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Effect of Dietary Energy Restriction on Vascular Density during Mammary Carcinogenesis

Henry J. Thompson, John N. McGinley, Nicole S. Spoelstra, Weiqin Jiang, Zongjian Zhu, and Pamela Wolfe

Cancer Prevention Laboratory, Colorado State University, Fort Collins, Colorado

ABSTRACT

Inhibition of mammary carcinogenesis by dietary energy restriction is associated with a decrease in cell proliferation and the induction of apoptosis. Although changes in the metabolism of insulin-like growth factor I and glucocorticoids have been proposed to modulate these cellular processes, limitations in blood supply could induce similar effects. To investigate this possibility, female Sprague Dawley rats were given an injection of 1-methyl-1-nitrosourea and fed purified diets *ad libitum* or at 60% *ad libitum* intake, *i.e.,* 40% dietary energy restriction. Premalignant mammary pathologies and mammary adenocarcinomas obtained from these rats were processed for vascular density analysis via CD-31 immunostaining. Vascular density, measured as vessels/unit area, of premalignant mammary pathologies and adenocarcinomas from dietary energy restriction rats was reduced 31 and 39%, respectively ($P < 0.01$). This effect, which was observed in a 50-μm wide band of tissue surrounding each pathology, was exerted on blood vessels > 25 μm^2. Conversely, intratumoral vascular density was unaffected by dietary energy restriction. cDNA microarray and Western blot analyses of adenocarcinomas for evidence of dietary energy restriction-mediated effects on vascularization revealed that only the level of vascular endothelial growth factor receptor protein Flk-1 was significantly reduced ($P < 0.001$). It appears that dietary energy restriction imposes limitations in the supply of blood to developing pathologies, an effect that could directly inhibit the carcinogenic process. The vascular density data imply that dietary energy restriction inhibited the growth of endothelial cells but leave unresolved the question of whether dietary energy restriction had a specific effect on angiogenesis. The factors that account for the failure of dietary energy restriction to limit intratumoral vascularization are not obvious and merit additional investigation.

INTRODUCTION

Dietary energy restriction is a powerful physiological approach to protecting animals against cancer in experimentally induced model systems. The cancers against which dietary energy restriction provides protection are wide ranging and include spontaneously occurring (1) and chemically and virally induced cancers at multiple organ sites, including the mammary gland (2, 3). Cancers occurring in genetically engineered models also are inhibited by dietary energy restriction (4, 5). Until recently, relatively little has been known about the mechanisms that account for protection. However, as reviewed in Refs. 6, 7, that situation is changing. Evidence continues to accumulate indicating that dietary energy restriction inhibits cell proliferation and induces apoptosis, activities that appear to have at least some degree of specificity for cells in premalignant and malignant pathologies (8–10), and these effects may be mediated by the modulation of insulin-like growth factor I and glucocorticoid metabolism (6). However, relatively little is known about the effects of dietary energy restriction on the process of vascularization, particularly during premalignant stages of the carcinogenic process. Investigation of the effects of dietary energy restriction on the vascular density associated with premalignant and malignant mammary pathologies occurring in a chemically induced model for breast cancer was the focus of the experiments reported in this study.

The 1-methyl-1-nitrosourea (MNU)-induced mammary carcinogenesis model in the rat has been used to evaluate the cancer inhibitory activity of chemopreventive agents, various nutrients, and other dietary factors; dietary energy restriction has strong inhibitory activity in this system (11). This model also is used to study the mechanism(s) by which protective agents exert their effects. Although considerable attention has been directed to investigating mechanisms directly linked to cell proliferation and apoptosis, there is now increasing recognition that some cancer preventive agents may act by blocking the process of angiogenesis (12). On the basis of the early work of Maiorana and Gullino (13) and Brem *et al.* (14) in which mammary tissue from MNU-treated rats as well as MNU-induced mammary carcinomas were observed to stimulate vascularization in the rabbit iris angiogenesis assay, it appears that the MNU model is well suited for the investigation of agents that may affect blood vessel formation and/or growth.

On the basis of the reports from our laboratory (15, 16), experiments were conducted to determine how dietary energy restriction modulated blood vessel density during premalignant and malignant stages of mammary carcinogenesis. The effects of dietary energy restriction on blood vessel density prompted the assessment of mammary carcinomas by cDNA microarray analyses to determine whether genes associated with blood vessel development and growth were differentially regulated by dietary energy restriction. Western blotting was used to selectively investigate effects of dietary energy restriction on proteins involved in angiogenesis.

MATERIALS AND METHODS

Chemicals and Reagents

MNU was obtained from Ash Stevens (Detroit, MI). Donkey serum (Jackson ImmunoResearch, West Grove, PA) was used as blocking serum to prevent nonspecific binding of primary antibodies. Goat anti-CD31 antibody was obtained from Research Diagnostics (Flanders, NJ). Horseradish peroxidase-conjugated streptavidin (Dako Corp. Carpinteria, CA) and stable 3,3′-diaminobenzidine (Research Genetics, Huntsville, AL) were used in the visualization of blood vessels.

For Western blotting determination, antivascular endothelial growth factor (anti-VEGF), anti-Flt-1 (also designated VEGF-R1), anti-Flk-1 (also designated VEGF-R2 or KDR), and goat antimouse immunoglobulin- and goat antirabbit immunoglobulin-horseradish peroxidase-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology Corp. (Santa Cruz, CA); anti-β-actin antibody was obtained from Sigma Chemical Co. (St. Louis, MO); enhanced chemiluminescence detection system was obtained from Amersham Life Science Inc. (Arlington Heights, IL). The Cancer/Angiogenesis-1 GEArray kit (SuperArray, Inc.) was used to detect gene expression. The kit included 23 target genes plus two housekeeping genes and one internal control gene.

Carcinogenesis Experiment

Seventy-eight female Sprague Dawley rats were obtained from Taconic Farms (Germantown, NY) at 20 days of age. At 21 days of age, animals were injected with 50 mg MNU/kg body weight (i.p.) as described previously (17).

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Requests for reprints: Henry J. Thompson, Cancer Prevention Laboratory, Colorado State University, 1173 Campus Delivery, Fort Collins, CO 80523. Phone: (970) 491-7748; Fax: (970) 491-1004; E-mail: henry.thompson@colostate.edu.

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Rats were housed individually in stainless steel metabolic cages with wire mesh bottoms. The cages were equipped with adjustable width external tunnel feeders that permitted accurate quantification of food intake. Animal rooms were maintained at 22 ± 1°C with 50% relative humidity and a 12-h light/12-h dark cycle. Rats were weighed three times/week throughout the study. From 16 days postcarcinogen until study termination, rats were palpated twice/week for detection of mammary tumors. The study was terminated 77–78 days postcarcinogen.

Feeding Protocol

One day postcarcinogen, rats were randomized into two groups; 24 rats were assigned to the *ad libitum* group and 54 rats were assigned to the 40% dietary energy restriction group. The reason for the unequal number of rats/group was due to the potency of the inhibitory activity of dietary energy restriction and the objective to have a sufficient number of mammary pathologies of each type to evaluate. After randomization and individual housing, animals were adapted to the caging system for 4 days. From 5 days postcarcinogen, all rats were meal fed and given two meals/day (6:00–9:00 a.m. and 2:00–5:00 p.m.) 7 days/week to reduce possible confounding because of intergroup variation of meal timing, meal number, and duration of fasting between meals. Dietary energy restriction was started at 9 days postcarcinogen with 10% dietary energy restriction, increased to 20% dietary energy restriction on the next day (10 days postcarcinogen), and finally increased to 40% dietary energy restriction at 11 days postcarcinogen. This level of dietary energy restriction (40%) was maintained for the remainder of the study. Measured amounts of food were provided to rats at each meal. In the *ad libitum* group, the amount of food remaining after each meal was determined and used to calculate the amount eaten. This information was used to compute the amount of food to be given to the dietary energy restriction group at each subsequent meal to restrict intake to 60% of the *ad libitum* group. A modified AIN-93G diet was used as described previously (11). The diet fed to 40% dietary energy restriction animals was formulated to ensure an intake of all nutrients equivalent to the *ad libitum* group while limiting total dietary calories by reducing carbohydrate.

Necropsy

All grossly visible mammary pathologies were excised at necropsy; a portion of each was fixed in 10% neutral buffered formalin, and when an adequate amount of tissue was available, the remainder was snap frozen in liquid nitrogen for subsequent molecular analyses. All lesions were further processed and histologically classified. In addition, whole mounts of abdominal-inguinal mammary gland chains were prepared, and tissue was fixed in 10% neutral buffered formalin. The fixed tissues were subsequently stained, and stained whole mounts were photographed and then evaluated under ×2 magnification for detection of any abnormality that might be a mammary pathology as previously described in detail (17). All abnormalities were excised from whole mounts and processed for histological classification as described in Ref. 18. Premalignant pathologies and adenocarcinomas were additionally evaluated for vascular density as outlined in a subsequent paragraph. The following pathologies were evaluated: intraductal proliferations, ductal carcinoma *in situ* (DCIS), and adenocarcinomas.

Assessment of Vascular Density

Paraffin sections of all lesions in formalin-fixed mammary gland whole mount preparations were prepared, stained with H&E, and histologically classified. In turn, paraffin blocks from lesions diagnosed as premalignant or malignant mammary pathologies were further sectioned and processed for vascular density analysis as described below. Only pathologies with fully intact epithelium and that were circumscribed by a minimum of a 50-μm width band of fully intact stroma were evaluated for vascular density. Consequently, an unequal number of intraductal proliferations and DCIS were evaluated/treatment group. A total of 41 carcinomas that met the criteria for vascular density analysis was recovered from the dietary energy restriction group, and therefore, a similar number of carcinomas (tumor size matched) were evaluated from the ad libitum group.

The method for immunostaining of mammary tissue for blood vessel detection has been reported in detail (15). Briefly, paraffin sections from formalin fixed mammary pathologies were cut at 4-μm and mounted onto 3-aminopropyltriethoxysilane-treated slides. Sections were then processed for immunostaining of blood vessels using antiserum directed against the CD31 epitope. Census counting of all blood vessels was undertaken. In assessing hyperplasias and DCIS, blood vessels were not found within these morphological structures; therefore, blood vessel density was quantified in a 50-μm width band of mammary tissue circumscribing each structure. For mammary carcinomas, vascular density was determined both within the tumor and in a 50-μm width band of mammary tissue circumscribing each carcinoma. Vessels were counted using images of immunostained sections captured with a Kodak DCS-420 digital camera (Eastman Kodak, Rochester, NY) mounted on a Zeiss Axioskop microscope using a ×10 objective. The photographic coupler has a ×10 ocular, and the charge-coupled device imaging sensor on the DCS-420 has a focal magnification of ×2.5 yielding images with an approximate total magnification of ×250. The resolution of each image was 1012 × 1524 or 1.5 megapixels. Images were acquired using a 32-bit Kodak TWAIN driver v5.02 within Adobe Photoshop v4.0 graphic software (Adobe Systems, Inc., San Jose, CA) running on a 300 MHz Pentium III PC with 128 MB of RAM. Lesions that exceeded the size of a single imaging area were captured by photographing contiguous microscopic fields in a raster pattern. Each captured image was merged using a layer technique in Adobe Photoshop to form a single composite image for analysis. All vessels were circumscribed manually using a digitizing pen to rule out inclusion of artifact or background immunostain. Criteria established by Weidner *et al.* (19) were used to identify blood vessels in immunostained sections. Specifically, positively stained endothelial cells or endothelial cell clusters, regardless of size or shape, that were clearly separate from adjacent blood vessels, mammary epithelial cells, or other connective tissue elements were counted. Vessel lumens, although usually present, were not necessary for a structure to be defined as a blood vessel, and RBCs were not used to define a vessel lumen. As outlined in Ref. 16, vessels were assigned to one of five categories based on size: ≤ 10, >10 and ≤ 25, >25 and <50, >50 and <75, and >75 μm^2. We have proposed that microvessels that are <25 μm^2 in cross-sectional area and, particularly those <10 μm^2, have a high probability of being newly formed; having vessel count data categorized by size improves the ability to make inferences about angiogenic activity from vascular density data (20).

RNA Isolation

Total RNA was isolated from mammary carcinomas using RNeasy Mini kit (Qiagen, Valencia, CA) according to the manufacturer's directions. Briefly, carcinomas were homogenized using a Polytron homogenizer (Brinkman Instruments) in Qiagen buffer of RLT, and cell lysate was collected by centrifugation for 3 min at 15,850 × *g*. Then, the lysate was transferred onto a Qiagen-shredder column sitting in the 2-ml collection tube and centrifuged for 2 min at 15,850 × *g*. The same volume as the collected lysate of 70% ethanol was added to the homogenized lysate and mixed. The mixture was transferred to an RNeasy mini spin column sitting in a 2-ml collection tube and centrifuged for 15 s at 15,850 × *g*. After the RNeasy minicolumn was washed with Qiagen buffers of RW1 and RPE, RNA was washed out from the RNeasy column with RNase-free water and collected in a 1.5-ml collection tube. The quality of total RNA was determined by measuring the absorbance at 260 nm ($A_{260\ nm}$) and 280 nm ($A_{280\ nm}$) in a spectrophotometer. The $A_{260\ nm}/A_{280\ nm}$ ratio of samples was 1.9–2.1.

Synthesis of cDNA Probes

Total RNA was used as a template for biotinylated probe synthesis using Nonrad-GEArray kit (SuperArray, Inc., Bethesda, MD). Five μg of total RNA were annealed with GEAprimer Mix at 70°C for 2 min and cooled to 42°C. Then, the RNA was labeled with labeling mixture (Nonrad-GEAlabeling buffer, biotin-16-dUTP, RNase inhibitor, and reverse transcriptase) at 42°C for 120 min. The reaction was stopped, denatured, and neutralized by specific solutions offered by SuperArray, Inc. The resulting cDNA probe was ready to be used for hybridization.

Hybridization and Chemiluminescent Detection

GEArray membrane (SuperArray, Inc.) was prehybridized with GEAhyb Hybridization Solution (SuperArray, Inc.) containing denatured sheared salmon sperm DNA (100 μg DNA/ml; Life Technologies, Inc., Grand Island,

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NY) at 68°C for 2 h and hybridized in the Hybridization Solution (SuperArray, Inc.) containing denatured cDNA probe of the samples at 68°C overnight. After washing the membrane twice with wash solution 1 (300 mM sodium chloride, 30 mM sodium citrate, and 1% SDS) and twice with wash solution 2 (15 mM sodium chloride, 1.5 mM sodium citrate, and 0.5% SDS) for 20 min each at 68°C, the membrane was blocked in GEAblocking solution (SuperArray, Inc.) for 40 min and incubated in the same solution containing alkaline phosphatase-conjugated streptavidin (1:5000 dilution) for another 40 min at room temperature. After the membrane was washed in a washing buffer (SuperArray Inc.) three times and rinsed in a rinsing solution (SuperArray Inc.), the membrane was incubated with chemiluminescent substrate and exposed to X-ray film. Signals were quantified by scanning the film with ScanJet (Hewlett Packard), and the intensity of the spots was analyzed by using the Image-Pro Plus software (Media Cybernetics). β-Actin and glyceraldehyde-3-phosphate dehydrogenase were used as positive controls, and bacterial plasmid (pUC18) was used as a negative control.

Expression of Angiogenesis Regulatory Molecules by Western Blotting

Mammary carcinomas were homogenized in lysis buffer [10 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 0.2 mM sodium vanadate, 0.2 mM phenylmethylsulfonyl fluoride, 0.5% NP40, and 0.2 unit/ml aprotinin] using Polytron tissue homogenizer (Brinkman Instruments, Westbury, NY). The lysate was collected by centrifugation for 15 min in an Eppendorf centrifuge at 4°C, and protein concentration in the clear supernatant was determined by the Bio-Rad protein assay. For Western blotting of angiogenesis regulatory molecules, 40 μg of protein lysate/sample were denatured with SDS-PAGE sample buffer [63 mM Tris-HCl (pH 6.8), 10% glycerol, 2% SDS, 0.0025% bromphenol blue, and 5% 2-mercaptoethanol], subjected to SDS-PAGE on an 8 or 12% gel, and the protein bands blotted onto a nitrocellulose membrane (Invitrogen, Carlsbad, CA). The levels of VEGF, Flt-1, Flk-1, and β-actin were determined using the specific primary antibodies designated above, followed by treatment with the appropriate peroxidase-conjugated secondary antibody and visualized by the enhanced chemiluminescence detection system. Signals were quantified by scanning the film with ScanJet (Hewlett Packard, Palo Alto, CA), and the intensity of the bands was analyzed by using Image-Pro Plus software (Media Cybernetics, Silver Spring, MD).

Statistical Analyses

Carcinogenesis Data. Differences among groups in cancer incidence were evaluated by a χ^2 test of homogeneity across groups (21). Differences among groups in the number of mammary carcinomas/rat (multiplicity) were evaluated by ANOVA after square root transformation of the count data as recommended in Ref. 22.

Vascular Density Data. Because of the variable and in some cases limited number of pathologies evaluated in a particular histopathological diagnosis, we first asked whether dietary energy restriction affected the vascular density of premalignant pathologies irrespective of type and then proceeded to explore whether effects on specific categories of pathologies were apparent. Probability plots of the log-transformed data showed the data were not log-normal. A general nonparametric approach that relies only on the empirical distribution for determining *P* values is the bootstrap, resampling with replacement from the observed data under the assumption that the null hypothesis is true. Bootstrap *P* values, which are based on the empirical distribution of the errors, were computed for both the log of the count and the log of the area data by size class and for the total across size classes (23). Trends in central tendency across severity in the premalignant data were evaluated by the Jonckheere-Terpstra test for ordered alternatives (24). This test can be used when the groups are independent; the alternative hypothesis is stated $\tau_1 \leq \tau_2 \leq \ldots \leq \tau_k$ and at least one strict inequality holds. τ_i is the median of the *i*th group.

Because five vessel size categories were evaluated for each morphological structure, measurements within morphological structure were not independent, *i.e.,* measurements within a given structure were more alike than measurements between morphological structures of the same type; correlations were strongest in adjacent size categories. Because each collection of five size classes and their total can be considered a family and the resulting statistical tests will not be independent, we controlled the family-wise error rate with the step-down adjustment for multiple comparisons, which is an option in the multiple testing procedure in SAS. All *P* values reported were based on bootstrap results unless stated otherwise.

cDNA Microarray Analyses. Data derived from cDNA microarrays were normalized for differences in the expression of housekeeping genes. Differences in gene expression levels were estimated using a bootstrap procedure with a step-down adjustment for multiple comparisons (23). This procedure incorporates the distributional characteristics of the data, as well as the dependence structure between the tests.

Western Blot Analyses. Western blot analyses represent semiquantitative estimates of the amount of a specific protein that is present in a cell extract. This fact was taken into account in the statistical evaluation of the data. The data displayed in the graphs are reported as means ± SDs of the actual scanning units derived from the densitometric analysis of each Western blot. Treatment effects were evaluated by the nonparametric Kruskal-Wallis test. To control for multiple comparisons, the *P* value accepted as statistically significant was $P = 0.017$ ($P = 0.05/3$, because three comparisons were made).

Exploratory assessment, graphs, and the resampling were done in SAS version 8.1 and SYSTAT version 10.

RESULTS

Body Weight and Carcinogenic Response. As intended, dietary energy restriction significantly reduced the rate of body weight gain and final body weights (207 *versus* 162 g, $P < 0.01$, *ad libitum versus* dietary energy restriction, respectively). Dietary energy restriction also reduced the incidence (96 *versus* 59%, $P < 0.01$, *ad libitum*

Table 1 *Effect of DER on the vascular density of premalignant mammary pathologies*

Vessel size category*	Unit of measure†	Treatment group‡	
		AL	DER
≤10	Count	1.9 ± 4.6 (0.0, 0.0, 0.0)	3.3 ± 11.1 (0.0, 0.0, 0.0)
>10 and ≤25	Count	40.4 ± 39.9 (8.9, 32.3, 54.5)	34.5 ± 40.5 (0.0, 14.2, 54.7)
>25 and ≤50	Count	79.4 ± 54.5 (40.2, 68.4, 110.9)	50.8 ± 37.1 (23.3, 46.8, 81.4)
>50 and ≤75	Count	40.9 ± 32.4 (16.3, 32.7, 57.3)	28.5 ± 30.6 (0.0, 13.1, 51.6)
>75	Count	85.48 ± 79.03 (45.3, 68.2, 96.1)	55.2 ± 40.9 (25.0, 49.7, 84.0)
Total	Count	248.03 ± 158.56 (152.9, 225.9, 310.8)	172.3 ± 97.9 (97.6, 139.5, 250.3)
≤10	Area	12.81 ± 32.55 (0.0, 0.0, 0.0)	25.9 ± 84.7 (0.0, 0.0, 0.0)
>10 and ≤25	Area	789.7 ± 785.6 (181.3, 617.5, 1,100.5)	616.2 ± 704.5 (0.0, 304.1, 1,056.9)
>25 and ≤50	Area	2,863.1 ± 1,990.6 (1,517.5, 2,457.5, 3,867.6)	1,846.0 ± 1,389.2 (861.0, 1,621.2, 2,917.6)
>50 and ≤ 75	Area	2,486.3 ± 1,972.5 (950.7, 1907.3, 3476.7)	1,766.9 ± 1,890.5 (0.0, 844.4, 3,149.3)
>75	Area	27,847.2 ± 32,487.5 (8,624.6, 15821.5, 31,142.8)	11,342.7 ± 10,891.9 (3,918.0, 8,242.5, 15,662.5)
Total	Area	33,999.2 ± 33,899.0 (13,449.2, 21,356.5, 38,165.3)	15,597.7 ± 12,245.1 (6,576.8, 11,874.9, 20,683.8)

Abbreviations: DER, dietary energy restriction; AL, *ad libitum*.

* Vessel size categories are in units of μm^2.

† Count data are average number of blood vessels/mm^2; area data are average area in μm^2/mm^2. Values are means ± SD. Numbers in parentheses are as follows: (25th percentile, median, 75th percentile). *N* was 60 premalignant pathologies in the AL group and 37 in the DER group.

‡ DER reduced overall vascular density of premalignant mammary pathologies when density was expressed as either vessel counts ($P = 0.02$) or vessel area ($P = 0.0002$); the predominate effect of DER in reducing vessel counts and area was on vessels > 25 μm^2 ($P < 0.025$ and $P < 0.005$, respectively).

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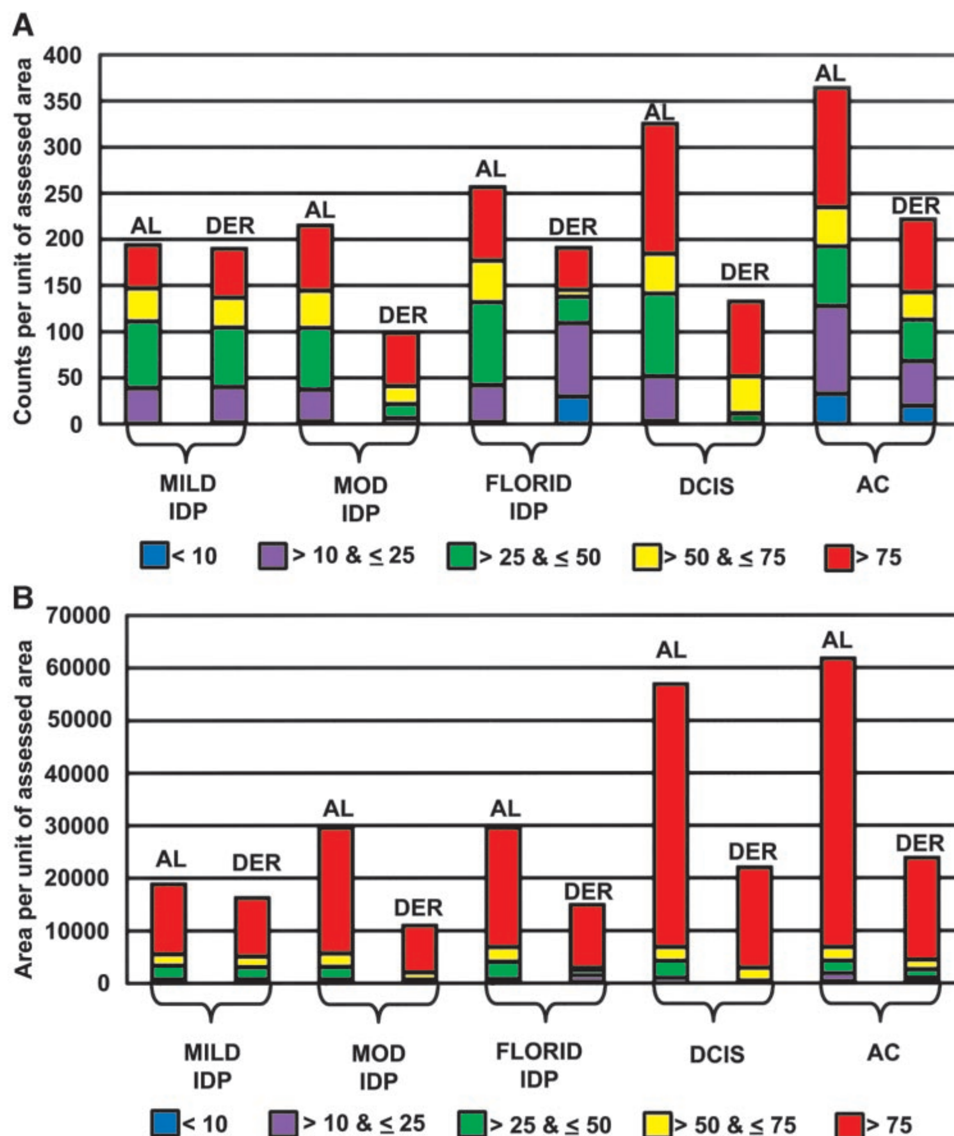

Fig. 1. Effect of dietary energy restriction (DER) on vascular density measured as blood vessel counts (*A*) or blood vessel area (*B*) in a 50-μm width band of fully intact stroma circumscribing each pathology. Stacked bar graphs permit visualization of the effects of DER on blood vessels within five size categories: 1, $\leq$10; 2, $>$10 and $\leq$25; 3, $>$25 and $\leq$50; 4, $>$50 and $\leq$75; and 5, $>$75 μm^2. These effects are shown for mild, moderate, and florid intraductal proliferations (IDPs), ductal carcinoma *in situ* (DCIS), and adenocarcinomas (ACs).

versus dietary energy restriction, respectively) and multiplicity (4.3 *versus* 1.0, $P < 0.001$, *ad libitum versus* dietary energy restriction, respectively) of palpable mammary carcinomas, a finding consistent with other studies (11, 25).

Effect of Dietary Energy Restriction on the Vascular Density on Premalignant Mammary Pathologies. The distribution of blood vessels associated with premalignant mammary pathologies has been reported (20). Dietary energy restriction significantly reduced overall vascular density of premalignant mammary pathologies when density was expressed as either vessel counts ($P = 0.02$) or vessel area ($P = 0.0002$) per unit of assessed area (Table 1). Further evaluation of these data by vessel size category indicated that the predominate effect of dietary energy restriction in reducing vessel counts and area was on vessels $> 25\ \mu m^2$ ($P < 0.025$ and $P < 0.005$, respectively). Additional hypotheses generating exploratory analyses indicated that dietary energy restriction had no significant effect on the density of blood vessels associated with mild hyperplasia (Fig. 1). On the other hand, dietary energy restriction resulted in a statistically significant reduction in both count and area for blood vessels that were associated with moderate or florid hyperplasia or DCIS, $P < 0.01$.

Effect of Dietary Energy Restriction on the Vascular Density of Mammary Carcinomas. The distribution of blood vessels associated with mammary carcinomas has been reported (16, 20). The density of blood vessels surrounding each carcinoma (extratumoral) and occurring within each carcinoma (intratumoral) was measured as blood vessel counts and area/unit of assessed area (Table 2).

Extratumoral Vascular Density. The overall density of extratumoral blood vessels was reduced by dietary energy restriction (counts and area, $P < 0.0001$). Additional analysis of these data showed that vascular density (counts or area) was numerically reduced in size categories 1–5 in dietary energy restriction-treated animals, but the effect was statistically significant only for vessel size categories 3–5 (all P values < 0.001).

Intratumoral Vascular Density. The intratumoral vascular density measured as counts or area of blood vessels in all vessel size categories except size category 5 was reduced in dietary energy restriction-treated animals, but none of these effects reached the level of statistical significance.

Effect of Dietary Energy Restriction on the Expression of Genes Involved in Blood Vessel Formation and Growth. As an initial assessment, total RNA was isolated from a limited number of carcinomas from each dietary treatment and transcript levels for 23 genes known be to involved in blood vessel formation and growth was assessed. The list of genes is shown in Fig. 2, and key results are summarized in Table 3. Transcript levels of only six of the genes assessed were above background (Fig. 2), and after normalization of

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Table 2 *Effect of DER on the vascular density of mammary carcinomas*

Vessel size category*	Unit of measure†	Extratumoral vascular density: Treatment group‡ AL	Extratumoral vascular density: Treatment group‡ DER	Intratumoral vascular density: Treatment group AL	Intratumoral vascular density: Treatment group DER
≤10	Count	32.8 ± 46.5 (0.0, 8.5, 57.5)	19.9 ± 46.2 (0.0, 1.0, 17.9)	6.70 ±12.9 (0.0, 1.5, 7.4)	9.9 ± 29.1 (0.0, 0.1, 4.6)
>10 and ≤25	Count	94.8 ± 111.9 (5.9, 45.4, 152.9)	47.9 ± 59.8 (1.0, 27.9, 72.4)	19.3 ± 24.9 (0.6, 7.0, 29.0)	15.6 ± 25.4 (0.3, 5.1, 16.2)
>25 and ≤50	Count	64.8 ± 37.2 (36.2, 62.6, 89.8)	45.4 ± 55.9 (6.5, 29.4, 53.0)	11.2 ± 8.4 (3.9, 10.4, 17.2)	11.5 ± 11.4 (1.4, 8.1, 20.7)
>50 and ≤75	Count	42.4 ± 29.5 (22.5, 37.3, 51.5)	29.6 ± 33.5 (9.6, 18.5, 36.5)	7.9 ± 9.1 (2.8, 6.1, 8.1)	6.8 ± 6.5 (2.1, 5.0, 8.3)
>75	Count	129.7 ± 52.9 (92.6, 119.0, 152.7)	79.2 ± 63.0 (33.5, 57.1, 106.0)	18.2 ± 18.9 (6.8, 11.9, 21.3)	16.5 ± 13.8 (8.6, 13.7, 21.1)
Total	Count	364.5 ± 188.8 (235.0, 294.3, 496.0)	222.0 ± 181.1 (88.4, 170.9, 262.1)	63.2 ± 44.1 (32.4, 56.2, 80.4)	60.4 ± 56.0 (25.4, 43.2, 85.4)
≤10	Area	239.3 ± 337.7 (0.0, 62.9, 441.8)	141.4 ± 321.0 (0.0, 8.4, 158.6)	48.2 ± 94.3 (0.0, 10.4, 58.8)	68.1 ± 187.3 (0.0, 0.8, 33.9)
>10 and ≤25	Area	1,583.5 ± 1,805.0 (120.2, 812.4, 2503.6)	807.5 ± 935.4 (22.0, 529.4, 1242.9)	319.5 ± 396.3 (11.5, 133.2, 500.1)	257.5 ± 380.7 (4.6, 112.4, 285.6)
>25 and ≤50	Area	2,320.4 ± 1,249.9 (1,362.1, 2,260.4, 3,054.4)	1,674.0 ± 2,103.7 (270.5, 1,018.1, 1,938.2)	407.1 ± 310.0 (153.7, 354.7, 617.7)	419.4 ± 428.4 (55.0, 286.4, 714.1)
>50 and ≤75	Area	2,629.8 ± 1,816.2 (1,361.6, 2,268.0, 3,251.6)	1,832.7 ± 2,050.2 (604.1, 1,184.5, 2,311.1)	492.7 ± 566.4 (176.6, 376.1, 517.2)	422.5 ± 399.2 (128.1, 305.7, 512.0)
>75	Area	54,937.0 ± 44,604.0 (28,547.5, 45,163.9, 63,097.6)	19,400.3 ± 15,459.3 (7,592.9, 13,722.0, 29,820.8)	3,873.9 ± 4,454.0 (1,050.4, 2,749.5, 5,310.8)	3,166.0 ± 2,818.6 (1,369.3, 2,984.0, 3,949.0)
Total	Area	61,710.0 ± 44,896.9 (35,211.9, 48,393.7, 72,276.9)	23,855.9 ± 19,032.1 (10,679.5, 16,885.7, 36,949.2)	5,141.5 ± 4,665.9 (2,112.5, 3,616.4, 7,564.4)	4,333.6 ± 3,347.3 (2,279.5, 3,612.5, 5,139.7)

Abbreviations: DER, dietary energy restriction; AL, *ad libitum*.

* Vessel size categories are in units of μm^2.

† Count data are average number of blood vessels/mm^2; area data are average area in $\mu m^2/mm^2$. Values are means ± SD. Numbers in parentheses are as follows: (25th percentile, median, 75th percentile). *N* was 45 carcinomas in the AL group and 41 in the DER group.

‡ Total density of extratumoral blood vessels was reduced by DER (counts and area, $P < 0.0001$); vascular density (counts or area) was numerically reduced in size categories 1–5 in DER-treated animals, but the effect was statistically significant only for vessel size categories 3–5 (all P values < 0.001). The intratumoral vascular density measured as counts or area was reduced in DER-treated animals, but none of these effects reached the level of statistical significance.

expression data for differences in the expression of housekeeping genes and a step-down adjustment of statistical results for multiple comparisons, none of the differences observed between *ad libitum* and dietary energy restriction was statistically significant.

Effect of Dietary Energy Restriction on the Expression of Proteins Involved in Angiogenesis. Although differences in transcript expression for genes involved in blood vessel formation were not statistically significant, the greatest numerical difference between the *ad libitum* and dietary energy restriction group was for VEGF. Therefore, we decided to evaluate a series of mammary carcinomas for protein levels of VEGF and two of its receptors, Flt-1 and Flk-1, via Western blotting. Representative blots and a summary of the results of these analyses are shown in Fig. 3. Although the levels of VEGF and Flt-1 in the carcinomas from dietary energy restriction-treated rats were 34.5 and 38.1%, respectively, of levels observed in carcinomas from *ad libitum*-treated rats, these differences were not statistically significant. However, levels of Flk-1 in carcinomas from dietary energy restriction-treated rats were 29.4% of those observed in *ad libitum*-treated rats, and this effect was statistically significant even after adjustment for multiple comparisons, with $P < 0.001$.

DISCUSSION

Using a rat model for breast cancer that is implemented in young growing animals, our laboratory has recently reported that blood vessel density increases during the morphological progression of transformed mammary epithelial cells through mild, moderate, and florid hyperplasia to DCIS and adenocarcinoma, effects that are likely to be because of both new blood vessel formation as well as the growth of existing blood vessels (20). Interestingly, dietary energy restriction has been reported to inhibit the occurrence of both premalignant and malignant mammary pathologies in the same model system, an effect associated with an inhibition of cell proliferation and an induction of apoptosis in epithelial cell populations within these pathologies (8–10). Although we have hypothesized that dietary energy restriction-mediated changes in the metabolism of insulin-like growth factor I and glucocorticoids directly alter rates of cell proliferation and apoptosis in transformed populations of epithelial cells (6), limitations in the supply of nutrients and in the elimination of metabolic wastes could induce similar effects. To our knowledge, there are no reports on the effects of dietary energy restriction on vascularization of premalignant or malignant mammary pathologies. In this discussion, we address three issues: (*a*) did dietary energy restriction affect vascular density; (*b*) if vascular density was affected, what does it mean relative to the cancer inhibitory activity of dietary energy restriction; and (*c*) what candidate mechanism(s) could account for the effects observed.

Premalignant Pathologies. Because vascular density increases across the morphologically defined steps in the progression of mammary carcinogenesis (20), the study hypothesis formulated was that dietary energy restriction would inhibit this pathology-associated increase in vascular density. The data shown in Table 1 and Fig. 1 are consistent with this hypothesis. Specifically, dietary energy restriction reduced blood vessel area/unit of assessed area by 54% and blood vessel counts by 31%. Although the impact of these differences on the diffusion of nutrients and metabolic wastes to and from the associated pathologies is a matter of speculation, the magnitude of these differences suggests that the effects are likely to be meaningful and merit additional investigation.

Discussion of candidate mechanisms that could account for the observed reduction in vascular density attributed to dietary energy restriction is somewhat problematic given the difficulty of inferring mechanisms from vascular density data collected from a single time point at the end of a study (20, 26, 27). However, as outlined in (20), the procedures used for vascular density analysis, *i.e.,* the classifica-

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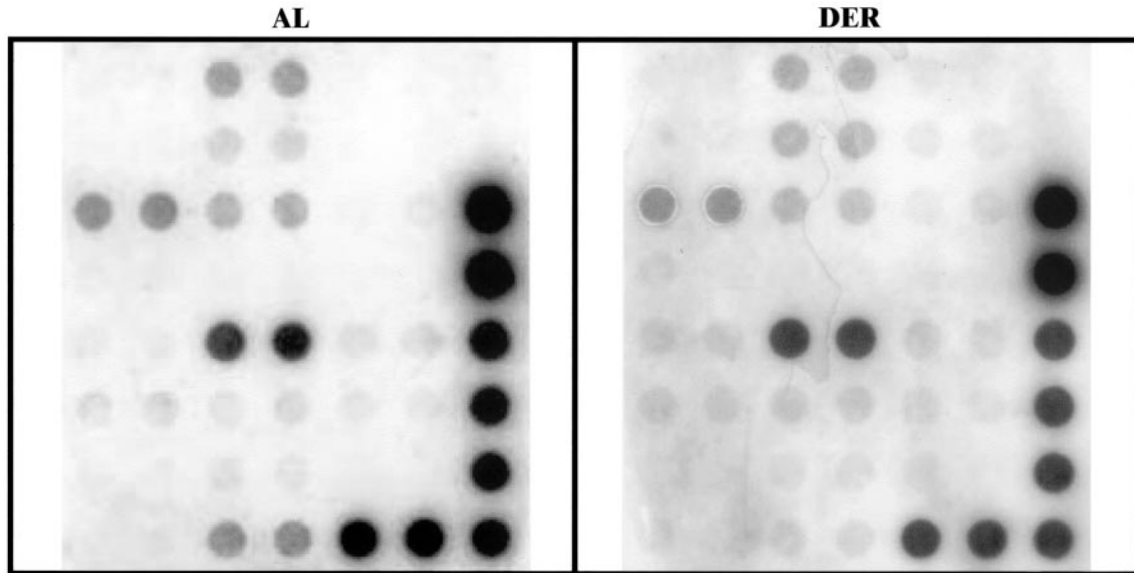

Distribution of genes

Angiopoietin 1	Angiopoietin 1	Angiopoietin 2	Angiopoietin 2	Angiogenin	Angiogenin	*pUC18*
bFGF	bFGF	Endostatin	Endostatin	Endothelin R type A	Endothelin R type A	*pUC18*
FGF-1	FGF-1	FGFR3	FGFR3	FLK1	FLK1	*β-actin*
FLT1	FLT1	FLT4	FLT4	Grol	Grol	*β-actin*
PEDF	PEDF	Pleiotrophin	Pleiotrophin	Smad5	Smad5	*GAPDH*
Smad7	Smad7	STK5	STK5	Tie 1	Tie 1	*GAPDH*
Tie 2 (TEK)	Tie 2 (TEK)	VEGF-B	VEGF-B	VEGF-C	VEGF-C	*GAPDH*
VEGF-D	VEGF-D	VEGI	VEGI	GAPDH	GAPDH	*GAPDH*

Fig. 2. The Cancer/Angiogenesis-1 GEArray kit (SuperArray) was used to detect the expression of genes involved in angiogenesis. The kit included 23 target genes plus two housekeeping genes and one internal control gene. Among 23 target genes, the expression of only six genes was above background. Three carcinomas (chips)/condition were hybridized. Results from a representative chips are shown. AL, *ad libitum* fed; DER, dietary energy restriction.

tion of blood vessels into size categories and census counting of vessels, were developed to address these limitations. There were 31% fewer blood vessels in the dietary energy restriction *versus ad libitum* group, *i.e.,* 76 fewer blood vessels/unit (mm^2) of assessed area. There are at least two mechanisms that could account for the observed decrease in blood vessel number: inhibition of angiogenesis or a reduction in the size of the pathologies such that their encroachment on existing blood vessels was reduced. On the basis of the vessel count data (Table 1), there was no evidence that angiogenesis was inhibited by dietary energy restriction. Specifically, the statistical evaluation of that data showed that the effects of dietary energy restriction were limited to blood vessels $> 25\ \mu m^2$ while we have operationally defined current angiogenic activity to be correlated with the occurrence of blood vessels $\leq 25\ \mu m^2$ (20). Nonetheless, it could be argued that a small reduction in neovascularization over the time course of this experiment could result in a greater effect than suggested by the data presented. On the other hand, data reported in Ref. 20 indicate that angiogenesis above the level observed in the terminal end bud, the morphological structure from which premalignant pathologies emerge, was not required for progression of premalignant mammary pathologies. An alternative reason for the 31% reduction in blood vessel number is that differences between treatment groups in the encroachment of premalignant pathologies on existing blood vessels resulted in differences in vascular density. Consistent with this explanation, the premalignant pathologies assessed in the dietary

Table 3 *Effect of DER on gene expression in mammary carcinomas*

Gene name	AL*	DER	Fold difference
FGF-1	0.753 ± 0.099	0.393 ± 0.065	1.9
FGFR-3	0.440 ± 0.075	0.270 ± 0.026	1.6
VEGF	0.537 ± 0.207	0.115 ± 0.039	4.6
Angiopoietin 2	0.560 ± 0.114	0.330 ± 0.102	1.7
Pleiotrophin	1.223 ± 0.153	0.983 ± 0.032	1.2
Endostatin	0.127 ± 0.015	0.253 ± 0.015	0.5

NOTE. The Cancer/Angiogenesis-1 GEArray kit (SuperArray) was used to detect the expression of genes involved in angiogenesis. The kit included 23 target genes plus two housekeeping genes and one internal control gene. Among 23 target genes, the expression of only six genes was above background.

Abbreviations: DER, 40% dietary energy restriction; AL, *ad libitum* fed; FGF-1, fibroblast growth factor 1; FGFR-3, fibroblast growth factor receptor 3.

* Each value is expressed in relative density units and is a mean ± SD. Differences in gene expression between groups were not statistically significant.

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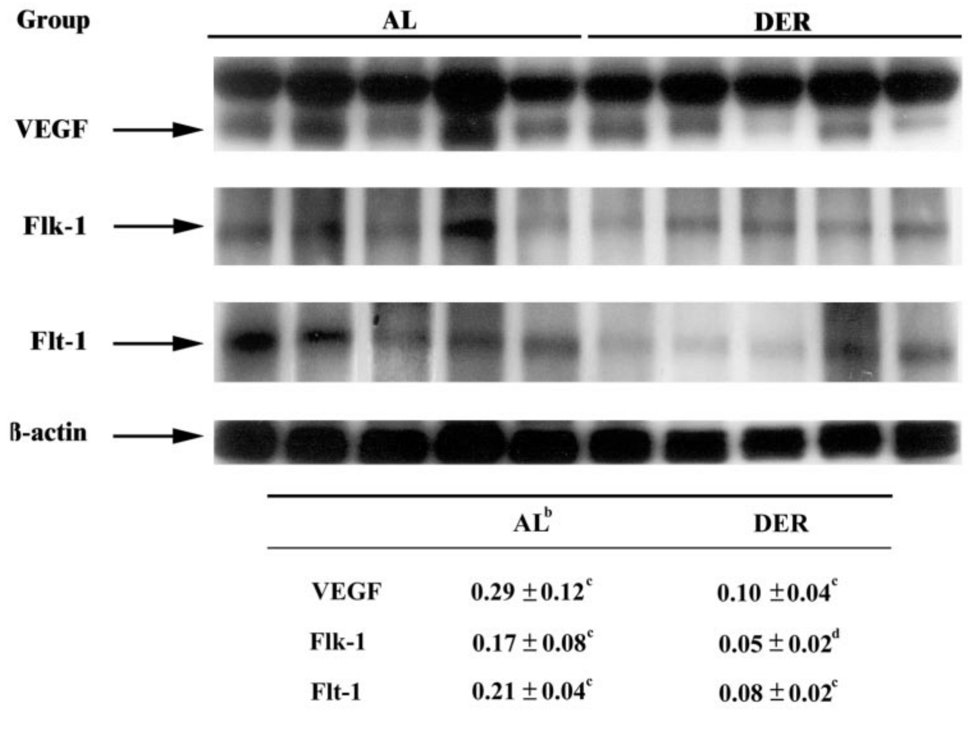

	AL[b]	DER
VEGF	0.29 ± 0.12[c]	0.10 ± 0.04[c]
Flk-1	0.17 ± 0.08[c]	0.05 ± 0.02[d]
Flt-1	0.21 ± 0.04[c]	0.08 ± 0.02[c]

Fig. 3. Representative Western blot analyses for vascular endothelial growth factor (VEGF) and its receptors, Flt-1 and Flk-1. A total of 16 carcinomas from each treatment group was evaluated: AL, *ad libitum* fed; DER, 40% dietary energy restriction. Each value is a mean ± SD, $n = 16$/treatment group. Data were normalized to β-actin. Values in a row with different alphabetical superscript ([c] and [d]) were significantly different ($P < 0.001$). To control for multiple comparisons, the P value accepted as statistically significant was $P \leq 0.017$ ($P = 0.05/3$, because three comparisons were made).

energy restriction group were smaller than those assessed in the *ad libitum* group (170.2 *versus* 90.5 mm^2, *ad libitum versus* dietary energy restriction; $P < 0.001$), and as noted above, dietary energy restriction reduced the number of blood vessels $> 25\ \mu m^2$, *i.e.,* established blood vessels.

The effect of dietary energy restriction on vascular density was ~20% greater on blood vessel area than counts. Although part of this effect could be explained by differences in the rate of disease progression mediated by dietary energy restriction, it is also possible that the growth of blood vessels was suppressed via direct effects of dietary energy restriction on endothelial cell proliferation and/or apoptosis. A direct test of this candidate mechanism will require additional studies and is likely to benefit from the use of cell isolation techniques such as laser capture microdissection.

Carcinomas. As reported in Ref. 20, the transition of premalignant mammary pathologies to adenocarcinomas is accompanied by a marked increase in blood vessels counts and area, particularly in vessel size categories $\leq 25\ \mu m^2$, the size categories that we operationally defined to reflect the induction of angiogenesis. This effect was observed in the assessment of both intratumoral and extratumoral vascular density, but as previously reported (16, 20), the magnitude of the effect was greater in the tissue immediately adjacent to carcinomas. On the basis of these observations, we hypothesized that dietary energy restriction would reduce the vascular density of mammary carcinomas in all vessel size categories. To test this hypothesis, a series of carcinomas from the *ad libitum* ($n = 45$) and dietary energy restriction ($n = 41$) groups were evaluated. The analysis was limited to carcinomas that were size matched to minimize differences in vascularization that are associated with size (20). The discussion, which follows, is separated into effects on extratumoral and intratumoral vascular density.

Extratumoral Vascular Density. As shown in Table 2 and Fig. 1, extratumoral vascular density was lower in carcinomas occurring in dietary energy restriction; an overall reduction of 39% was observed in the number of blood vessels/unit of assessed area and blood vessel area was reduced by 61%. These findings are consistent with the stated working hypothesis and parallel the findings reported for premalignant pathologies; the magnitude of the effects was similar, which supports the conclusion that the inhibition of vascularization by dietary energy restriction is likely to impose limitations on the supply of blood to associated carcinomas, a factor that needs to be considered when investigating the mechanisms that account for the cancer inhibitory activity of dietary energy restriction.

The data shown in Table 2 do not support all components of our study hypothesis. To our surprise, the effect of dietary energy restriction reached the level of statistical significance only for blood vessels $> 25\ \mu m^2$. Strictly speaking, this finding indicates that according to our criteria, angiogenesis was not inhibited by dietary energy restriction. However, additional scrutiny of the data (Table 2) indicates that caution is warranted in interpreting the statistical results. There is a difference in extratumoral vascular density of 143 blood vessels/mm^2 of assessed area. Of these blood vessels, 28% are in size categories $\leq 25\ \mu m^2$. Although differences between treatment groups in the encroachment of pathologies on existing blood vessels was cited as a possible explanation to account for the reduction by dietary energy restriction in blood vessel counts associated with premalignant pathologies, the carcinomas assessed were size matched, thus minimizing this possibility. The lack of significance in vessels $\leq 25\ \mu m^2$ may be due, at least in part, to the markedly higher variance associated with the count and area data in the dietary energy restriction group. Although the increase in variance could be attributed to differences in the measurement process, this is unlikely because carcinomas were assessed in a random sequence. Rather, we suspect that the variability reflects that clones of cells that were selected and that expanded and progressed to carcinomas despite the strong negative selection of pressure exerted by dietary energy restriction used different pathways

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to circumvent the inhibitory activity of dietary energy restriction; this resulted in variability in their ability to deal with limitations in blood supply. Interestingly, the largest numerical effect of dietary energy restriction was on blood vessel area not counts (61 *versus* 39% reduction), and the differences in area were greatest for vessels in size category 5, *i.e.,* vessels $> 75\ \mu m^2$. In our judgment, these data provide evidence that dietary energy restriction is likely to be affecting rates of cell proliferation and/or apoptosis in endothelial cell population in existing blood vessels. This possibility merits additional investigation.

Intratumoral Vascular Density. The data shown in Table 2 provide no support for the hypothesis that dietary energy restriction inhibited intratumoral blood vessel formation or growth. Specifically, no evidence was found that dietary energy restriction affected overall vascular density measured as either area or counts/unit of assessed area. These findings were unexpected and are in fact contrary to results in two reports we are aware of in which transplantable tumor model systems for prostate and brain were used to study the effects of dietary energy restriction on tumor angiogenesis. In that work (28, 29), dietary energy restriction was reported to inhibit tumor growth and to decrease mean intratumoral vascular density That differences would exist in transplantable *versus* chemically induced tumor models is not surprising, but comparisons of that work with our findings provided no obvious explanation for the differences noted. To further explore the lack of effect of dietary energy restriction on intratumoral vascular density, carcinomas from both treatment groups were subjected to analyses using cDNA microarrays and Western blotting to obtain a better understanding in tumors of the effects of dietary energy restriction on the synthesis of RNA and protein products known to be involved in blood vessel formation and growth

Molecular Analyses. Table 3 and Fig. 2 summarize an exploratory analysis of the effects of dietary energy restriction on expression of an array of genes involved in vascularization. Of the 23 genes assessed, the transcript levels of only six genes (refer to Table 3) were above background, and of these, differences in expression between *ad libitum* and dietary energy restriction did not reach the level of statistical significance, although a 4.6-fold difference in the transcript level of VEGF was noted. It was for this reason that Western blottings for VEGF and two of its receptors were performed on 16 carcinomas from each treatment group. Those assays revealed differences in levels of VEGF, Flt-1, and Flk-1 (Fig. 3), but other than the effect of dietary energy restriction in reducing Flk-1, the differences did not reach the level of statistical significance. In general, these results are consistent with the data on intratumoral vessel density.

Conclusions. Dietary energy restriction reduced the density of blood vessels associated with premalignant mammary pathologies as well as the density of blood vessels in immediate proximity to mammary carcinomas. Although it is a matter of speculation whether the magnitude of the observed differences in blood vessel density was affected by the age of the animals used in this model for breast, the data presented are consistent with the possibility that dietary energy restriction inhibited the growth of endothelial cells. It remains unresolved whether dietary energy restriction has a specific effect on angiogenesis. On the basis of the effects of dietary energy restriction on vascular density, it can be inferred that dietary energy restriction imposes limitations on the supply of nutrients to and elimination of wastes from developing pathologies; these limitations could exert direct effects on cell proliferation and apoptosis in transformed epithelial cell populations undergoing clonal selection and expansion. The factors that account for the failure of dietary energy restriction to limit intratumoral blood vessel formation and growth are not obvious and highlight the potential importance of studying the mechanisms that account for resistance to the protective effects of dietary energy restriction, an area of investigation that has been largely neglected.

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