2. Gross anatomy depicting absence of prostate tumors in the animals supplemented with antioxidants. Blood sample was first obtained by direct heart puncture. All animals were examined at necropsy for gross organ abnormalities, kidney, liver, spleen, bladder, seminal vesicle, and prostate. A portion of the tissue was placed in formalin and processed for histopathological evaluation. A, standard diet; B, standard diet + antioxidants; C, 40% calorie diet; D, 40% calorie diet + antioxidants.

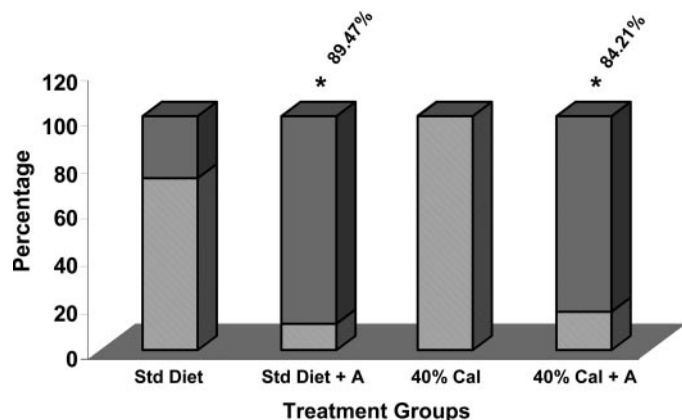


Fig. 3. Effect of antioxidants on the prevention of prostate tumors in *Lady* mice. Antioxidants (vitamin E, selenium, and lycopene) in the diet reduces the incidence of prostate tumors. The organs collected at necropsy were prepared for pathological evaluation. Results shown are the numbers of mice with prostate tumors as compared with the animals that were normal after antioxidant treatment. A defined as antioxidants (vitamin E, selenium, and lycopene); \*,  $P < 0.0001$ .

chemiluminescent emission, which is measured by a photomultiplier. Results were determined via a calibration curve.

**Statistical Method.** Tumor development and the disease-free data were plotted for the various groups and the  $P$  value calculated using  $\chi^2$ . All calculations were performed using statistical software.

## RESULTS

**Antioxidant Administered in the Diet to *Lady* Mice Is Non-Toxic.** Body weight was not significantly altered in the groups fed diets containing antioxidants. Although the diets were isocaloric, there was a 20% increase in the body weight of the animals on a 40% calorie diet (with or without additives) as compared with the animals on a standard diet (Fig. 1).

**Effect of Antioxidant Consumption in Prostate of *Lady* Mice.** Transgenic animals on a standard diet or a 40% calorie diet (groups 1 and 3) developed prostate tumors by 28 to 32 weeks. Animals on the

supplemental diet with antioxidants (groups 2 and 4) had grossly normal prostates.

Antioxidants in the diet significantly reduced the incidence of prostate cancer in the *Lady* transgenic mice. These differences were striking and readily apparent on gross anatomy (Fig. 2;  $P < 0.0001$ ). At the termination of the experiment, we observed that 73.68% (14 of 19 animals) of the animals from the standard diet (group 1) and 100% (19 of 19 animals;  $P < 0.046$ ) of the animals on the high-fat diet (group 3) developed prostate cancer. In striking contrast, the micronutrient-treated animals demonstrated tumors in 10.53% (2 of 19 animals) and 15.79% (3 of 19 animals; groups 2 and 4 respectively;  $P < 0.0001$ ; Fig. 3). A proportion of the animals (in groups 1 and 3) exhibited liver as well as seminal vesicle tumors along with the development of prostate tumors. Wet prostate weight of the tumors ranged from 2 to 4 g compared with the micronutrient treated animals whose prostates weighed 0.02–0.16 g. All cancers were poorly differentiated carcinoma. The liver in these animals had metastasis. This was associated with the presence of lipid deposits in the hepatocytes of the animals in the high-fat diet. Remarkably, most animals on an antioxidant supplemented diet (groups 2 and 4) had histologically normal prostates and livers. There were lipid vacuoles in the livers of the animals on a 40% calorie diet plus antioxidants (Fig. 4).

**Administration of Antioxidants and the Expression of Proliferative Marker (PCNA) in the Prostate of *Lady* Mice.** Antioxidant consumption resulted in a marked reduction in PCNA protein expression in the prostate of *Lady* mice compared with the non-antioxidant treated controls (Fig. 5).

**Immunohistochemistry of Prognostic Tissue Marker p27<sup>Kip1</sup> in the Prostate of *Lady* Mice Supplemented with Antioxidants.** Immunohistochemistry performed on formalin-fixed paraffin-embedded tissue demonstrated a strong correlation between disease-free state and increased levels of p27<sup>Kip1</sup>. Expression of p27 varied between samples within the group. However, a uniformly intense immunoreactivity for p27<sup>Kip1</sup> was localized in both the groups (groups 2 and 4) treated with the additives (Fig. 5). p27 staining was primarily cytoplasmic (80%). Some p27 was expressed in the nucleus and the membrane of the secretory cells (20%). The staining intensity in the

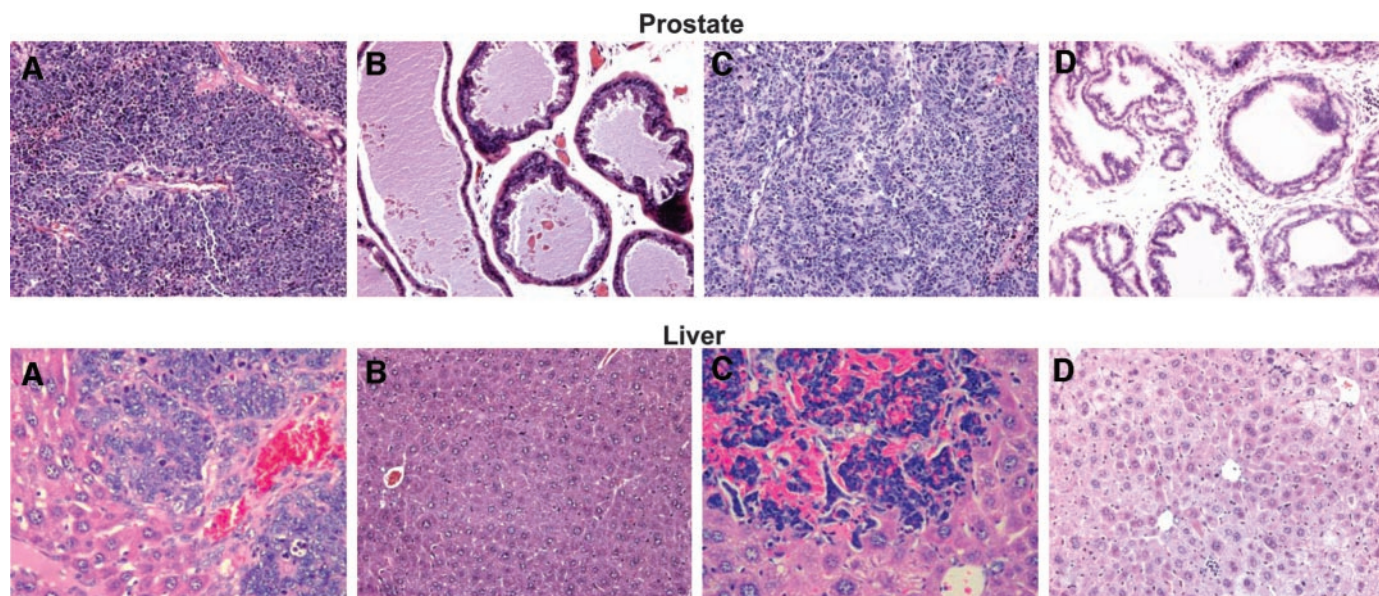


Fig. 4. Histopathology of 12T-10 *Lady* mice. Prostate and liver tissue from animals on the various diet regimen were fixed in 10% formalin, processed, and embedded in paraffin, cut at 5  $\mu\text{m}$  of thickness and stained with H&E. Antioxidant-treated animals demonstrated normal prostate and liver as compared with the animals on standard and high-fat diet. A, standard diet; B, standard diet + antioxidants; C, 40% calorie diet; D, 40% calorie diet + antioxidants. Prostate, A and C, poorly differentiated carcinoma; B and D, normal prostate. Liver, A and C, metastatic poorly differentiated carcinoma; B and D, normal liver.

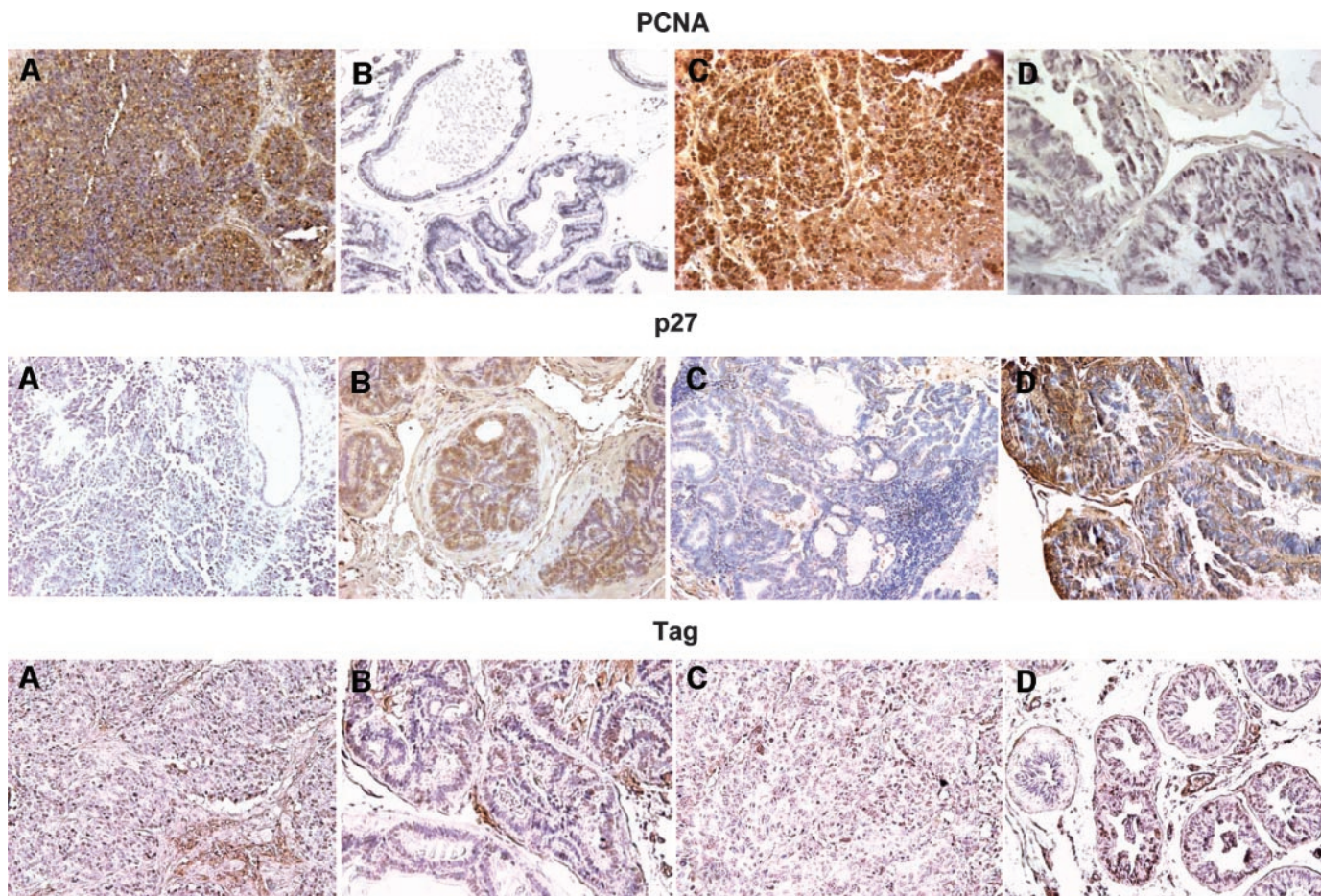


Fig. 5. Immunohistochemistry. Immunostaining was performed on 5  $\mu\text{m}$ -thick paraffin sections of the prostate. Sections were treated as described in "Materials and Methods" and reacted with the following antibodies: rabbit polyclonal PCNA antibody (FL-261; Santa Cruz Biotechnology); rabbit polyclonal p27 antibody (C-19; Santa Cruz Biotechnology); or mouse monoclonal SV40 Tag (AB-2) antibody (Oncogene Research). A, standard diet; B, standard diet + antioxidants; C, 40% calorie diet; D, 40% calorie diet + antioxidants.

antioxidant treated animals (groups 2 and 4; graded by two blinded individuals) was 2+ in 90% of the samples and 3+ in 10% ( $P < 0.0001$ ). The untreated animals from groups 1 and 3 that developed tumors had low (<1+) expression in 80% and absence of p27 expression in 20% ( $P < 0.0001$ ).

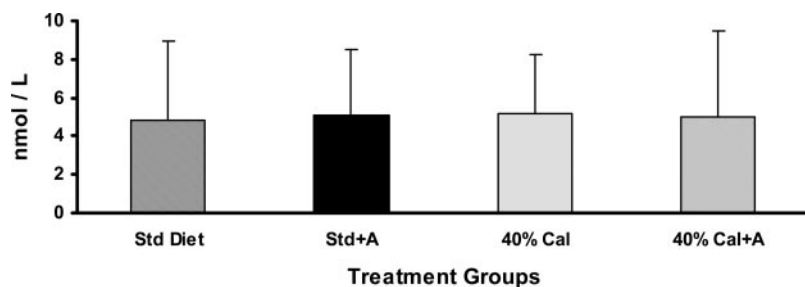
**Expression of Large T Antigen in Prostate of *Lady* Mice.** Expression of SV40 large T antigen (Tag) was detected in all groups with and without the supplementation of antioxidants (groups 1–4; Fig. 5). Importantly, animals treated with micronutrients that had no evidence of prostate cancer had expression of the SV40 Tag in their prostates.

**Antioxidants and Serum Testosterone Levels.** Administration of antioxidants in the diet was associated with no alterations in serum testosterone levels between the groups (Fig. 6). Serum testosterone values ranged from 4.82 to 5.15 nmol/L.

## DISCUSSION

Nutrition is a major risk factor responsible for the difference in global distribution of clinical prostate cancer (31). Human and animal studies indicate that certain dietary ingredients can modulate the growth rate of prostate cancer cells (32–36). The risk of developing clinically significant prostate cancer appears to be affected by dietary fat, fatty acids, obesity, dietary fiber, fruits, vegetables, antioxidants, and soy protein intake. The *Lady* mice displaying focal and invasive adenocarcinomas and neuroendocrine cancer of the prostate between 20 to 30 weeks of age afford an opportunity to characterize the earlier events in prostate cancer progression (29). Beginning at 5 to 6 weeks of age, these animals were fed a diet with or without antioxidants. Body weights of the animals showed no antioxidant-related toxicity. The addition of antioxidants (vitamin E, selenium, and lycopene) in

Fig. 6. Serum testosterone of *Lady* mice. The Roche Diagnostics Elecsys 2010 Immunoassay System was used. Results shown are serum testosterone levels in each experimental group  $\pm$  SD. A defined as antioxidants (vitamin E, selenium, and lycopene).



the diet reduced the incidence of prostate tumors dramatically (89.47% and 84.21% of the treated animals were normal; groups 2 and 4). The antioxidants added were in proportion to the human equivalent of (per day) 800 IU vitamin E ( $\alpha$ -tocopherol succinate), 200  $\mu$ g of selenium (seleno-DL-methionine) and 50 mg of lycopene.

Several studies have shown that antioxidants prevent the growth of prostrate cancer cells *in vitro* (5, 7–9, 37, 38). We demonstrate that this effect also occurs *in vivo*. The *Lady* (12T-10) model develops precursor lesions more analogous to human high-grade prostatic intraepithelial neoplasia, without associated prominent stromal hypercellularity. In addition, this line predictably develops invasive carcinoma with glandular differentiation (adenocarcinoma) as well as neuroendocrine prostate cancer that commonly metastasizes (30). Using this model we were able to demonstrate that the addition of vitamin E, selenium, and lycopene to the diet dramatically reduced the incidence of prostate tumors in these animals.

Total fat intake has been associated with an elevated occurrence of prostate cancer. The addition of a high-fat diet (40% of calories from fat) increased the incidence of prostate cancer in these animals (group 3). However, this effect was blocked by antioxidants in the diet (group 4). Vitamin E is a potent intracellular antioxidant with demonstrable antitumor properties in a variety of cancer models (39–41). Our laboratory has shown that vitamin E inhibits the growth of prostate cancer cells *in vitro* mediated by alteration in cell cycle regulatory molecules, specifically p27 (9). A second study carried out in our laboratory revealed that a combination of antioxidants (vitamin E and selenium) is shown to potentiate or act in synergy, thereby enhancing the proportion of cancer prevention (42).

Antioxidant consumption for 28 to 32 weeks resulted in marked reduction in the levels of expression of the proliferative marker, PCNA. PCNA serves as a requisite auxiliary marker for increased proliferation for DNA polymerase  $\delta$ -driven DNA synthesis and is cell cycle regulated (43). We further confirmed that antioxidants mediate their effect through p27. The reduction of p27 protein is of prognostic importance in cancers (44–47). Our results indicate that frequent loss of cyclin-dependent kinase inhibitor p27<sup>Kip1</sup> in prostate cancer cells correlates with advancing biological aggressiveness, implicating deregulation of p27<sup>Kip1</sup> in prostate tumor progression. Supplementation of diets with antioxidants augmented the expression of p27 similar to that seen in normal tissue.

Expression of SV40 T antigen was observed in all of the animals regardless of the type of supplemental diet. The absence of prostate tumors and histological normal prostates with the supplementation of antioxidants confirms the fact that this was an antioxidant-mediated effect and not a consequence of SV40 Tag down-regulation. Analysis of serum testosterone demonstrated no alteration within the groups, implying that the antioxidants were not interacting with the gonad-pituitary axis.

This model affords an opportunity to dissect the preventive effect of micronutrients in prostate cancer. The relative contributions of the three micronutrients used in this study (vitamin E, selenium and lycopene) remain to be elucidated. A central issue is whether these act by preventing initiation or progression of prostate cancer. These questions warrant additional study.

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## REFERENCES

- National Heart, Lung, and Blood Institute. Clinical guidelines on the identification, evaluation, and treatment of overweight and obesity in adults—the evidence report [review]. *Obes Res (Suppl 2)*:51S–209S, 1998. Erratum in: *Obes Res* 1998 Nov;6:464.
- Fleshner NE, Bagnell PS, Klotz LH, Venkateswaran V. Dietary fat and prostate cancer. *J Urol* 2004;171:S19–24.
- Zock PL. Dietary fats and cancer. *Curr Opin Lipidol* 2001;12:5–10.
- Fleshner NE, Kucuk O. Antioxidant dietary supplements: Rationale and current status as chemopreventive agents for prostate cancer. *Urology* 2001;57:90–4.
- Menter DG, Sabichi AL, Lippman SM. Selenium effects on prostate cell growth. *Cancer Epidemiol Biomark Prev* 2000;9:1171–82.
- Kucuk O, Sarkar FH, Sakr W, et al. Phase II randomized clinical trial of lycopene supplementation before radical prostatectomy. *Cancer Epidemiol Biomark Prev* 2001;10:861–8.
- Gysin R, Azzi A, Visarius T. Gamma-tocopherol inhibits human cancer cell cycle progression and cell proliferation by down-regulation of cyclins. *FASEB J* 2002;16:1952–4. Epub 2002 Oct 04.
- Venkateswaran V, Klotz LH, Fleshner NE. Selenium modulation of cell proliferation and cell cycle biomarkers in human prostate carcinoma cell lines. *Cancer Res* 2002;62:2540–5.
- Venkateswaran V, Fleshner NE, Klotz LH. Modulation of cell proliferation and cell cycle regulators by vitamin E in human prostate carcinoma cell lines. *J Urol* 2002;168:1578–82.
- Fleshner N, Fair WR, Huryk R, Heston WD. Vitamin E inhibits the high-fat diet promoted growth of established human prostate LNCaP tumors in nude mice. *J Urol* 1999;161:1651–4.
- Patterson RE, White E, Kristal AR, Neuhauser ML, Potter JD. Vitamin supplements and cancer risk: the epidemiologic evidence. *Cancer Causes Control* 1997;8:786–802.
- Wang Y, Ichiba M, Oishi H, Iyadomi M, Shono N, Tomokuni K. Relationship between plasma concentrations of beta-carotene and alpha-tocopherol and life-style factors and levels of DNA adducts in lymphocytes. *Nutr Cancer* 1997;27:69–73.
- Albanes D, Heinonen OP, Huttunen JK, et al. Effects of alpha-tocopherol and beta-carotene supplements on cancer incidence in the alpha-tocopherol beta-carotene cancer prevention study. *Am J Clin Nutr* 1995;62(6 Suppl):1427S–30S.
- Rautalahti MT, Virtamo JR, Taylor PR, et al. The effects of supplementation with alpha-tocopherol and beta-carotene on the incidence and mortality of carcinoma of the pancreas in a randomized, controlled trial. *Cancer (Phila)* 1999;86:37–42.
- Woodson K, Tangrea JA, Barrett MJ, Virtamo J, Taylor PR, Albanes D. Serum alpha-tocopherol and subsequent risk of lung cancer among male smokers. *J Natl Cancer Inst* (Bethesda) 1999;91:1738–43.
- Clark LC, Combs GF Jr, Turnbull BW, et al. Effects of selenium supplementation for cancer prevention in patients with carcinoma of the skin. A randomized controlled trial. Nutritional Prevention of Cancer Study Group. *J Am Med Assoc* 1996;276:1957–63.
- Clark LC, Dalkin B, Krongrad A, et al. Decreased incidence of prostate cancer with selenium supplementation: results of a double-blind cancer prevention trial. *Br J Urol* 1998;81:730–4.
- Conn PF, Schach W, Truscott TG. The singlet oxygen and carotenoid interaction. *J Photochem Photobiol B* 11:41–7, 1991. Erratum in: *J Photochem Photobiol B* 1993;17:89.
- Di Mascio P, Kaiser S, Sies H. Lycopene as the most efficient biological carotenoid singlet oxygen quencher. *Arch Biochem Biophys* 1989;274:532–8.
- Rao AV, Fleshner NE, Agarwal S. Serum and tissue lycopene and biomarkers of oxidation in prostate cancer patients: a case-control study. *Nutr Cancer* 1999;33:159–164.
- Horvath PM, Ip C. Synergistic effect of vitamin E and selenium in the chemoprevention of mammary carcinogenesis in rats. *Cancer Res* 1983;43:5335–41.
- Ip C, White G. Mammary cancer chemoprevention by inorganic and organic selenium: single agent treatment or in combination with vitamin E and their effects on *in vitro* immune functions. *Carcinogenesis (Lond)* 1987;8:1763–6.
- Polyak K, Lee MH, Erdjument-Bromage H, et al. Cloning of p27<sup>Kip1</sup>, a cyclin-dependent kinase inhibitor and a potential mediator of extracellular antimitogenic signals. *Cell* 1994;78:59–66.
- Porter PL, Malone KE, Heagerty PJ, et al. Expression of cell-cycle regulators p27<sup>Kip1</sup> and cyclin E, alone and in combination, correlate with survival in young breast cancer patients. *Nat Med* 1997;3:222–5.
- Loda M, Cukor B, Tam SW, et al. Increased proteasome-dependent degradation of the cyclin-dependent kinase inhibitor p27 in aggressive colorectal carcinomas. *Nat Med* 1997;3:231–4.
- Thomas GV, Szigeti K, Murphy M, Draetta G, Pagano M, Loda M. Down-regulation of p27 is associated with development of colorectal adenocarcinoma metastases. *Am J Pathol* 1998;153:681–7.
- Esposito V, Baldi A, De Luca A, et al. Prognostic role of the cyclin-dependent kinase inhibitor p27 in non-small cell lung cancer. *Cancer Res* 1997;57:3381–5.
- Tsihlias J, Kapusta LR, DeBoer G, et al. Loss of cyclin-dependent kinase inhibitor p27<sup>Kip1</sup> is a novel prognostic factor in localized human prostate adenocarcinoma. *Cancer Res* 1998;58:542–8.
- Greenberg NM, Demayo FJ, Finegold M, et al. Prostate cancer in a transgenic mouse. *Proc Natl Acad Sci USA* 1995;92:3439–43.
- Masumori N, Thomas TZ, Chaurand P, et al. A probasin-large T antigen transgenic mouse line develops prostate adenocarcinoma and neuroendocrine carcinoma with metastatic potential. *Cancer Res* 2001;61:2239–49.

31. Cai X, Haleem R, Oram S, et al. High fat diet increases the weight of rat ventral prostate. *Prostate* 2001;49:1–8.
32. Carroll KK, Khor HT. Dietary fat in relation to tumorigenesis. *Prog Biochem Pharmacol* 1975;10:308–53.
33. Ross RK, Shimizu H, Paganini-Hill A, Honda G, Henderson BE. Case-control studies of prostate cancer in blacks and whites in southern California. *J Natl Cancer Inst* (Bethesda) 1987;78:869–74.
34. Kolonel LN. Nutrition and prostate cancer. *Cancer Causes Control* 1996;7:83–4.
35. Whittemore AS, Kolonel LN, Wu AH, et al. Prostate cancer in relation to diet, physical activity, and body size in blacks, whites, and Asians in the United States and Canada. *J Natl Cancer Inst* (Bethesda) 1995;87:652–61.
36. Giovannucci E, Rimm EB, Colditz GA, et al. A prospective study of dietary fat and risk of prostate cancer. *J Natl Cancer Inst* (Bethesda) 1993;85:1571–9.
37. Thompson TA, Wilding G. Androgen antagonist activity by the antioxidant moiety of vitamin E, 2,2,5,7,8-pentamethyl-6-chromanol in human prostate carcinoma cells. *Mol Cancer Ther* 2003;2:797–803.
38. Bhamre S, Whitin JC, Cohen HJ. Selenomethionine does not affect PSA secretion independent of its effect on LNCaP cell growth. *Prostate* 2003;54:315–21.
39. Heinonen OP, Albanes D, Virtamo J, et al. Prostate cancer and supplementation with alpha-tocopherol and beta-carotene: incidence and mortality in a controlled trial. *J Natl Cancer Inst* (Bethesda) 1998;90:440–6.
40. McCormick DL, Rao KV. Chemoprevention of hormone-dependent prostate cancer in the Wistar-Unilever rat. *Eur Urol* 1999;35:464–7.
41. Nakamura A, Shirai T, Takahashi S, Ogawa K, Hirose M, Ito N. Lack of modification by naturally occurring antioxidants of 3,2'-dimethyl-4-aminobiphenyl-initiated rat prostate carcinogenesis. *Cancer Lett* 1991;58:241–6.
42. Venkateswaran V, Fleshner NE, Klotz LH. Synergistic effect of vitamin E and selenium in human prostate cancer cell lines. *Prostate Cancer Prostatic Dis* 2004;7:54–6.
43. Prosperi E. Multiple roles of the proliferating cell nuclear antigen: DNA replication, repair and cell cycle control. *Prog Cell Cycle Res* 1997;3:193–210.
44. Yang RM, Naitoh J, Murphy M, et al. Low p27 expression predicts poor disease-free survival in patients with prostate cancer. *J Urol* 1998;159:941–5.
45. Foulkes WD, Brunet JS, Stefansson IM, et al. The prognostic implication of the basal-like (cyclin E high/p27 low/p53+/glomeruloid-microvascular-proliferation+) phenotype of BRCA1-related breast cancer. *Cancer Res* 2004;64:830–5.
46. Vis AN, Noordzij MA, Fitoz K, Wildhagen MF, Schroder FH, van der Kwast TH. Prognostic value of cell cycle proteins p27(kip1) and MIB-1, and the cell adhesion protein CD44s in surgically treated patients with prostate cancer. *J Urol* 2000;164:2156–61.
47. Halvorsen OJ, Haukaas SA, Akslen LA. Combined loss of PTEN and p27 expression is associated with tumor cell proliferation by Ki-67 and increased risk of recurrent disease in localized prostate cancer. *Clin Cancer Res* 2003;9:1474–9.