Nutrition 28 (2012) 67–75

Contents lists available at ScienceDirect

Nutrition

journal homepage: www.nutritionjrnl.com

Basic nutritional investigation

Dietary walnuts inhibit colorectal cancer growth in mice by suppressing angiogenesis

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ARTICLE INFO

Article history:
Received 15 September 2010
Accepted 20 March 2011

Keywords:
Nuts
Linseed
Colon cancer
Xenograft
VEGF

ABSTRACT

Objective: Animal studies have demonstrated that dietary supplementation with flaxseed oil inhibits colorectal cancer growth. Recent data indicate that walnuts have strong antiproliferative properties against colon cancer cells in vitro but no previous study has assessed the effects of walnuts in vivo or performed a joint evaluation of flaxseed oil and walnuts. The aim of the present study was to examine the effect of dietary walnuts on colorectal cancer in vivo and to comparatively evaluate their efficacy in relation to flaxseed oil.
Methods: HT-29 human colon cancer cells were injected in 6-wk-old female nude mice. After a 1-wk acclimation period, mice ($n = 48$) were randomized to diets containing ~19% of total energy from walnuts, flaxseed oil, or corn oil (control) and were subsequently studied for 25 d.
Results: Tumor growth rate was significantly slower in walnut-fed and flaxseed-fed mice compared with corn oil-fed animals ($P < 0.05$) by 27% and 43%, respectively. Accordingly, final tumor weight was reduced by 33% and 44%, respectively ($P < 0.05$ versus control); the differences between walnut and flaxseed diets did not reach significance. We found no differences among groups in metabolic and hormonal profile, serum antioxidant capacity, or inflammation ($P > 0.05$). However, walnuts and flaxseed oil significantly reduced serum expression levels of angiogenesis factors, including vascular endothelial growth factor (by 30% and 80%, respectively), and approximately doubled total necrotic areas despite smaller tumor sizes ($P < 0.05$ versus control). Dietary walnuts significantly decreased angiogenesis (CD34 staining; $P = 0.017$ versus control), whereas this effect did not reach significance in the flaxseed oil group ($P = 0.454$ versus control).
Conclusion: We conclude that walnuts in the diet inhibit colorectal cancer growth by suppressing angiogenesis. Further studies are needed to confirm our findings in humans and explore underlying mechanisms.

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Introduction

Colorectal cancer is the third most common type of cancer worldwide, with mortality being approximately one half that of the incidence [1]. There are several established risk factors for colorectal cancer, many of which, such as dietary habits, are modifiable [2]. It has been estimated that 30% to 50% of colorectal cancer in men and ~20% in women could be prevented by adoption of a prudent diet and other lifestyle changes [3,4]. Nevertheless, although a "Western" dietary pattern is typically associated with increased colorectal cancer risk [5], there is still much uncertainty regarding the putative protective role of individual foods and nutrients [2].

The results of several large-scale epidemiological studies in humans show an inverse association between nut and seed consumption and the incidence of colorectal cancer [6–9]. With one exception [10], animal studies examining the effect of flaxseed (linseed) supplementation on the formation of precancerous colorectal lesions [11–13] and the growth of chemically induced colorectal tumors [14–16] reported beneficial effects. However, the underlying mechanisms remain unclear and poorly

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0899-9007/$ - see front matter Published by Elsevier Inc.
doi:10.1016/j.nut.2011.03.004

described. Furthermore, the recent observations that walnuts retard the growth rate of breast cancer cells implanted in mice [17] and that walnut extracts have dose-dependent inhibitory effects on the growth of colon cancer cells in vitro [18] raise the possibility that dietary walnuts could also be beneficial against colorectal cancer in vivo.

The purpose of the present study, therefore, was to comparatively investigate the effect of isoenergetic amounts of walnuts and flaxseed oil in the diet on colorectal cancer growth rate in mice in vivo and to explore relevant underlying molecular mechanisms.

Materials and methods

Animals

Six-week-old female athymic nude (nu/nu) mice were obtained from Charles River Laboratories (Wilmington, MA, USA). They were acclimatized on the control diet for 10 d prior to implantation of tumor cells. Mice were individually numbered for unique identification and were housed, four per cage, in a barrier mouse facility (temperature controlled at 24°C, 12-h light/dark cycles) at the Beth Israel Deaconess Medical Center (BIDMC) Animal Research Facility (Boston, MA, USA). All animal use and handling were approved by the BIDMC Institutional Animal Care and Use Committee.

Tumor induction

HT-29 human colon cancer cells (American Type Culture Collection, Manassas, VA, USA) were cultured in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Five million cells were injected subcutaneously in one flank of each mouse. Seven days after tumor cell injection, tumors had reached 3-5 mm in diameter. Injection of tumor cells into animals results in variable tumor sizes; to reduce variability in this experiment, outliers (25% of all mice with tumors in the highest and lowest quartiles) were excluded before diet randomization.

Dietary treatment

Seven days after tumor cell injection, 48 mice were randomized after stratification for body weight to either control, walnut, or flaxseed oil diets ($n = 16$ per group), which were prepared by Research Diets (New Brunswick, NJ, USA). The control diet was modeled after the standard AIN-76 diet for mice. The walnut diet was formulated to contain the equivalent of two servings of walnuts per day in humans, which provides 376 calories or 18.8% of a 2000 calorie/d diet; this amount of energy was substituted for corn oil (Welch, Holme & Clark, Newark, NJ, USA) in the control diet, or lignan-containing flaxseed oil (Barlean's Organic Oils, Ferndale, WA, USA). The walnut diet was prepared from whole walnut kernels (kindly provided by the California Walnut Commission), including the brown husk but not the shell, which were finely ground in a food processor and immediately mixed with the remainder of the dry ingredients of the diet. The diets were pelleted and dried for 2 d in a temperature- and humidity-controlled room to remove excess water. Thereafter, diets were sterilized with 10-20 kGy of gamma irradiation (Cobalt 60) and stored in sealed bags at –20°C to prevent fat oxidation and bacterial growth. Mice had free access to water and were fed fresh food twice weekly. Mice were fed the experimental diets for 25 d, from day 7 after tumor cell injection until the end of the study on day 32.

Body weight and tumor growth

Body weight and tumor size were measured three times weekly. An electronic scale was used to weigh the mice, and a vernier caliper was used to measure tumor size. Tumor volume was calculated using the formula: volume = 0.5 × length × width × depth.

Sacrifice and tissue handling

The experiment was terminated after 25 d of diet treatment, when the tumor of at least one control-fed mouse reached the maximum allowable size of 1500 mm^3 [17]. Blood was collected from the submandibular vein and the serum was frozen at −80°C until further analyses. Mice were sacrificed using carbon dioxide inhalation. The tumor, liver, and visceral fat were weighed upon removal. The tumors were dissected and a cross-section was fixed in 10% neutral buffered formalin followed by paraffin embedding; 4 μm-thick sections were placed on microscope slides for histological and immunohistochemical staining. Portions of the tumor were flash frozen in liquid nitrogen and stored at −80°C until protein extraction.

Determination of hormones, substrates, markers of inflammation, and serum antioxidant capacity

Commercially available ELISA kits were used for measuring serum concentrations of adiponectin, leptin, insulin, and C-reactive protein (all from ALPCO Diagnostics, Salem, NH, USA) and insulin-like growth factor (IGF) 1 (R&D Systems, Minneapolis, MN, USA). Free fatty acid concentration was determined with the NEFA-HR(2) enzymatic colorimetric assay (Wako Diagnostics, Richmond, VA, USA). Serum antioxidant capacity was assayed as previously described [17]. Glucose concentration was measured by using the LifeScan One Touch Ultra glucose meter (Johnson & Johnson, New Brunswick, NJ, USA).

Western blot analyses

Protein extraction was performed in lysis buffer containing 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 0.1 mM PMSF, 0.05% aprotinin, and 0.1% Igepal. SDS-PAGE (4-12%) was performed using 70 μg protein, blotted onto nitrocellulose membranes (Schleicher & Schuell, Keene, NH, USA). Antibodies against p-Erk (Tyr204, sc-7976), Erk (sc-93), p-AMPK (Thr 172, sc-101630), AMPK (sc-19126), p-STAT3 (tyr 705, sc-7993), STAT3 (sc-8019), p-Akt (Ser473, sc-101629), Akt (sc-5298), and actin (sc-69879), and secondary antibodies (anti-mouse, sc-2005; anti-rabbit, sc-2357; anti-goat, sc-2354) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against p-S6 (Ser 235/236, #2211S) and S6 (#2317) were purchased from Cell Signaling (Beverly, MA, USA). The membranes were blocked for 1 h in TBS containing 1% BSA (p-AMPK, AMPK, p-Akt, Akt, p-STAT3, STAT3) or 5% milk (p-Erk, Erk, p-S6, S6, p53, actin), and incubation with primary antibodies was performed in TBS-Tween containing 1% BSA or 5% nonfat dry milk, respectively. Following overnight incubation, blots were washed three times with TBS-Tween and then incubated with horseradish peroxidase-labeled secondary antibodies for 2 h. Enhanced chemiluminescence (Amersham, Arlington Heights, IL, USA) was used for detection. Measurement of signal intensity was performed using ImageJ processing and analysis software (National Institutes of Health, Bethesda, MD, USA).

Immunohistochemical analyses

Representative tumors from each diet group were chosen for further analyses. Hematoxylin and eosin (H&E) staining was performed by the Histology Core at BIDMC (Boston, MA, USA). Immunohistochemical staining for Ki67, CD34, and Mac2 was performed at the Immunohistochemical Core Facility at Brigham and Women's Hospital (Boston, MA, USA). The number of Ki67-positive cells was counted in five representative images (×20) to evaluate proliferation ($n = 4$ per group). TUNEL staining was performed using Millipore's Apoptag kit using tonsil as positive control to assess apoptosis ($n = 4$ per group). Mac2-positive cells were counted in five visual fields at ×40 magnification to evaluate tumor macrophage infiltration ($n = 4$ per group). To assess angiogenesis, areas of highest tumor vessel density were determined in each tumor and five representative images were taken at ×40. Images were evaluated using a horizontal grid by marking every point where CD34-stained vessels crossed the grid ($n = 7$ for the control and walnut groups, $n = 8$ for the flaxseed group). All analyses were performed in a blinded manner.

Angiogenesis proteome profile array

Vascular endothelial growth factor (VEGF) and IGF-binding proteins (IGFBP) -1, -2, and -3, as well as other angiogenesis-related proteins, were measured from pooled serum samples by using the semiquantitative Proteome Profiler Mouse Angiogenesis Array Kit (R&D Systems). X-ray films were developed using Pierce ECL (Thermo Scientific, Waltham, MA, USA) after 1-min exposure. Mean pixel density was measured for each spot and normalized to the positive control using ImageJ software (National Institutes of Health, Bethesda, MD, USA). Relative differences of 25% or more from control values were considered significant.

Statistical analysis

Data were tested for normality by using the Shapiro-Wilk procedure. Non-normally distributed data were log-transformed for analysis, or ranked when no suitable transformation was possible. To evaluate differences in food intake, body weight, and tumor volume over the course of the study, multilevel modeling was used allowing for random intercepts and slopes among mice; fixed effects included time and diet and their interaction. One-way ANOVA followed by Tukey's post-hoc test were used to evaluate differences among the three groups in tumor weight and all serum parameters at sacrifice. Results are presented as means and 95% confidence intervals for normally distributed variables, or medians and quartiles for ranked variables. Statistical significance was set at