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Dietary lipids and calorie restriction affect mammary tumor incidence and gene expression in mouse mammary tumor virus/v-Ha-*ras* transgenic mice

(breast cancer/ω3 lipids/ω6 lipids/p53/food restriction)

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ABSTRACT **We have studied the effects of food restriction (FR) and substitution of fish oil (FO; ω3) for corn oil (CO; ω6) on breast tumor incidence and survival in mouse mammary tumor virus/v-Ha-*ras* transgenic (Onco) mice. The diets were as follows: group 1, 5% (wt/wt) CO fed ad libitum (AL); group 2, 5% CO, restricted calories (40% fewer calories than AL; FR); group 3, 20% CO fed AL; and group 4, 20% FO fed AL. After 3 years, 40% of FR Onco (group 2) mice were alive, whereas there were no survivors in the other three groups. Similarly, tumor incidence was reduced to 27% (5 out of 18) in FR animals (group 2), whereas it was 83% (11 out of 13) in group 1 mice, 89% (16 out of 18) in group 3 mice, and 71% (10 out of 14) in group 4 mice. These protective effects of FR on survival and tumor incidence were paralleled by higher expression of the tumor suppressor gene p53 (wild type) and free-radical scavenging enzymes (catalase and superoxide dismutase) in breast tumors. Immunoblotting showed less *ras* gene product, p21, and increased p53 levels in the tumors of FR mice. In addition, FR decreased RNA levels of c-*erbB-2*, interleukin 6, and the transgene v-Ha-*ras* in tumors. In contrast, analysis of hepatic mRNA from tumor-bearing FR mice revealed higher expression of catalase, glutathione peroxidase, and superoxide dismutase. Survival and tumor incidence were not influenced significantly by dietary supplementation with FO in place of CO. Taken together, our studies suggest that moderate restriction of energy intake significantly inhibited the development of mammary tumors and altered expression of cytokines, oncogenes, and free-radical scavenging enzymes.**

Food restriction (FR) decreases the incidence of spontaneous tumors in rodents and reduces the incidence of breast tumors in C3H/Bi, C3H/He, and C3H/Ou mice (1–5). The latter develop breast tumors due to transmission of the mouse mammary tumor virus (MMTV) during nursing (4). In these animals, FR decreases prolactin levels and anti-MMTV antibody levels, which correlate closely with decreased viral (A and B) particles in mammary glands in C3H/Bi mice (4). ras is a critical component of intracellular mitogenic signaling pathways, and its activated forms can transform cells (6, 7). Transgenic mice serve as a powerful tool to study the effects of tissue-specific expression of activated genes (8, 9), and the MMTV/v-Ha-*ras* Onco mouse provides a model in which to investigate the effects of ras on breast tumors in mice (10). This mouse has been derived by fusing the MMTV long terminal repeat as a promoter of v-Ha-*ras* containing activating mutations in codons 12 (Gly → Arg) and 59 (Ala → Thr). The MMTV promoter is glucocorticoid and prolactin inducible and is tropic for breast tissue; the result is that a high level of transgene expression occurs in the mammary glands and to a variable extent in other organs (10).

We examined the influence of FR and also the source [corn oil (CO) and fish oil (FO)] and level [5% or 20% (wt/wt)] of dietary lipid on tumor incidence and expression of genes reported to be associated with the initiation of breast tumors and their transformation to malignancy. The study was carried out for a period of 3 years by using the commercially available transgenic Onco mouse first constructed by Leder and coworkers (10). A preliminary report of this work has been presented (45).

METHODS

Animals and Diets. Weanling female MMTV/v-Ha-*ras* transgenic mice were purchased from DuPont. Mice positive for the transgene by Southern blot analysis of genomic DNA isolated from epithelial cells of the tails of the mice were utilized for the study. The mice were fed nutritionally adequate semipurified diets containing CO, which has higher levels of oleic acid (18:1ω6) and linoleic acid (18:2ω6), or FO, which is richer in palmitic acid (16:0), eicosapentaenoic acid (20:5ω3), and docosahexanoic acid (22:6ω3). The dietary regimen was initiated when the mice were weaned at 1 month of age and contained four groups: group 1, 5% (wt/wt) CO fed ad libitum (AL); group 2, 5% CO (40% calorie restriction as compared to group 1; FR); group 3, 20% CO fed AL; and group 4, 20% FO fed AL. The composition of the semipurified diet is given in Table 1. Diets were prepared weekly, and a known amount of food was provided daily. Precautions were taken to avoid lipid peroxidation. The mice were maintained in plastic cages, and a 12-h light/12-h dark cycle was followed. Body weights were recorded monthly. Animals with large tumors were killed, and both tumors and livers were collected aseptically, snap frozen in liquid nitrogen, and stored at −80°C until further use.

Isolation of RNA and Northern Blot Analysis. Total RNA was extracted from mammary tumors (five mice per group) and livers (three mice per group) by the acid/guanidinium isothiocyanate/phenol/chloroform extraction procedure (11). Twenty micrograms of total RNA was electrophoresed, electroblotted onto nitrocellulose (Schleicher & Schuell), and fixed to the membrane by UV irradiation (Stratalinker 1800; Stratagene). The blots were prehybridized, hybridized, and washed according to the procedure described in detail else-

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Abbreviations: CO, corn oil; AL, ad libitum; FR, food restriction; FO, fish oil; CAT, catalase; SOD, superoxide dismutase; GSH-Px, glutathione peroxidase; MMTV, mouse mammary tumor virus; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL-6, interleukin 6.

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Table 1. Diet components

Ingredients	Weight, % 5% CO AL	5% CO FR	20% CO AL	20% FO AL
CO	5.00	5.00	20.00	1.00
FO	—	—	—	20.00
Casein	20.00	20.00	24.60	24.60
Corn starch	15.00	13.50	13.60	12.60
Sucrose	50.00	47.00	29.50	29.50
Fiber	5.00	5.00	6.10	6.10
DL-Methionine	0.30	0.30	0.40	0.40
Vitamin mix	1.00	2.00	1.20	1.20
Mineral mix	3.50	7.00	4.30	4.30
Choline bitartrate	0.20	0.20	0.30	0.30
Total	100.00	100.00	100.00	100.00

The FR mice were given 60% of the calories of the mice fed the 5% CO AL diet. CO was obtained from Harlan Bioproducts (Indianapolis), FO (deodorized menhaden oil) was obtained through the National Institutes of Health from Charleston Laboratories (Charleston, SC), and the rest of the dietary components including the vitamin mix (vitamin diet fortification) and mineral mix (AIN mineral mixture 76) were from ICN.

where (12). All blots were exposed at −80°C to Kodak XAR-5 film with DuPont intensifying screens. The intensity of autoradiographic bands was determined semiquantitatively by video image analysis using the National Institutes of Health IMAGE 1.4 program. The densitometric intensity of a specific RNA band was normalized to that of the constitutively expressed glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA. The data were expressed as the ratio of the intensity of the specific RNA band to that for GAPDH. The cDNA probes (American Type Culture Collection) used in this study were interleukin 6 (IL-6; 5.2 kb, *Sst* I), murine v-Ha-*ras* (2.2 kb, *Bam*HI–*Eco*RI), human c-*erbB-2* (1.45 kb, *Eco*RI), catalase (CAT; 1.2 kb, *Xho* I), human superoxide dismutase (SOD; 0.6 kb; *Pst* I), human glutathione peroxidase (GSH-Px; 0.55 kb, *Eco*RI), human GAPDH (1.2 kb, *Pst* I), and p53 (1.4 kb, *Xba* I; a generous gift from Richard M. Elledge, University of Texas Health Science Center, San Antonio). The cDNA probes were labeled with [α-^{32}P]dCTP (3000 Ci/mmol; 1 Ci = 37 GBq; Amersham) by random prime labeling (Boehringer Mannheim) to a specific activity of 0.8–1.6 × 10^9 cpm/μg.

Western Blot Analysis. Western blot analysis was carried out essentially as described (13). Equal amounts of total tumor homogenate were subjected to Tris/glycine/SDS/16.5% PAGE. Proteins were electroblotted onto nitrocellulose membranes, and nonspecific binding sites were blocked with 10% (vol/vol) normal goat serum for 1 h at 23°C. The blots were then processed essentially as described (12, 13). The antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) used were anti-v-Ha-Ras (non-neutralizing, rat anti-mouse Ha-Ras p21; does not cross-react with v-K-Ras or c-K-Ras p21) and anti-p53 (a monoclonal antibody that reacts with both wild-type and mutant p53, raised against amino acids 14–389). After extensive washing, the blots were exposed to Kodak XAR-5 film at −80°C with intensifying screens.

Statistical Analyses. The values are expressed as the mean ± SEM. Statistical analyses of the data were carried out using Student's *t* test, and $P < 0.05$ was considered significant.

Histology. Histological sections of tumors were prepared by standard methods and stained with hematoxylin/eosin. Tumors were classified as small-cell, intermediate-cell, and large-cell types as described (14).

RESULTS

Transgene Expression. Before the start of the study, all mice were tested, and the presence of the v-Ha-*ras* transgene was confirmed by Southern blot analysis of DNA isolated from epithelial cells of the tails. Further, the analysis was repeated in animals that died spontaneously with or without tumors. In FR mice, the transgene persisted even at 3 years of age. Hence, the variation in life span and tumor incidence noted was not due to loss of the transgene.

Survival, Histopathology, Tumor Incidence, and Body Weight. The composition of the FR diet was similar to that of 5% CO (group 1), but the availability of the diet was restricted so that only 60% of the energy was available as compared to that of AL fed mice. FR at this level does not produce malnutrition, and calorie intake, when analyzed per gram of body weight, was similar in all the groups. The distribution of tumor cell subtypes was not altered by dietary intake. Small-cell tumors constituted 6–8%, intermediate-cell tumors constituted 6–16%, and large-cell tumors constituted 12–23% of the tumors.

Twelve to 18 mice per group were analyzed to compare the influences of source and level of dietary lipid and/or FR. Fig. 1*B* shows the survival analysis of Onco mice. When fed AL diets that contained CO (5% or 20%) or FO (20%), the mice exhibited decreased survival as compared to FR mice. By 16 months of age, ≈50% of AL fed mice died, and by 32 months, 100% had died. In each AL fed group, 2 or 3 mice died spontaneously without tumors while others were sacrificed when found moribund with tumors. By contrast, only 33% of FR mice had died (vs. AL; $P < 0.01$) at 16 months of age, and 50% were still alive at 32 months of age (vs. AL; $P < 0.002$). When tumor incidence was analyzed, only 27% (5 out of 18) of FR mice had developed tumors. Further, these tumors, though visible, were generally markedly reduced in size as compared to tumors in the AL groups. The AL fed mice had tumor incidences of 71% or more (5% CO, 11 out of 13, 83%; 20% CO, 16 out of 18, 89%; 20% FO, 10 out of 14, 71%).

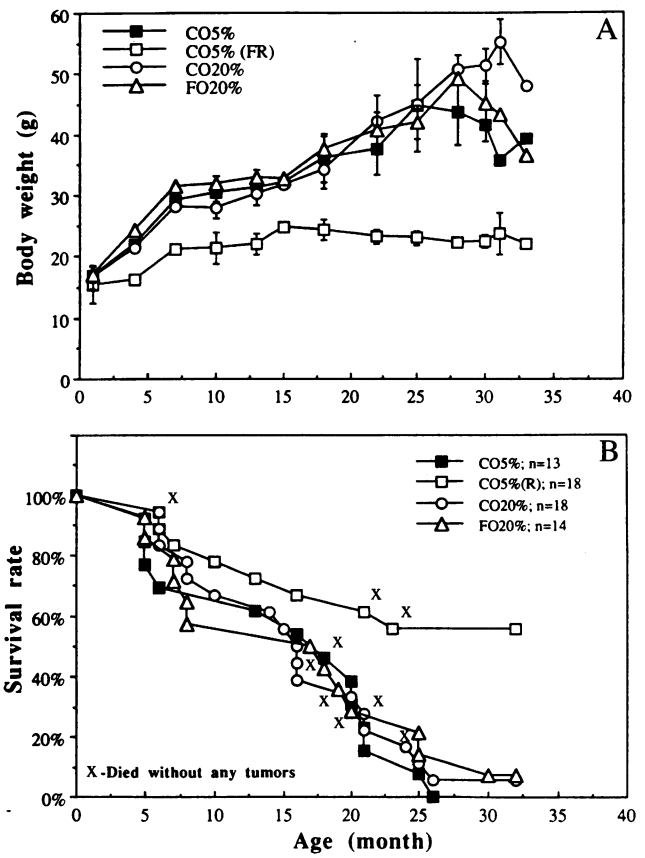

FIG. 1. Effect of FR and/or level and source of dietary lipids on body weight (*A*) and survival rate (*B*) of Onco mice.

Several AL fed mice developed multiple tumors, whereas FR mice all had a single breast tumor. Besides visible breast tumors, a few mice developed lacrimal gland tumors. Each mouse with a lacrimal gland tumor developed early and rapidly growing breast tumors, whereas mice free of lacrimal gland abnormalities developed tumors late in life.

Fig. 1*A* presents an analysis of body weights in the different groups. At the start of the study, the average body weight was ≈17 g. The body weights of AL fed mice increased steadily and significantly up to 25 months of age. The body weights of FR mice increased steadily up to 7 months of age and maintained a stable plateau thereafter until the end of the 3-year study period. Their weights were consistently lower than those of mice in the AL fed groups.

Northern Blot Analysis of RNA Isolated from Tumors. A solid piece of tumor was collected several weeks after detection in AL or in FR groups. Total RNA isolated from these tumors was analyzed by Northern blot to evaluate alterations in expression of v-Ha-*ras*, p53, c-*erbB-2*, IL-6, and the antioxidant enzymes CAT and SOD. Fig. 2 compares the autoradiographs and densitometric analyses of bands for gene expression in tumors from 5% CO AL and 5% CO FR mice. FR significantly decreased the expression of v-Ha-*ras* (1.4-fold; $P < 0.02$) and c-*erbB-2* (1.8-fold; $P < 0.05$). These are oncogenes potentially involved in malignant transformation. Interestingly, expression of p53, a tumor suppressor gene, was increased in the FR mice (2.54-fold; $P < 0.05$ vs. that of AL, in tumors of FR mice) (Fig. 2).

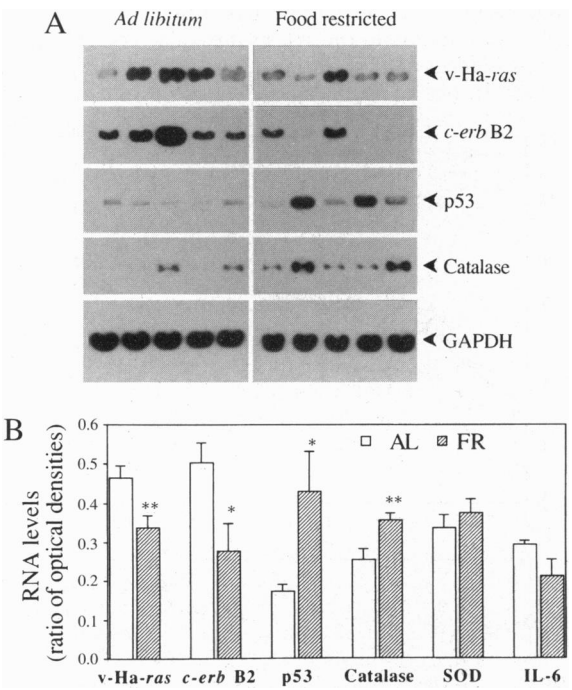

FIG. 2. (*A*) Northern blot analysis of mRNA isolated from tumors from Onco mice. Twenty micrograms of total RNA was size fractionated, electroblotted onto a nitrocellulose membrane, fixed by UV irradiation, and hybridized with the cDNA probes as indicated. The same membrane was used after stripping off the previous probe. mRNA sizes were determined in comparison to the relative mobility of mRNA standards and to that of 28S and 18S rRNA. The autoradiographic exposure time was 4–6 days for all samples except was GAPDH, in which case it was 1.5 days. (*B*) Densitometric analysis. Video image analysis was performed using the National Institutes of Health IMAGE 1.4 program on the autoradiograms shown in *A*. Values (mean ± SE) were represented as a ratio (arbitrary numbers obtained for a specific RNA band to that of GAPDH). *, $P < 0.05$; **, $P < 0.02$ significantly different when compared to AL fed mice.

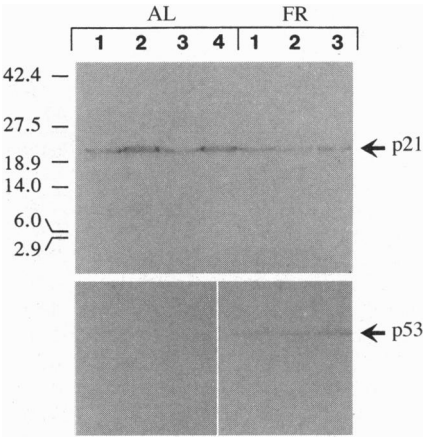

FIG. 3. Immunoblotting. Equal amounts of protein per well were separated on either Tricine/SDS/16.5% PAGE (p21) or SDS/7.5% PAGE (p53) and electroblotted at 4°C onto a nitrocellulose membrane. Nonspecific protein binding sites were blocked with 10% (vol/vol) normal goat serum and then incubated sequentially with monoclonal antibodies against v-Ha-Ras (p21) or p53, secondary antibody, and ^{125}I-labeled protein A, washing after each incubation. Autoradiographic exposure time was 6 days. No signal was detected in AL fed mice even after exposing the membrane for a prolonged period. Prestained molecular weight standards (kDa) are shown on the left. The arrows indicate the specific proteins detected by the antibodies.

Beneficial influences of FR have been linked to reduction in reactive oxygen intermediates as a consequence of increased expression of free-radical scavenging enzymes. Hence we studied the expression of the antioxidant enzymes CAT and SOD in the tumors. As shown in Fig. 2, FR increased expression of CAT in tumors (1.4-fold; $P < 0.02$). By contrast, expression of SOD (Fig. 2*B*) was not altered by FR. Expression of IL-6, which may be involved in induction and progression of tumors, was lower in tumors from FR mice, but the differences did not reach significance when compared to 5% CO AL mice (Fig. 2*B*). FR seemed to be a key element in regulation of gene expression in tumors since gene expression in tumors from 20% CO AL and 20% FO AL mice was similar to that observed in 5% CO AL mice (data not shown).

Immunoblotting. Equal amounts of protein from the tumors of 5% CO AL and FR mice were electrophoresed through SDS/PAGE and analyzed by immunoblotting to study the level of the transgene as well as the level of p53 protein. Fig. 3 shows that the level of v-Ha-*ras* p21 protein was considerably lower in tumors from FR mice. By contrast, p53 expression was detected only in FR mice, and even after prolonged exposure, no p53 expression could be detected in tumors from the mice fed AL (Fig. 3).

Northern Blot Analysis of RNA Isolated from Livers. Total RNA was isolated from livers of tumor-bearing mice and analyzed by Northern blot to study the expression of CAT, GSH-Px, and SOD. Fig. 4 shows autoradiograms and densitometric analyses of antioxidant enzyme gene expression. The levels of CAT (1.4-fold; $P < 0.05$) and GSH-Px (4.1-fold; $P < 0.001$) were significantly higher in FR mice as compared to those of the groups fed AL.

DISCUSSION

These findings establish that mammary tumorigenesis is inhibited in FR transgenic Onco mice by life-long (3 years) moderate energy restriction. Further, this action is paralleled by a reduced expression of the transgene-v-Ha-*ras*, the oncogene c-*erbB-2*, and IL-6. In contrast, FR maintained higher levels of expression of the tumor suppressor gene p53 and

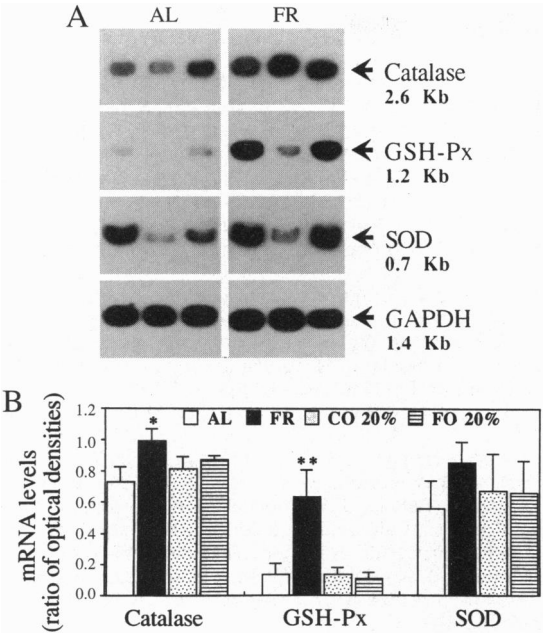

FIG. 4. (*A*) Northern blot analysis of total RNA isolated from livers of tumor-bearing Onco mice consuming either FR or AL diets. Twenty micrograms of total RNA was size fractionated, electroblotted onto a nitrocellulose membrane, fixed by UV irradiation, and hybridized with the cDNA probes indicated. The same membrane was used after stripping off its previous label. mRNA sizes were determined in comparison to the relative mobility of 28S and 18S rRNA and mRNA standards. The autoradiographic exposure time was 4 days for CAT, GSH-Px, and SOD and 1.5 days for GAPDH. (*B*) Densitometric analysis of autoradiographic bands obtained by Northern blot analysis of total RNA isolated from tumors from Onco mice on FR or AL diets supplemented with either CO or FO at 20% (wt/wt) (autoradiographic data on 20% diets not shown). Video image analysis was performed essentially as described in *Methods.* Values (n = 3) (mean ± SE) are represented as ratios (numbers obtained for CAT, GSH-Px, and SOD to that of GAPDH). The autoradiographic exposure time was 4 days for CAT, GSH-Px, and SOD and 1.5 days for GAPDH.

antioxidant enzymes in tumors (CAT and SOD) and liver (CAT, GSH-Px, and SOD).

Several studies have demonstrated reduced tumor incidence in rodents fed either a low calorie or a low fat diet (15–19). The exact mechanisms by which malignancies are lowered or prevented by diet have not yet been fully elucidated. Earlier observations emphasized changes in endocrine hormones, inhibition in expression of viral particles, and changes in immune functions (15–21). Recent studies have revealed that FR may decrease malignancy and extend the life span by inhibiting free-radical formation, which reduces DNA damage, and inhibiting secretion of proinflammatory cytokines (15, 22–25). In keeping with earlier observations, reduced tumor incidence has been linked to reduced accumulation of viral transcripts in FR animals (4, 24). A challenging question has been whether FR will reduce development of mammary tumors in transgenic animals. Our study clearly establishes that FR reduces tumor incidence in MMTV/v-Ha-*ras* mice by significantly altering the expression of genes that are linked to oncogenesis.

Oncogenesis and carcinogenesis appear to be a multistep process that involves activation of cellular protooncogenes and inactivation of tumor suppressor genes. One group of oncogenes that has been studied extensively is the ras family. ras encodes a GTP-binding and GTPase-activating protein of M_r 21,000 ($p21^{ras}$) (6, 7). The proteins are located mainly in the plasma membrane and to a minor extent in the cytoplasm and are involved in the transduction of signals for cellular proliferation (26). Mutations of the *ras* gene lead to $p21^{ras}$ products deficient in GTPase activity, with loss of control of cellular proliferation (27). The role of ras in initiation or progression of breast cancer remains controversial. However, expression of *ras* mRNA and protein occurs in normal breast epithelium, and its overexpression has been noted in breast tumors (28). Thus activated *ras* may be involved in the etiology or progression of breast cancer. Further, the $p21^{ras}$ gene product has been shown to mediate autocrine production of growth factors in transformed cells and can also promote an estrogen-independent aggressive phenotype (29, 30).

In the present study, increased *ras* gene and protein expression were observed in all three AL groups, and the expression was significantly inhibited in the tumors from FR mice. In addition to the *ras* oncogene, c-*erbB-2/neu* protooncogene expression was decreased in tumors from the Onco mice studied herein. Overexpression of *neu*, a M_r 185,000 (p185) transmembrane protein with tyrosine kinase activity, is associated with decreased survival of humans with breast cancer as well as increased lymph node metastasis in breast cancer patients (31, 32). The results presented here indicate that FR decreases expression of c-*erbB-2/neu* and suppresses tumorigenesis in Onco mice. This influence seemed to be relatively specific to decreased energy intake. The other three dietary groups included groups in which ω3 highly unsaturated dietary lipids were substituted for CO and also increased consumption of CO from 5% to 20%. Only FR reduced the expression of c-*erbB-2/neu.*

Decreased expression of IL-6 by FR in parallel with increased survival in the Onco mouse suggests that this multifunctional cytokine may play a role in tumor growth (33). IL-6 might also play a role in migration and metastasis of solid tumors (34, 35). Neutralizing antibodies to IL-6 suppress the growth of solid tumors, and IL-6 promotes migration and metastasis of breast carcinoma cells (34, 35).

The p53 gene codes for a potent tumor suppressor gene, and its activity appears to be mediated by the transcriptional activation of p53 responsive genes (36). Further, reintroduction of wild-type p53 into tumor cells containing a mutant p53 can inhibit growth depending on the extent of DNA damage that these cells have accumulated (37). In human breast cancer, the p53 tumor suppressor gene is frequently inactivated by mutation or gene loss (38). In the context of multistep carcinogenesis, it seems likely that p53 loss or mutation may play a critical role early in deregulation of cell proliferation. It is interesting to note that FR maintained higher p53 levels in tumors while suppressing expression of *ras.* The expression of *ras* in the Onco mouse is linked to its MMTV promoter and may therefore be driven by hormonal factors that, in turn, may be influenced by diet (4, 24, 39).

However, diet also regulates p53 gene expression (40). When a cohort of $p53^{-/-}$ knockout mice were fed an FR diet, both tumor development and tumor-related mortality were reduced as compared to AL fed knockout mice, indicating that dietary manipulations can modify genomic lability and subsequent tumorigenesis. FR in $p53^{-/-}$ mice prevented premature entry of splenic lymphocytes into S phase, suppressing possible mutations in other oncogenes (40). Since wild-type p53 is a negative regulator of cell proliferation (arrests cells and codes for G_1 phase) and a molecule that promotes apoptosis (41), suppression of tumorigenesis in FR Onco mice could be due to increased apoptosis of the cancer cells. It has recently been postulated that FR retards aging and tumorigenesis by upregulating apoptosis and by enhancing expression of free-radical scavenging enzymes (42). FR has been reported to curtail hepatic carcinogenesis by enhancing apoptosis of initiated or preneoplastic/cancerous cells, thus suppressing both tumor initiation and proliferation (43, 44). In the present study, molecular events that are associated with suppression of tumorigenesis have been observed. FR preserved expression of p53 and suppressed expression of v-Ha-*ras* and also the

oncogene c-*erbB-2*. In contrast, FR mice maintained higher expression of key antioxidant enzymes—CAT and SOD in tumors and CAT and GSH-Px in livers—implying that maintenance of efficient free-radical scavenging mechanisms may also be relevant to decreased tumorigenesis and perhaps prolongation of life as well.

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