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Conjugated linoleic acid (CLA) is a naturally occurring component of the food supply that inhibits mammary carcinogenesis. Data obtained on this project indicate: 1) that the anticancer activity of CLA is unlikely to be explained based soley on its antioxidant activity; 2) that CLA is unlikely to inhibit carcinogenesis by directly antagonizing linoleic acid metabolism; 3) that CLA inhibits carcinogenesis irrespective of the presence or absence of the codon 61 mutation in the Ha-ras gene. Candidate genes that are over-expressed specifically in mammary carcinomas have been identified. The effects of CLA on the expression of these genes will be investigated to determined if they are involved in the inhibition of mammary carcinogenesis by CLA.

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Henry J. Thompson 10/8/97

PI - Signature / Date

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INTRODUCTION

PROPOSAL RELEVANCE. In 1981 Doll and Peto (1) estimated that nutrients and other dietary factors could account for a significant percentage of the risk for epithelial cancers in the United States and recently Doll (2) has suggested that approximately 35% of these cancers may be preventable via changes in dietary behaviors. Of the nutritional and dietary factors considered with regard to the risk for breast cancer, the role that the amount and type of dietary fat and calories play in the disease process has received prominent attention. This work has recently been reviewed (3,4). Two facts that have surfaced in this area of investigation are particularly relevant to the experiments being conducted. First, the level of caloric intake has a prominent effect on mammary tumorigenesis (3) and second, dietary fat has a specific effect on mammary tumorigenesis, but this effect is observed only when caloric intake is ad libitum (3). Our laboratory was one of the first to report the requirement for ad libitum intake for a fat specific effect on mammary tumorigenesis to be manifest (5), an observation that has recently been confirmed by others (6). It appears that this observation applies over a range of dietary fat concentrations. Given that a major health concern in the United States continues to be the consequences of intake of calories in excess of energy needs, it is probable that fat specific effects are being exerted in the U.S. population and other societies in which there is a surfeit of dietary calories.

As part of an overall public health initiative, Americans are being encouraged to eat less and exercise more in order to maintain "ideal" body weight, and to reduce the percent of dietary calories that they consume as fat (7). This advice is given with greatest specificity for prophylaxis of diseases of the heart, but these recommendations also apply to cancer, especially of the breast and colon. In general, it is recommended that dietary fat intake be reduced to $\leq$ 30% dietary calories with $\leq$ 10% provided as saturated fat, 10% as monounsaturated fat and 10% as polyunsaturated fat. An opportunity exists, therefore, to make recommendations about the specific fats that provide these calories. With regard to cancer, a principal interest lies in altering the type of polyunsaturated fatty acids (PUFA) that are being ingested. The question now receiving particular attention is whether all families of PUFA have similar effects on tumorigenesis and if individual fatty acids have selective effects on the mammary gland. ***The program of research being conducted on this grant specifically addresses this issue. We are investigating the cancer preventive activity of a specific fatty acid, conjugated linoleic acid (CLA), and we are studying various mechanisms that may account for its protective activity.***

CLA , a collective term that refers to conjugated dienoic derivatives of linoleic acid, is a naturally occurring substance in dairy products and in animal tissues. In a number of recent publications evidence has emerged indicating that CLA fed in the diet is a potent inhibitor of chemically-induced mammary carcinogenesis in the rat (8-11). This effect of CLA is in sharp contrast to that of linoleic acid which has been shown to stimulate the carcinogenic process in the same tumor model system in a dose dependent manner. Of added interest is the apparent potency of CLA in cancer prevention in comparison to other fatty acids reported to have cancer inhibitory activity. The most prominent among these are the fatty acids in fish oil. However, the amount of fish oil needed for cancer inhibitory activity usually exceeds 10% (w/w) in the diet. Recent work indicates that a level of CLA as low as 0.1% (w/w) was sufficient to produce a significant inhibition of mammary carcinogenesis. Thus, CLA is considerably more potent than any other fatty acid in inhibiting tumor development.

The potential relevance of these observations for cancer prevention in humans is considerable. In a direct extrapolation of the laboratory animal data to a 55 kg person, the amount of CLA required for cancer prevention would be equivalent to 2.8 g per day. The current estimate of

CLA consumption per day in the United States is 1 gram. The difference in these values is relatively small. Given that dietary levels of at least 1.5% CLA (w/w) can be fed chronically without adverse consequences, it appears that achieving a protective level of CLA consumption is quite feasible. CLA offers great potential as a preventive agent and could even be provided at effective levels via the food supply either via designer foods or as a dietary supplement.

In the work currently being conducted on this grant we are investigating the biological activity(s) of CLA that accounts for its cancer preventive activity. Our working hypothesis is that CLA affects the processes of clonal expansion and/or clonal selection via modulating genetic and/or epigenetic mechanisms obligatory for, or permissive to the carcinogenic process. This hypothesis is being evaluated by determining the effect of CLA on the expression of molecular markers relevant to the process of mammary carcinogenesis. These investigations may identify critical molecular events that can be targeted for cancer prevention.

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TECHNICAL OBJECTIVES OF THIS PROJECT

Objective 1. ***Does CLA inhibit the formation of oxidative damage to DNA?***

CLA has been reported to be a potent antioxidant in test tube assays, but its biological activity as an antioxidant is unclear. We have reported that feeding CLA reduced lipid peroxidation in mammary gland measured as malondialdehyde but had no effect on DNA oxidation measured as 8-hydroxydeoxyguanosine (8-OHdG). During the past year we have developed methods to enhance sensitivity to detect effects of CLA on DNA oxidation and to determine if CLA affects the transcriptional activity of the redox sensitive protein, NF-kappa B, the activation of which has been implicated in carcinogenesis.

Objective 2. ***Does CLA alter the process of clonal expansion that occurs in the mammary gland in response to carcinogenic insult?***

During this reporting period a new approach to assessing the effects of CLA on mammary gland morphology was developed to assess the apparent ability of CLA to modify the developmental potential of a subset of target cells that are normally susceptible to carcinogen-induced transformation.

Objective 3. ***Does CLA affect the process of clonal selection such that the pathogenetic pathway leading to mammary tumor formation is altered?***

The hypothesis that forms the basis for this objective is that CLA inhibits tumor occurrence by modulating the "activity" of specific genes, whose misregulation is central to the carcinogenic process. The key issue is to identify the genes that CLA modulates, and whether the effect is direct or indirect. During this reporting period methods were developed to study the effects of CLA on the expression of two cell cycle regulatory proteins, cyclin D1 and p27. Our decision to focus on these proteins was based on our observation that CLA suppresses mammary gland development, that CLA inhibits cell proliferation, and that deregulation of the cell cycle is involved in the processes clonal expansion and selection of transformed cells. Our goal is to determine if cyclin D-1 is down-regulated and/or p27 up-regulated by CLA. Such effects to be mediated by modulation of a transcriptional activator such as NF kappa B.

BODY OF PROGRESS REPORT

The effort during the third year of funding was directed to continuing work on Technical Objective 1, and on work requisite to evaluation of the effects of CLA on clonal selection and clonal expansion (technical objectives 2 and 3). The following sections detail the methods that were developed to meet the goals stated in these objectives and the results obtained.

Materials and Methods

New Methods Developed During This Reporting Period.

Mammary Gland Epithelial Cell Enrichment We decided to implement a procedure for isolation of mammary epithelial cells from whole gland so as to increase the specificity and sensitivity of our assays of cellular oxidation. We have adopted a modified procedure from Moon, et al (1) in order to efficiently and effectively harvest mammary epithelial cells from the gland.

Approximately a 1 cm square of fresh excised mammary tissue is introduced to 10 mL of sterile incubation medium (medium 199, 2.8% sodium bicarbonate, penicillin streptomycin) and 35 mg of collagenase. This solution is incubated in a Dubnoff shaking incubator at 37^0 for 1-1 1/2 hours with exposure to ultrasonic generator following ½ - ¾ hour of incubation. The mixture is subjected to sonication at 100mA for 2-3 minutes and continues it's incubation in the Dubnoff incubator. The tissue is observed to safeguard against complete disruption which can lead to incomplete separation of epithelial from adipose cells. The mixture is centrifuged at 400 x g for 20 minutes following the transfer into a 15mL centrifuge tube. Proceeding the centrifugation, tissue was observed to be floating in the incubation medium which was removed by aspiration. The walls of the centrifuge tube were cleaned with Kimwipes and the subnatant was exposed to 10 mL of 0.9% sodium chloride solution. The resulting mixture is inverted repeatedly to re-suspend the subnatant and then centrifuged for 10 minutes at 400 x g. Following the fourth wash , the supernatant is carefully aspirated, the walls of the tube wiped with Kimwipes, and the pellet is re-suspended in PBS.

References: Moon, R.C., Janss, D.H., Young, S. (1969) Preparation of Fat Cell-"Free" Rat Mammary Gland. The Journal of Histochemistry and Cytochemistry, 17, 182-186.

Comet Assay For comet analysis, mammary epithelial cells are harvested from the whole gland as discussed above. Cell membranes are further disrupted by use of a non-ionic detergent (nonidet P40) in a dounce homogenizer and nuclei are removed from suspension by centrifugation. Our comet analysis method is based on that of Singh *et al.*(1). Isolated nuclei are suspended in phosphate buffered saline (PBS) at a concentration of ~200,000/ml and then mixed 1:1 with 1.0% low melting point (LMP) agarose in PBS at 37°C. 40 ul of the resulting suspension is quickly delivered onto fully frosted slides precoated with 60ul of 0.5% normal melting point agarose and then covered with a 25mmx30mm #1 coverslip. After the agarose has set a third layer of 40ul of 0.5%LMP agarose is delivered on top and again allow to set. Slides are then incubated in a lysis buffer (1% Triton X-100, 10% DMSO, 1% Na-N-lauroyl sarcosine, 10mM Tris, 100mM Na_2EDTA, 2.5M NaCl, pH 10.0) for one hour. If endonuclease III treatment is desired, slides are washed 3x5min in endonuclease III buffer (.4mM HEPES-KOH, 100mM KCl, .5mM EDTA, .2mg/ml BSA fraction V, pH 8.0) and then covered with 50ul of either endonuclease III in buffer (.05U/ul) or buffer alone, and then incubated for 30 minutes at 37°C. Slides are then placed in

electrophoresis buffer (1mM Na_2EDTA, 300 mM NaOH, pH 13.0) for 40 minutes to allow time for DNA unwinding. After unwinding slides are placed in a single row on a horizontal electrophoresis tank with enough fresh buffer to just cover the slides. After electrophoresis 20min at 25V and 300mA, the slides are neutralized with 3, 5 minute washes (400mM Tris buffer, pH7.5) and then stained with 30ul of a 1/50 dilution of Oli-Green DNA fluorescent dye. Slides are visualized and photographed on a Zeiss Axioskop 20 fluorescence microscope with an excitation filter of 450nm-490nm and a barrier filter of 520nm.

Reference: Sasaki, Y.F., Izumiyama, F., Nishidte, E., Matsusaka, N., Tsuda, S. (1997) Detection of rodent liver carcinogen genotoxicity by the alkaline single-cell gel electrophoresis (COMET) assay in multiple mouse organs (liver, lung, spleen, kidney, and bone marrow). Mutation Research, 391, 201-214.

Assessment of the NF kaapa B protein complex and NF kappa B transcriptional activity.

NF kaapa B protein complex by western blot analysis Frozen tissue stored at -80°C is used for protein extraction. 50mg of sample was homogenized in 150ul ice cold RIPA buffer with protease inhibitors (100mM PMSF, 1.9ug/ml Aprotinin, 1M DTT). The volume was brought to 1ml with ice-cold RIPA. The homogenate was incubated on ice for 30 minutes. Sample was centrifuged 2X for 20 minutes and 1X for 5 minutes to obtain a clear supernatant of whole cell lysate (16,000Xg, 4°C). Supernatant was frozen and stored at -80°C. Protein was quantified using Bio-Rad Bradford assay kit. We used a dose response, run on a 10% protein gel at 0, 5, 10, 20, and 40ug, to decide that 40ug of protein was the optimum to conserve antibody and to concentrate any less abundant proteins. The dose response with duplicate loading was also used to determine our reproducibility. Samples were run on a 10% running gel (37.5:1 bis/acrylamide) and a 5% stacking gel. Protein samples were added to 15ul 2X loading buffer and denatured by boiling 5 minutes and placed on ice. Rainbow marker (Amersham RPN 756) and mammary tumor protein was used as positive controls. After exposure, each membrane is stripped and re-hybridized for -actin to confirm gel loading and sample integrity. Proteins were transferred from gel to nitrocellulose membrane by semi-dry transfer method. All incubations are performed in a hybridization oven at 37°C. 5% milk solution is prepared with non-fat dry milk in 1XTBS/0.1% Tween-20 (TTBS) solution and used for blocking (1hour) and antibody dilutions. Then, membrane was blocked with 20ml of milk solution for 1 hour. Add primary antibody in 5ml 5% milk solution and incubate for 1 hour. All antibodies were ordered from Santa Cruz and used at the following dilution factors: NF B p65 at 1:1000, IkB- at 1:200, -actin at 1:1000. We are trying to achieve better results for NF B p50. Wash membrane 3X with 15ml of TTBS. Add HRP-conjugated secondary antibody for 1 hour (Sigma anti-rabbit IgG 1:10K). Wash membrane 3X with 15ml of TTBS. ECL was performed using Amersham kit (RPN 2106).

Detection of DNA-protein interaction by electrophoretic mobility shift assay

Electrophoretic mobility shift assay (EMSA)

Proteins from cell extracts from either the nucleus or cytoplasm are detected by their ability to retard mobility of a labeled DNA fragment during electrophoresis through a nondenaturing gel. This method is known as **electrophoretic mobility shift assay (EMSA)**. The protocol of EMSA include four stages:

- Preparation of the nuclear or cytoplasmic extracts from mammalian cells
- Preparation of a radioactivity labeled DNA probe containing a particular protein binding site
- A binding reaction in which a protein mixture is bound to the DNA probe

- Electrophoresis of protein-DNA complexes through the nondenaturing gel, which is then dried and autoradiographed

We are using Promega's Gel Shift Assay System (Promega Catalog # E3300). Nuclear extracts are prepared from fresh rat tissue using the protocol in Current Protocol in Molecular Biology (edited by Ausubel, F.M., et al) with minor modifications. NF-κB consensus oligo (Promega Catalog # E3291) is labeled by T_4 polynucleotide kinase reaction with γ-^{32}PdATP (3000 Ci/mmol, 10 μCi/μl). Unincorporated label is removed by ethanol precipitation. In our DNA binding reactions, competition assays, i.e., negative control (probe along), positive control (using Hela nuclear extract, Promega Catalog # E3521), specific competitor (unlabeled competitor oligo) and nonspecific competitor (unlabeled non competitor) are included. Competition mobility shift assay and antibody super shift assays are used because most protein preparations will contain both specific and nonspecific DNA binding proteins. The competition shift assay will also use a NF-κB mutant oligonucleotide (Santa Caruz Biotechnology Catalog # sc-2511) which is identical to the probe fragment except for a mutation in the binding site that is known to disrupt function and presumably binding activity. In brief, 2 μl of Hela nuclear extract or 12-15 μg crude extract from rat tissue is incubated with 5,000-20,000 cpm radiolabeled NF-κB probe, and appropriate competitor in 1X Gel Shift Binding Buffer in a volume of 10 μl. The protein should be added last. The reaction is incubated at 30 ° for 30 min. 1 μl of 10X loading buffer is added to the negative control and 1 drop of glycerol is added to rest of the reactions. The protein-DNA complexes are electrophoresized on a prerunned 4% nondenaturing polyacrylamide gel (acrylamide:bisacrylamide=37.5:1) with 1.5 mm thick spacers and comb in 1XTBE buffer until the appropriate separation of bands is achieved. The gel is run at 200 V in a cold room to minimize overheating and denaturation of the probe during electrophoresis. The gel is then transferred to a filter paper, dried on a gel dryer, and exposed to X-ray film at -80°C with an intensifying screen.

Antibody supershift assay If the protein that forms the complex is recognzed by the antibody, the antibody can either block complex formation, or it can form an antibody-protein-DNA ternary complex and thereby specifically result in a further reduction in the mobility of the protein-DNA complex. In the present work, antibodies of NF-κB p65 (A), p65 (C-20), p50 (NLS), p50 (D-17) and p52 (K-27) from Santa Cruz (Catalogue # sc-109-G, sc-372-G, sc-114-G, sc-1192, and sc-298) are used for the supershift assay.

Measurement of mammary gland growth All whole mounts of the abdominal-inguinal mammary gland chains were photographed at 2 X magnification and the resulting images were scanned and digitized. Measurements of ductal extension of the mammary gland into the fat pad and of the amount of ductal branching were performed on the digitized images of entire abdominal-inguinal mammary gland chains using "IMAGE-PRO PLUS" software (Media Cybernetics). The length of mammary gland between the upper most lymph node and mammary branch border is quantified as a measure of ductal extension (DE). Images are then further processed to remove the lymph nodes and lesions from the mammary gland. This processed image is then evaluated for total area of the mammary gland fat pad into which mammary ductal epithelium has extended. This area is defined by drawing a line around the 360 degree perimeter of a mammary gland chain established by the point to point connection of a line from the outer most extending end bud one to the other. The area within this perimeter is then determined by subtracting the area occupied by the lymph nodes and lesions. We next use digital filters to assess the percent of this area that is occupied by mammary epithelium, i.e. the amount of ductal branching.

BrdU labeling and counting A pulse labeling technique is used to assess the rate of cell proliferation. Rats are injected with 50 mg bromodeoxyuridine/kg body weight i.p. exactly 3 hrs prior to euthanasia. The three hour period of labeling is selected since evidence indicates that this also is the time span represented in the quantification of cell death by apoptosis using morphological criteria. Uninvolved mammary gland duct and pre-malignant and malignant mammary gland lesions are stained for immunohistochemical analysis. Anti-BrdU antibody (Becton Dickinson, 1:40) was used to detect BrdU labeled nuclei. Labeled and unlabeled cells are imaged and counted at 400X with a computer assisted image analyzer (CAS-200), using the Quantitative Nuclear Antigen Program Version 3.0 (Becton-Dickinson/Cellular Imaging Systems, San Jose, CA). Two cameras with two bandpass filters, one at 620 nm, which measures all nuclei stained with hematoxylin (with or without DAB staining) and the other at 500 nm, which measures only nuclei stained with DAB, allow for excellent spectral discrimination between the brown (DAB chromoagen) and blue (hematoxylin). Both nuclear and antibody thresholds are set with a negative control antibody cocktail slide. The nuclear threshold is set to the value that best discriminates between the nuclei and cytoplasm. The antibody threshold is set to the value at which no stain can be detected in the nuclei of the negative control slide. Standardization is established through the use of control tumor tissue in each assay, which is treated in the same manner as the sample tissue. The Quantitative Nuclear Antigen software application is used to measure the percentage of cell nuclei in the tissue sections that contain BrdU labeled antigen. The proliferation index, the percentage of labeled cells over total cells counted, is determined by counting 20 fields per each slide (approximately 2000 cells).

Apoptotic cell counting The same sections used for determining proliferation index are stained using a standard H&E protocol. Apoptotic cells are identified by morphological criteria. The apoptotic index, the percentage of apoptotic cells over total cells counted, was determined on the same 20 fields subjected to analysis for cell proliferation.

Cyclin D1 staining and quantification Uninvolved mammary gland duct and pre-malignant and malignant mammary gland lesions are stained for immunohistochemical analysis of cyclin D1. Anti-cyclin D1 antibody (NeoMarkers, 1:40) is used to detect cyclin D1 labeled nuclei. Labeled and unlabeled cells are imaged and counted at 400X with a computer assisted image analyzer (CAS-200), using the Quantitative Nuclear Antigen Program Version 3.0 (Becton-Dickinson/Cellular Imaging Systems, San Jose, CA) (xx). Two cameras with two bandpass filters, one at 620 nm, which measures all nuclei stained with hematoxylin (with or without DAB staining) and the other at 500 nm, which measures only nuclei stained with DAB, allow for excellent spectral discrimination between the brown (DAB chromoagen) and blue (hematoxylin). Both nuclear and antibody thresholds were set with a negative control antibody cocktail slide. The nuclear threshold was set to the value that best discriminated between the nuclei and cytoplasm. The antibody threshold was set to the value at which no stain could be detected in the nuclei of the negative control slide. Standardization is established through the use of control tumor tissue in each assay, which is treated in the same manner as the sample tissue.

The Quantitative Nuclear Antigen software application is used to measure the percentage of cell nuclei in the tissue sections that contain cyclin D1 labeled antigen by counting 20 fields per each slide (approximately 2000 cells), and three parameters were taken. These are: (1) positive area, which means the percentage of the nuclear area that is positive for the cyclin D1; (2) positive stain, which represents the summed optical density for the positive area divided by the summed optical density for the entire nuclear area, and give an indication of the cyclin D1 density in the positive area; (3) positive optical density (POD) per positive cell (PC), which represents the average summed optical density for the cyclin D1 staining within each cell, and indicates the amount of cyclin D1 per each cell.

P27 staining and quantification Uninvolved mammary gland duct and pre-malignant and malignant mammary gland lesions are stained for immunohistochemical analysis of p27. Anti-p27 antibody (NeoMarkers, 1:40) is used to detect p27 labeled nuclei. Labeled and unlabeled cells are imaged and counted at 400X with a computer assisted image analyzer (CAS-200), using the Quantitative Nuclear Antigen Program Version 3.0 (Becton-Dickinson/Cellular Imaging Systems, San Jose, CA) (xx). Two cameras with two bandpass filters, one at 620 nm, which measures all nuclei stained with hematoxylin (with or without DAB staining) and the other at 500 nm, which measures only nuclei stained with DAB, allow for excellent spectral discrimination between the brown (DAB chromoagen) and blue (hematoxylin). Both nuclear and antibody thresholds are set with a negative control antibody cocktail slide. The nuclear threshold was set to the value that best discriminated between the nuclei and cytoplasm. The antibody threshold was set to the value at which no stain could be detected in the nuclei of the negative control slide. Standardization was established through the use of control tumor tissue in each assay, which is treated in the same manner as the sample tissue.

The Quantitative Nuclear Antigen software application is used to measure the percentage of cell nuclei in the tissue sections that contain p27 labeled antigen by counting 20 fields per each slide (approximately 2000 cells), and three parameters were taken. These are: (1) positive area, which means the percentage of the nuclear area that is positive for the p27; (2) positive stain, which represents the summed optical density for the positive area divided by the summed optical density for the entire nuclear area, and give an indication of the p27 density in the positive area; (3) positive optical density (POD) per positive cell (PC), which represents the average summed optical density for the p27 staining within each cell, and indicates the amount of p27 per each cell.

Source and composition of CLA and other dietary fats. The method of CLA synthesis from 99+% pure linoleic acid is detailed in reference (6, listed above). CLA was custom ordered from Nu-Chek, Inc. (Elysian, MN). Gas chromatographic analysis showed that three particular isomers, *c*9,*t*11-,*t*9,*c*11- and *t*10,*c*12-CLA, constituted about 90% of the total. There were minimal variations in isomer distribution from batch to batch. Other fats used included: Mazola brand corn oil was obtained from Best Foods, Somerset, NJ, lard was purchased from Harlan Teklad, Madison, WI, menhaden oil was obtained from Marine Oil Test Program, U.S. Department of Interior, and palm oil was obtained from the Edible Oils Institute.

Animals and Diets

Animals. Female Sprague Dawley rats were used in the work reported. They were obtained from either Taconic Farms (Germantown, NY) or Charles River, Wilmington, Delaware. All rats were certified pathogen free.

Diet Formulations. A variety of diet formulations were used depending on the research question being addressed. All diets were modifications of the AIN-76A formulation and were designed to meet or exceed the known nutrient requirements of the rat unless otherwise specified.

Previously Reported Methods The Use of Which Continues.

Analysis of urinary malondialdehyde (MDA). Following acid hydrolysis to release the bound form, MDA was derivatized with thiobarbituric acid (TBA) and the MDA-TBA adduct quantified by reverse phase HPLC with visible absorbance detection at 535nm. MDA content is expressed as nmol/mg creatinine.

In detail, 0.5 ml urine was combined with 5ul of an antioxidant solution containing 0.3M 2dp and 2% BHA in ethanol, and 40 ul concentrated HCL. The mixture was heated in a dry block at 96-99° for 4 and 3/4 hours. After samples had cooled slightly, 2 ml of TBA solution (1.11 % TBA in 74mM KOH) was added and the samples were heated at 96-99° for another 45 minutes. After cooling and immediately before HPLC analysis, samples were adjusted to a pH of 1.8 - 4.0 with 12N KOH. Previous method validation has confirmed that the presence of 2dp and BHA in urine samples during acid hydrolysis and TBA derivitzation prevents artifactual MDA contribution from food contamination in the urine, even with extreme contamination by menhaden oil containing diet. Creatinine was measured spectrophotometrically (Procedure 555, Sigma Diagnostics, St. Louis, MO 63178).

Determination of 8-OHdG and malondialdehyde in mammary tissue.

8-OHdG. For the assay of 8-OHdG, the various procedures of DNA purification from the mammary gland, the enzymatic digestion of DNA to deoxynucleosides, the isocratic separation of 8-OHdG and dG by HPLC, and the quantitation of 8-OHdG with an electrochemical detector were described in detail in a recent publication from our laboratory (11). Detector response was linear from 10 to >800 pg per injection for 8-OHdG and from <500 to 6000 ng for dG. Results are reported as residues of 8-OHdG per 10^6 residues of dG. The simultaneous analysis of both deoxynucleosides on a single HPLC injection abrogated the need for a recovery standard.

Malondialdehyde (MDA). Tissue malondialdehyde was quantified as its thiobarbituric acid derivative with reverse phase HPLC and photometric absorbance detection at 535nm. In detail, mammary gland was homogenized with a Polytron in water containing 1% antioxidant solution (AOS: 0.3M dipyridyl and 2% BHA, in ethanol), 1 part mammary gland to 9 parts water (wt/vol). Homogenized samples were centrifuged at 6500 x g and fat plugs were removed, followed by further homogenization to re-suspend the pellet. As optimum reaction conditions were found to vary with protein concentration, an amount of homogenate containing approximately 1.25 mg protein was prepared for hydrolysis. The homogenate was combined, in glass tubes, with 7.5 ul AOS and enough water to bring the volume to 1.47 ml. 7.5 ul 5N HCl was added, and covered tubes were heated to 96° C for 3 hours. Tubes were cooled quickly in tap water, and 30 ul sodium tungstate (Na_2WO_4) per tube was added to facilitate precipitation of protein. Tubes were centrifuged at 6500 x g for 10 min, and 1 ml of supernatant was then transferred from each to clean glass tubes. (The remaining supernatant and pellet were discarded.) 0.75 ml thiobarbituric acid (TBA) solution (1.11% TBA in 74 mM KOH) was added to each tube, and tubes were heated for 90 min for derivatization (to form TBA-MDA adduct). Samples were quickly cooled and the pH adjusted, if necessary, to between 2.5 and 4.0. The MDA-TBA adduct was separated using a 4.6 x 150 mm C18 column (Beckman Ultrasphere ODS) and a mobile phase consisting of 32.5% methanol in 50mM potassium phosphate buffer, pH 6.0 delivered at 1.5 ml/min. Photometric absorbance detection was at 535nm. MDA was quantified by comparison of sample peak heights to those of standards, prepared from 1,1,3,3-tetramethoxypropane (TMP). To aliquots of stock standard were added water to 1.5 ml, 5 ul AOS, 1 ml TBA solution and 40 ul concentrated HCl. Standards were heated at 96° C for 14 min, cooled, and their pH adjusted to between 2.5 and 4.0 with 12N KOH. Final results were expressed as nmol MDA/mg protein. Protein in tissue homogenates was quantified by the Bradford method using a commercial dye reagent (Bio-Rad Protein Assay, Bio-Rad Laboratories, Richmond, CA).

Determination of 8-OHdG concentration in liver DNA . The procedures described exhaustively herein contain significant changes from those previously described by us. The changes, such as eliminating phenol from the DNA isolation and adding BHT and 2-dp to buffers have been instrumental in reducing the contribution of artifacts to measured 8-OHdG.

The importance of guarding against artifacts and their mistaken interpretation can not be over stated.

Isolation and enzymatic digestion of DNA from rat liver. DNA was isolated from liver with a phenol free process and was subsequently digested enzymatically to nucleosides for chromatographic analysis. In detail, 10ul of 26.4 mg/ml BHT was added to a 13 ml polypropylene screw cap tube, followed by 3 ml digestion buffer (100mM NaCl; 10mM Tris, pH8.0; 0.5% sodium dodecyl sulphate, pH 8.0, 400 ug/ml proteinase K (30 mAnson units/mg, cat # 24568, EC 3.4.21.14, from EM Science)) and approximately 75 mg frozen pulverized liver. The tube was inverted repeatedly to mix and incubated in a 50° water bath for16-20 hrs, after which it was removed from the bath and allowed to cool briefly before adding 1 ml 7.5M ammonium acetate and mixing thoroughly. The resulting precipitate was removed from suspension by centrifugation at 19000g for ten minutes at 4°, and the supernatant decanted and extracted twice with 24:1 chlroform/isoamyl alchohol. Nucleic acids were precipitated by the addition of 3 ml isopropanol, transferred to 1 ml silanized glass vials (Type I, Class A borosilicate glass, Waters Associates, Milford, MA) and the precipitate was washed with 70% EtOH before dissolution in 340 ulTE buffer (10mM Tris; 1mM EDTA; pH 8.0) containing 5mM dp. RNA contamination was reduced by treating samples with RNase (55 ug in H_2O) for 1 hour at room temperature in the dark. After addition of 10 ul of 5M NaCl, DNA was precipitated by the addition of 350 ul isopropanol. While the presence of ribonucleosides does not interfere with the assay per se, removal of most of the RNA by treatment with RNase results in samples which are more readily digested to nucleosides and chromatographed. The DNA pellet was washed with 70% EtOH, dried briefly under reduced pressure without heat, and dissolved in100ul of 20mM sodium acetate, pH 4.8, containing 5mM DP. Dissolution was allowed to proceed overnight at room temperature in the dark prior to enzymatic digestion to nucleosides.

Chromatography of liver hydrolysate. 8-OHdG and dG were separated isocratically on a 4.6 X 250 mm Rainin Microsorb C18 column (5um, 100A) with a mobile phase of 8.2% methanol in 50 mM potassium phosphate buffer, pH 5.5, delivered at 1 ml/min. Detection of 8-OHdG was achieved on an ESA Coulochem Model 5100 A electrochemical detector equipped with a model 5011 analytical cell and a model 5020 guard cell. Detector potentials were set as follows: guard cell +0.43 V, detector one +0.12 V, detector two +0.38 V. 8-OHdG was measured as current at detector two. dG was monitored by absorbance at 290 nm with a Shimadzu SPD-10AV spectrophotometric detector installed downstream from the electrochemical detector. Results were reported a residues 8-OHdG per million residues dG. The simultaneous analysis of both analytes from a single HPLC injection provided excellent precision without rigorously quantitative sample handling.

RNA isolation Total RNA was extracted from carcinomas and tissues by acidic phenol extraction using a commercial kit from BIOTECX Laboraotries, Inc. (Houston, TX). For differential display of mRNA, the total RNA preparations were digested with RNase-free DNase (GenHunter Corporation, Nashville, TN) in order to remove contaminating genomic DNA. For cDNA library construction, poly A(+) mRNA was enriched by oligo (dT)-cellulose column.

Differential display Differential display of mRNA was carried out with the RNAimage™ kit (GenHunter Corporation, Nashville TN) according to manufacturer's instructions with two minor modifications: 1) One tenth of the recommended amount of total RNA was used for the reverse transcription step in order to minimize an inhibitory activity(s) present in the mammary tissue RNA preparation; 2) It was found that an annealing temperature of 42 °C for PCR was optimal in Denver, CO to yield reproducible display patterns. Duplicate reactions were run for each primer combination. The PCR products (labeled by alpha-^{32}P-dATP) from the 3 mammary carcinomas (T_1, T_2 and T_3), the uninvolved mammary tissue and kidney were contrasted side-

by-side on sequencing gels. Only those bands that were present in carcinoma lanes, but absent in mammary gland and kidney lanes were cut and re-amplified by PCR. The PCR products were size separated on low melting point agarose gel and band(s) of the expected size was eluted. The gel-purified PCR bands were used as templates to generate ^{32}P-labeled probes for Northern blot detection of gene expression on a screening panel of RNA preparations comprised of two kidney samples, two liver samples, the 3 mammary tumors that were used for the original differential display. In addition, the uninvolved mammary gland tissue and mammary gland tissue excised from a day-1 post-partum female rat were included in the screening panel. The RNA samples were size-separated by electrophoresis and transferred onto Nylon membrane for Northern blot detection of gene expression. GAPDH or cyclophylin gene was probed as an internal control for loading correction.

Cloning and Sequencing Those PCR bands that detected carcinoma-specific gene expression were cloned into pGEM-T vector (Promega, Madison, WI). For each band, four clones were inoculated and the plasmid DNA was isolated by an alkaline mini-prep procedure and at least two clones are sequenced on both strands by the dideoxy chain termination method of Sanger (7) using a kit from US Biochemicals (St Louis, MO). A commercial service utilizing thermal cycle sequencing (Cornell DNA Service, Ithaca, NY) was also used to confirm the sequence of a few of the clones. The cloned gene fragments were used as templates to generate randomly labeled probes for Northern detection again to confirm that the cloned sequences corresponded to the genes originally detected by the PCR products from differential display gels. Sequence search was done using the BLASTN algorithm (8) with GenBank nr databases and expressed sequence databases dbEST.

Cloning full length cDNA A cDNA library was constructed with pooled poly(A)+ mRNA isolated from mammary carcinomas using the Marathon cDNA construction kit (Clontech, Inc, Palo Alto, CA). The average length of the library was 1.5 kb. Based on the sequence information obtained for each gene fragment, a gene-specific primer was synthesized (Integrated DNA Technology, Inc, Coralville, IA) as the down stream primer. A universal upstream primer that annealed to the adapter which had been ligated into the cDNA library and the gene-specific primer were used for long distance PCR using KlenTaq (a combination of Taq and Vent polymerases) to increase fidelity of cloning (ClonTech, Palo Alto, CA). The PCR fragments were cloned into the pGEM-T vector and sequenced as described above.

Ha-*ras* codon 12 mutation detection in mammary carcinomas The paraffin-embedded tumor blocks were cut into 5-*u*m sections. These sections were mounted on plastic slides coated with polylysine and stained with hematoxylin and eosin (H&E). Each section was viewed without a cover slip under light microscopy and marked for tissue retrieval. Pieces of a section (approximately 2x2 mm) were carefully cut with scissors from the marked area. The scissors were soaked in 10% Chlorox bleach and heat sterilized between samples to prevent carry-over. Each piece was incubated with proteinase K (400 :g/ml in 100 mM Tris-HCl, 2 mM EDTA) at 50°C for 3 h. After the proteinase K was inactivated by heating at 95°C for 8 min, 5%-10% of the extract was used as the source of DNA for PCR amplification. This tissue collection procedure permits sampling of different regions of a carcinoma for PCR analyses. It is important to note that when this tissue sampling procedure was tested in independent experiments more than one hundred samples have been repeated at different times to check the reproducibility of the assay and to ensure the absence of carry-over and all of the repeated measures showed reproducible results.

The mutational status of Ha-*ras* codon 12 was determined by a modified polymerase chain reaction-generated restriction fragment length polymorphism (PCR-RFLP) method (16,17). The upstream primer (5'AGTGTGATTCTCATTGGCAG-3') was placed in intron-1 to avoid amplifying the Ha-*ras* pseudogene. The G → A mutation along with two mismatches in the

downstream primer (5'-AGGGCACTCTTTCgaACGCC-3') introduces a XmnI restriction site into the PCR product (116 bp), which, upon digestion with the XmnI endonuclease of the PCR products, generates a fragment of 98 bp that is diagnostic for the mutation. A tracer amount of alpha-^{32}P-dCTP was used to label the PCR products. The digested products were separated by electrophoresis on a 6% polyacrylamide gel and detected by autoradiography using Kodak X-ray films.

Results and Discussion

Further analyses of the cellular effects of CLA on oxidative damage indices

We have reported that feeding CLA reduce lipid peroxidation in mammary gland measured as malondialdehyde but had not effect on DNA oxidation measured as 8-OHdG.This was an unanticipated observation. Given the heterogeneity of the mammary gland and the potential loss of sensitivity to detect small but meaningful changes in oxidative DNA base damage in the target mammary epithelial cells, we have developed the methods necessary to specifically evaluate the question, "Does CLA protect mammary epithelial cells per against oxidative DNA base damage? After considering several approaches we decided to isolated epithelial cells from mammary gland and then subject to a single cell electrophoresis analysis for assess oxidative DNA base damage. In following paragraphs we report our progress in assay development.

We have developed a modified alkaline single cell gel electrophoresis assay (comet assay) to estimate DNA damage in nuclei isolated from mammary gland tissue. Our method is a variation of that described previously by Singh *et al.* (1). Briefly, nuclei are lysed with high ionic strength buffer containing ionic and non-ionic detergents after embedding in a thin agarose gel on microscope slides. Alkaline conditions facilitate unwinding and areas of relaxed supercoiling in DNA that has been damaged by strand breaks. During electrophoresis, the free ends and relaxed loops of DNA are free to migrate towards the anode, producing a tail (thus the name "comet assay") when visualized after staining with a fluorescent dye ***(figure 1)***. We have chosen to use the fluorescent DNA dye Oli-Green (Molecular Probes) due to its high affinity for ssDNA. In addition to detecting frank breaks and alkali labile sites we have used a DNA repair enzyme to detect specific oxidative damage. Incubation with endonuclease III converts specific oxidative damage to strand breaks by excising oxidized pyrimidines (3).

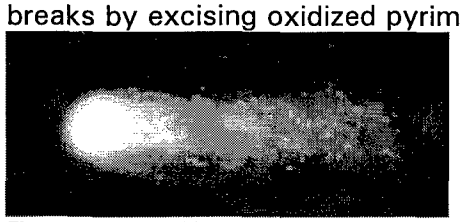

Figure 1: *Enlarged image of a comet from cultured lymphocytes (L1210 cell line). This image shows the strongly fluorescent nucleus/head and the tail produced by free ends and relaxed loops of DNA after electrophoresis.*

As a "positive contro" in assay development we have shown that treatment of cultured L1210 cells with H_2O_2 results in comets that are readily distinguishable from untreated controls by visual scoring ***(figure 2)*** and we have observed a positive dose/response correlation between H_2O_2 treatment and comet tail magnitude as well. However, a more precise estimation of DNA damage via comet assessment is anticipated for achieving the goals of this proposal. To that end, we are currently evaluating specialty software that facilitates a more discriminating estimation of DNA damage based on comet tail moment. Tail moment is a utile index of DNA damage that offers increased sensitivity and precision over other methods such as categorical scoring and tail length, and digital evaluation of tail moment has been shown to achieve even greater sensitivity(2).

a)

b)

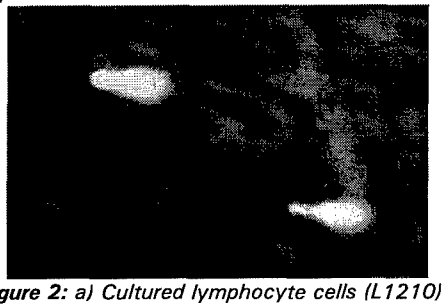

Figure 2: *a) Cultured lymphocyte cells (L1210) compared to b)cultures lymphocyte cells treated with 200uM H_2O_2 for 5 minutes on ice. The DNA damage induced by the H_2O_2 is clearly seen by the extensive tails in figure 2b).*

Because of the relative homogenity of liver we next applied the comet analysis to nuclei isolated from frozen solid tissue (rat liver) has shown extensive tailing that is inconsistent with expectation. We suspect this extensive tailing is due to artifacts of tissue preparation, probably from freezing. Data acquired from comet analysis of cultured lymphocytes that have been frozen in 10% DMSO indicate that frozen cells can yield comets with tails of low intensity, but that cells must be frozen in media or buffer containing DMSO such that membrane disruption and DNA strand breakage are minimized. Upon thawing, properly frozen samples show ~28% dead cells by trypan blue dye exclusion criteria, and subsequent analysis reveals two distinct populations of comets, one of which shows minimal comet tail magnitude and one consisting almost entirely of tails ***(figure 3)***. The relative proportions of these populations is comparable to those of live and dead cells respectively. In contrast, cells from fresh blood are uniformly possessed of minimal comet tails prior to endonuclease III treatment. Thus it appears that while cells from fresh tissue are optimal for comet analysis, the ability to identify and exclude dead cells from analysis will facilitate frozen storage of samples if such storage is necessary.

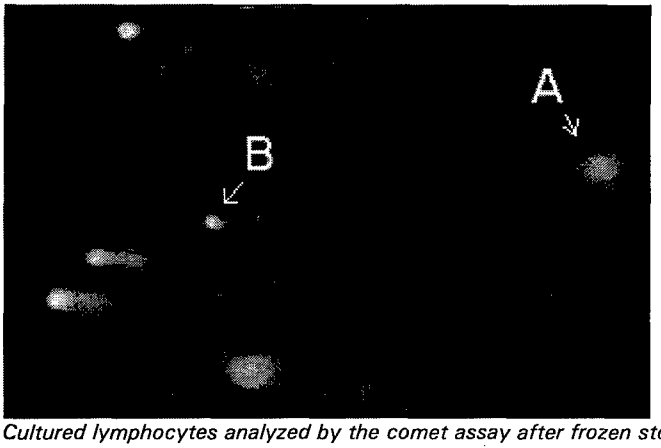

Figure 3: *Cultured lymphocytes analyzed by the comet assay after frozen storage in 10% DMSO. Examples of the two cell populations are labeled:* A *indicates a comet which is primarily tail and* B *indicates a comet with minimal damage.*

During year 4, mammary epithelial cells isolated from control and CLA fed animals will be be subjected to comet analysis. We will also determine comet activity in liver.

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Possible role of NF-κB in reduction of breast cancer risk by increased consumption of CLA

Our working hypothesis is that CLA increases the "reduced state" of the cell and thereby reduces the risk of mammary carcinogenesis by decreasing the transcriptional activation of NF kappa B. We anticipate that the comet assay described above will provide evidence of a "oxidation protected state" in mammary epithelial cells.

What is NF-κB? Originally defined as the nuclear factor that bound to the B site of the immunoglobin κ light chain gene enhancer in B lymphocytes, NF-κB is now known to be a family of dimeric transcription factors, with subunits that contain an amino-terminal stretch of approximately 300 amino acids that share homology with the v-Rel oncogene. Classical NF-κB is composed of a p50 and a p65 subunits. In addition, other Rel-related subunits have been identified, including c-Rel, RelB, and p52.

NF-κB is normally found in the cytosol as an inactive complex consisting of two subunits, p50 and p65, which are bound to an inhibitory subunit, termed IκB. Other members of the rel transcription factor family can also contribute to NF-κB complexes. Upon activation, NF-κB is released from IκB and translocates to the nucleus, where it binds its cognate DNA sequences and increases the transcription of specific genes.

Reactive oxygen intermediates and NF-κB Reactive oxygen intermediates (ROI), including hydrogen peroxide (H_2O_2) have been demonstrated to be potent activators of NF-κB. Consistent with these findings, antioxidants are effective inhibitors of NF-κB. In addition, administration of H_2O_2 and overexpression of glutathione peroxidase can each lead to induction or inhibition, respectively, of NF-κB activity. Therefore, the NF-κB activation pathway appears to be upstream of oxidative stress and ROI.

In a mouse model of aging, NF-κB was found to exist in a constitutively activated state in cells obtained from the major lymphoid organs of aged animals. Therapeutic treatment with dietary antioxidants or with agents capable of activating the peroxisome proliferator-activated receptor (PPAR)-α was able to correct the abnormal nuclear NF-κB activity, reduce lipid peroxide levels, and eliminate the dysregulated expression of cytokines and other genes under NF-κB control. These results suggest that abnormal activation of NF-κB in aging contributes to the dysregulated expression of certain pleiotropic cytokines.

How does modulation of NF-κB activity contribute to cancer risk?

Regulation of c-myc oncogene promoter by the NF-κB rel family

The promoter of the c-myc protooncogene contains two NF-κB binding sites. The effects on activation of a c-myc promoter/exon 1-CAT construct in NIH 3T3 cells by the individual members of the NF-κB family were tested. Classical NF-κB (p65/p50) was found to be a potent transcriptional activator of the c-myc promoter. Cotransfection with either p65 alone or p65 in combination with p50 mediated significant induction. In contrast, expression of either v-rel or chicken c-rel failed to transactivate, while murine c-rel induced c-myc promoter activity only slightly. Furthermore, induction by classical NF-κB was inhibited by coexpression of either v-rel or chicken c-rel. Thus, individual members of the rel family have differential effects on the c-myc oncogene promoter, which can modulate overall transcription activity and allow for precise regulation of this oncogene under diverse physiologic conditions. Activation of NF-κB may prevent c-myc-induced apoptosis, allowing neoplastic progression. NF-κB is activated in many different cell types by a variety of agents, suggesting that this factor is an important global transcriptional regulator, which is induced by numerous stimuli.

Several reports have suggested that proteins from the NF-κB or IκB families are involved in the development of cancer. v-Rel expressing viruses are highly oncogenic and cause aggressive lymphomas in young birds, while mutated c-Rel is transforming *in vitro*. The genes coding for c-Rel, p65, p50, p52 and Bcl-3 are located at sites of recurrent chromosomal translocations and genomic rearrangements in human cancers.

Increasing evidence supports the role of NF-κB/Rel family involvement in apoptosis. NF-κB controls the expression of growth factors or oncoproteins (c-Myc, Gro proteins), as well as of proteins derived from tumor suppressor genes such as p53. Therefore it is possible that, in different cellular environments, upregulated activation or inhibition of NF-κB activity could each lead to tumor formation. NF-κB also regulates the transcription of genes coding for extracellular proteases or adhesion molecules (urokinase, 92 kD type IV collagenase), and could therefore play a role in angiogenesis or invasiveness, two mechanisms required for tumor growth and the development of metastasis.

Detection of DNA-protein interaction by electrophoretic mobility shift assay

Proteins from cell extracts from either the nucleus or cytoplasm are detected by their ability to retard mobility of a labeled DNA fragment during electrophoresis through a nondenaturing gel. This method is known as **electrophoretic mobility shift assay (EMSA)**. The protocol of EMSA include four stages:

- Preparation of the nuclear or cytoplasmic extracts from mammalian cells
- Preparation of a radioactivity labeled DNA probe containing a particular protein binding site
- A binding reaction in which a protein mixture is bound to the DNA probe
- Electrophoresis of protein-DNA complexes through the nondenaturing gel, which is then dried and autoradiographed

The sensitivity of the EMSA enables femtomole quantities of DNA-bind proteins to be detected. This assay can be used to test binding of purified proteins or of uncharacterized factors found in the crude extracts. It also permits quantitative determination of the affinity, abundance, association rate constants, dissociation rate constants, and binding specificity of DNA-binding proteins.

Three additional protocols are available for competition assays to assess the sequence specificity of protein-DNA interactions. This is necessary because most protein preparations will contain both specific and nonspecific DNA binding proteins.

- Competition mobility shift assay
- Antibody supershift assay
- Multicomponent gel shift assays

Questions that will be addressed during the fourth year of the project using the comet assay and assays for NF kappa B :

- Does increased intake of CLA reduce DNA damage measured by COMET assay?
- Does increased intake of CLA exert a cancer-protective effect via modification of DNA-binding activity of NF-κB?
- Are levels of oxidative DNA base damage as measured by COMET assay positively associated with DNA-binding activity of NF-κB?

Morphological and biochemical status of the mammary gland as influenced by conjugated linoleic acid: implication for a reduction in mammary cancer risk. (Full length paper in appendix)

Previous research showed that treatment with CLA during the period of active mammary gland morphogenesis was sufficient to confer a lasting protection against subsequent mammary tumorigenesis induced by methylnitrosourea. The present study was designed to characterize certain morphological and biochemical changes of the mammary gland that might potentially render it less susceptible to cancer induction. Female Sprague Dawley rats were fed a 1% CLA diet from weaning until about 50 days of age. The mammary gland parameters under investigation included a) the deposition of neutral lipid, b) the identification and quantification of CLA and its metabolites, c) the density of the epithelium, and d) the proliferative activity of various structural components. Our results showed that CLA treatment did not affect total fat deposition in mammary tissue nor the extent of epithelial invasion into the surrounding fat pad , but was able to cause a 20% reduction in the density of the ductal-lobular tree as determined by digitized image analysis of the whole mounts. This was accompanied by a suppression of bromodeoxyuridine labeling in the terminal end buds and lobuloalveolar buds. The recovery of desaturation and elongation products of CLA in the mammary gland confirmed our prior suggestion that the metabolism of CLA might be critical to risk modulation. The significance of the above findings was investigated in a mammary carcinogenesis bioassay with the use of the DMBA model. When CLA was started at weaning and continued for 6 months until the end of the experiment, this schedule of supplementation produced essentially the same magnitude of mammary tumor inhibition in the DMBA model as that produced by one month of CLA feeding from weaning. The observation is consistent with the hypothesis that exposure to CLA during the time of mammary gland maturation may modify the developmental potential of a subset of target cells that are normally susceptible to carcinogen-induced transformation.

Is the modulate mammary gland development and mammary carcinogenesis by CLA reflected in changes in levels of cell cycle regulatory proteins?

Our unifying working hypothesis is that CLA inhibits clonal expansion and selection via keeping

NF kappa B in an inactive form in the cytosol. This could have the phenotypic effect reported in **Morphological and biochemical status of the mammary gland as influenced by conjugated linoleic acid: implication for a reduction in mammary cancer risk. (See paragraph above and appendix).** We anticipate that CLA , via this mechanism, will suppress the over expression of cyclin D-1 which is characteristic of cell cycle deregulation during mammary carcinogenesis, and that more cells in uninvolvedmammary gland and in pre-malignant and malignant mammary gland lesions will remain in a quiescent G(0)-G(1) state detected as increased expression of p27. The methods are in place to test this hypothesis during the fourth year of the grant.

The Appendix contains four papers supported by this grant and that were published within the last year.

CONCLUSIONS

Conjugated linoleic acid (CLA) is a naturally occurring component of the food supply that has been shown to inhibit the development of experimentally-induced breast cancer. During this reporting period we have obtained data that indicate that exposure to CLA feeding during the time of mammary gland maturation may modify the developmental potential of a subset of target cells that are normally susceptible to carcinogen-induced transformation. We are currently investigation the role of CLA in modifying the transcriptional activation of redox sensitive proteins and in the regulation of cell cycle transit.

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APPENDIX

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Retention of conjugated linoleic acid in the mammary gland is associated with tumor inhibition during the post-initiation phase of carcinogenesis

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Conjugated linoleic acid (CLA) has been reported to have significant activity in inhibiting mammary carcinogenesis. A major objective of this study was to evaluate how changes in the concentration of CLA in mammary tissue as a function of CLA exposure/withdrawal were correlated with the rate of occurrence of mammary carcinomas. Rats treated with a single dose of dimethylbenz[*a*]anthracene (DMBA) at 50 days of age were given 1% CLA in the diet for either 4 weeks, 8 weeks or continuously following carcinogen administration. No cancer protection was evident in the 4 or 8 week-CLA treatment groups. Significant tumor inhibition was observed only in rats that were given CLA for the entire duration of the experiment (20 weeks). Analysis of CLA in the mammary gland showed that the incorporation of CLA was much higher in neutral lipids than in phospholipids. When CLA was removed from the diet, neutral lipid- and phospholipid-CLA returned to basal values in about 4 and 8 weeks, respectively. The rate of disappearance of neutral lipid-CLA (rather than phospholipid-CLA) subsequent to CLA withdrawal paralleled more closely the rate of occurrence of new tumors in the target tissue. It appears that neutral lipid-CLA may be a more sensitive marker of tumor protection than phospholipid-CLA. However, the physiological relevance of CLA accumulation in mammary lipids is unclear and remains to be determined. A secondary goal of this study was to investigate whether CLA might selectively inhibit clonal expansion of DMBA-initiated mammary epithelial cells with wild-type versus codon 61 mutated *Ha-ras* genes. Approximately 16% of carcinomas in the control group (without CLA) were found to express codon 61 *ras* mutation. Although continuous treatment with CLA reduced the total number of carcinomas by 70%, it did not alter the proportion of *ras* mutant versus wild-type carcinomas, suggesting that CLA inhibits mammary carcinogenesis irrespective of the presence or absence of the *ras* mutation.

Introduction

Conjugated linoleic acid (CLA*) is a minor fatty acid found preferentially in red meat and dairy products (1). The biosynthesis of CLA in ruminants is accounted for by a rumen bacterium, which is known to convert linoleic acid to stearic acid via CLA (2). Over the past decade, research from several laboratories has shown that CLA expresses powerful activity in cancer protection in a number of animal models (3–7). Feeding diets containing ≤1% CLA results in a dose-dependent suppression of tumor development in the mammary gland (8). CLA appears to have a dual effect in the modulation of mammary carcinogenesis in rats. First, exposure to CLA during the window of active mammary gland morphogenesis may reduce the proliferation of epithelial end bud cells, thus conceivably rendering the target cell population less susceptible to carcinogen-induced neoplastic transformation (8,9). Second, CLA is also capable of inhibiting tumor promotion/progression (9); however, a continuous supply of CLA is required for this mechanism of action.

The above study regarding the effectiveness of CLA in blocking tumor progression was carried out in the methylnitrosourea (MNU)-induced mammary carcinogenesis model in rats fed a 5% corn oil diet (9). One objective of the experiments reported in this study was to confirm the necessity of maintaining CLA intake after cancer induction by using dimethylbenz[*a*]anthracene (DMBA)-treated rats fed a 20% corn oil diet. It was considered important to assess whether the requirement for continuous CLA feeding was dependent on the nature of the carcinogen and the fat content of the diet. Rats were therefore given CLA for a duration of either 4, 8 or 20 weeks, starting immediately after a single dose of DMBA, to evaluate the anti-carcinogenic efficacy of these various intervention regimens. The kinetics of mammary tissue CLA retention as a function of CLA exposure/withdrawal was also analyzed in order to determine the correlation between time-dependent changes in tissue concentrations of CLA and effectiveness of cancer protection.

Additionally, we were interested in finding out whether CLA might selectively inhibit the clonal expansion of DMBA-initiated cells carrying either the wild type or codon 61 mutated *Ha-ras* gene. Previous work from Thompson's laboratory has shown that high dietary levels of linoleic acid preferentially increased the number of wild type *Ha-ras* mammary tumors, but not the codon 12 mutant *Ha-ras* tumors, in the rat MNU model (10). In chemical carcinogenesis, specific *ras* mutations are induced and are believed to be involved in early stages of tumor development (11–14). Generally, *ras* mutation is considered to be permissive but not sufficient for carcinogenesis. Thus the *ras* genotype was used as a marker in the present study to identify subpopulations of neoplastically transformed cells that might be differentially modulated by CLA intervention.

Materials and methods

Pathogen-free female Sprague–Dawley rats were purchased from Charles River Breeding Laboratories at 45 days of age. They were fed a 20% corn oil diet (6) and were intubated with a single dose of 10 mg of DMBA at 50 days of age for the induction of mammary tumors. Supplementation of CLA (Nu-Chek, Elysian, MN) at 1% in the diet was started 4 days after carcinogen administration. A total of 90 rats were given CLA and were divided equally

*Abbreviations: CLA, conjugated linoleic acid; DMBA, dimethylbenz[*a*]anthracene; MNU, methylnitrosourea; PCR/RFLP, polymerase chain reaction-generated restriction fragment length polymorphism.

Table I. Time course of wild-type and mutant *ras* mammary tumor appearance in control and CLA-supplemented rats[a]

Treatment	*ras* Genotype	Total (%)
Control	Wild-type	49 (84%)
	Mutant	9 (16%)
4 weeks-CLA	Wild-type	49 (92%)
	Mutant	4 (8%)
8 weeks-CLA	Wild-type	43 (86%)
	Mutant	7 (14%)
Continuous-CLA	Wild-type	13 (81%)
	Mutant	3 (19%)

[a]These tumors were harvested from the mammary carcinogenesis experiment described in Figure 1.

into three groups according to the length of CLA treatment: 4 weeks, 8 weeks or continuously until the end of the experiment. Control rats (n = 30) were not given CLA at any time during the study.

Animals were palpated weekly for mammary tumors; the time of appearance and location of tumors in the mammary gland were recorded. The experiment was terminated 20 weeks after DMBA. By that time, the development of palpable tumors had plateaued for several weeks across all groups. Only histologically confirmed adenocarcinomas were reported in the results. Tumor incidences at the final time point were compared by chi squared analysis, and the total tumor yield between the control and CLA-treated groups was compared by frequency distribution analysis as described previously (15).

A total of 177 mammary adenocarcinomas were harvested from the above carcinogenesis bioassay. They were individually identified after excision so that each one could be tracked to its time of appearance in a particular rat. All 177 paraffin block-embedded tumors were analyzed for codon 61 *ras* mutation (CAA→CTA) by a modification of the polymerase chain reaction-generated restriction fragment length polymorphism (PCR/RFLP) method as described by Kumar and Barbacid (16). Two 5-μ sections were prepared side-by-side from the same paraffin block, one mounted on a plastic slide, the other on a glass slide, which was subsequently stained with hematoxylin and eosin for the identification of tumor cell foci under the microscope. The exact same area of interest was matched on the plastic slide and was then cut out for DNA extraction (17). The primers used for PCR amplification were 5′-GAGACGTGTTTACTGGACATCTT-3′ and 5′-GTGTTGTTGATGGCAAA-TACACAGAGG-3′ (synthesized by Integrated DNA Technologies, Coralville, IA), which yielded a 116 bp PCR product (18,19). The PCR reaction mixture contained 5 μl of DNA extract, 10 mM Tris–HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 15 μM deoxynucleotide triphosphate, 1 μCi of α-[^{32}P]dCTP, 0.1 μM upstream and downstream primers, and 0.5 units AmpliTaq DNA polymerase (Perkin–Elmer, Norwalk, CT). For each batch of PCR reaction, PCR-grade H_2O was used as a blank, DNA from a tumor bearing *Ha-ras* codon 61 mutation as a positive control, and DNA from normal mammary gland as a negative control. Amplification was carried out for 40 cycles at: 94°C for 30 s, 60°C for 30 s, and 72°C for 1 min using a GeneAmp PCR system 9600 (Perkin–Elmer). The codon 61 A→T mutation introduces a Xba I restriction site into the 116 bp PCR product, which upon digestion, generates two fragments of 80 and 36 bp that are diagnostic for the mutation. In contrast, the PCR product of the normal gene contains a sequence that is not susceptible to digestion by Xba I. The digested materials were separated by electrophoresis on a 6% polyacrylamide gel, and detected by autoradiography on X-ray film.

To study the kinetics of CLA retention in the mammary gland, a two-part experiment was conducted to examine (i) the rate of increase of tissue CLA following the start of CLA feeding, and (ii) the rate of disappearance of tissue CLA following CLA withdrawal. For the first part, 60-day-old rats (age-matched to those in the above carcinogenesis experiment but not given DMBA) were fed a 1% CLA diet and were killed at 1, 2, 4, 6 or 8 weeks later. For the second part, rats were fed a 1% CLA diet for 8 weeks, the treatment was discontinued and necropsy was timed at 1, 2, 4 or 6 weeks after CLA withdrawal. Total lipid was extracted from the mammary gland by chloroform/methanol. The separation of neutral lipids and phospholipids was achieved with the use of a Sep-Pak silica cartridge as described in an earlier publication (5). Gas chromatographic analysis of the CLA methyl ester was determined by the method reported previously by Chin *et al.* (1).

Results

Figure 1 shows the time course of mammary tumor development in control rats or rats fed CLA for various lengths of

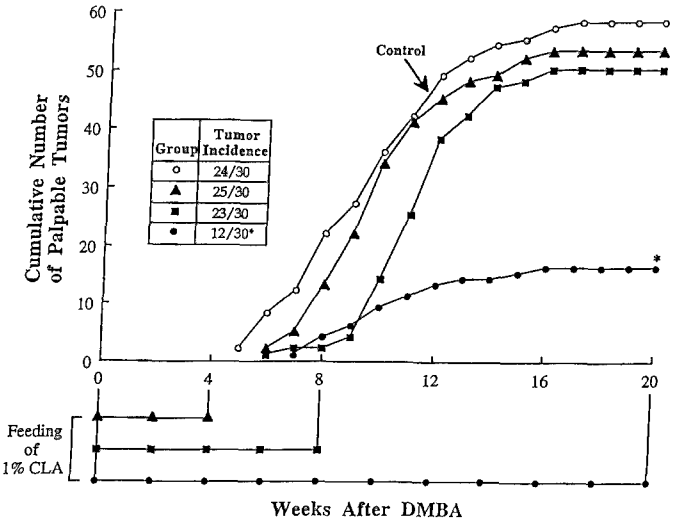

Fig. 1. Effect of interrupted versus continuous CLA feeding after DMBA administration on mammary carcinogenesis. The duration of CLA feeding in the three supplemented groups is indicated along the x-axis time line by the filled symbols, which match the time course of mammary tumor development on the main body of the diagram. Control group without CLA supplementation is represented by the open circle. The asterisk denotes statistically significant difference ($P < 0.05$) from the control data.

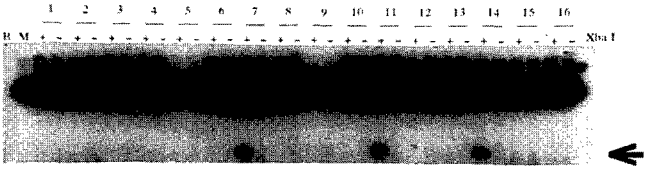

Fig. 2. Detection of *Ha-ras* codon 61 CAA→CTA mutation by PCR/RFLP method. The mutation produces a XbaI site in amplified 116-bp DNA fragment. Upon separation of the XbaI-digested product in 6% polyacrylamide gel, the presence of a 80-bp band (arrow) serves as a diagnostic marker for the mutation. PCR products were labeled with tracer amount of α-[^{32}P]dCTP and detected by autoradiography. Lane B, blank without template DNA; lane M, normal mammary gland DNA as a negative control; lane 1–16, mammary adenocarcinomas DNA. + and –, 5 μl of PCR product treated with or without 5 units of XbaI, respectively.

time. It can be seen that short-term feeding of CLA for only 4 or 8 weeks after DMBA administration was not effective in tumor inhibition. In the 8 week-CLA treatment group, the time course curve was shifted slightly to the right, suggesting a delay of about 2 to 3 weeks in the appearance of tumors. However, as soon as CLA was withdrawn, the rate of tumor appearance resumed at a rapid pace. At the time of necropsy, the difference in tumor occurrence between the control group and the 8 week-CLA treatment group was not statistically significant. In contrast, marked cancer protection, as judged by a 50% reduction in tumor incidence and a 70% reduction in the total number of tumors, was observed in rats that were given CLA for the entire duration of the study.

Figure 2 shows some representative electrophoresis autoradiograms of XbaI digested PCR products from tumors with either the wild-type or codon 61 mutant *ras* gene. The arrow in the diagram indicates the presence of a 80-bp band, which is diagnostic for the mutation. Table I summarizes the frequency distribution of both wild-type and mutant *ras* mammary

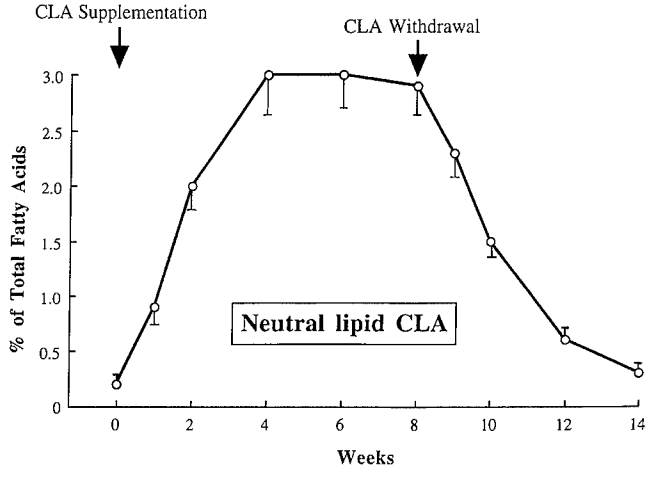

Fig. 3. The kinetics of CLA retention in neutral lipids of mammary gland following CLA supplementation and withdrawal. The results are expressed as the percentage of total fatty acids, mean ± SE (n = 6).

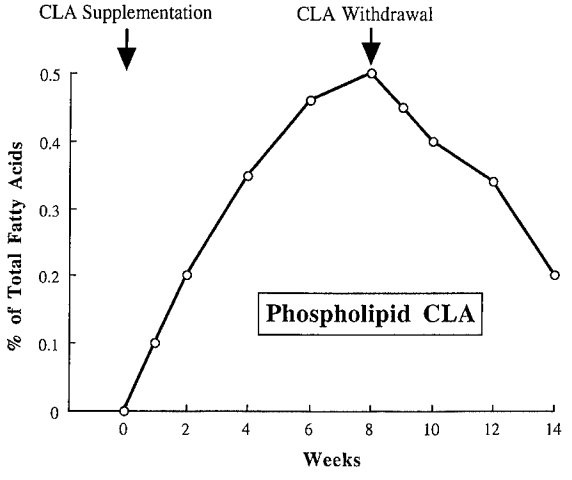

Fig. 4. The kinetics of CLA retention in phospholipids of mammary gland following CLA supplementation and withdrawal.

carcinomas from the above experiment. In the control group, 16% of the tumors expressed the mutant *ras* gene. Continuous feeding of CLA reduced the total number of carcinomas by 70%, but was found to suppress approximately the same proportion of wild-type and mutant *ras* carcinomas in comparison with the control group. Short-term treatment with CLA for 4 or 8 weeks did not decrease significantly the total number of carcinomas, nor did it alter markedly the distribution of carcinomas carrying either the wild-type or mutant *ras* gene. Overall, there was no unusual pattern in the time of appearance of the *ras* mutant tumors due to CLA intervention (data not shown). Thus our results indicate that CLA inhibited carcinogenesis irrespective of the presence or absence of the codon 61 *ras* mutation.

Figure 3 shows the rates of CLA accumulation and disappearance in the neutral lipid fraction of the mammary tissue following CLA administration and withdrawal. As the results indicate, the incorporation was rapid once CLA was added to the animals' diets. The level reached ~70% of maximum after 2 weeks of feeding, and plateaued after 4 weeks. At the peak, CLA was present at roughly 3% of total fatty acids in the neutral lipid fraction. In this experiment, some animals were given CLA for 8 weeks. The regimen was stopped, and the decrease in CLA concentration was then plotted in the same composite diagram. Figure 3 shows that as soon as CLA was discontinued, the rate of disappearance from the mammary tissue was equally fast, with a return to basal value in about 4 weeks.

Figure 4 shows the increases and decreases of mammary gland phospholipid CLA from the same experiment. It should be noted that during CLA supplementation, the concentration of CLA in phospholipids (expressed as percent of total fatty acids) was, on the average, an order of magnitude lower than the concentration in neutral lipids. Interestingly, the rate of change of phospholipid CLA in either the upswing or downswing of the exposure/withdrawal curve was slower compared with that observed with neutral lipid CLA. After the start of CLA feeding, the maximum level in phospholipids was not attained until about 6 to 8 weeks later. Similarly, a diminished but still detectable amount of CLA was present by 6 weeks subsequent to the removal of CLA from the diet.

Discussion

The present study confirms our previous report that a continuous supply of CLA is necessary for maximum tumor inhibition in the post-initiation phase of mammary carcinogenesis. As pointed out in the Introduction, the first experiment was done in MNU-treated rats fed a 5% corn oil diet (9), whereas the repeat experiment described here was carried out in DMBA-treated rats fed a 20% corn oil diet. Thus this characteristic of CLA in chemoprevention is apparently not dependent on specific genomic mutation induced at the time of initiation or the availability of linoleic acid fed to the animals during tumor progression. It might be instructive to contrast the effects of CLA and linoleic acid at this point. Our study here indicated that CLA inhibits mammary carcinogenesis irrespective of the presence or absence of *ras* mutation. Linoleic acid, on the other hand, has been demonstrated to promote selectively the development of the wild type *ras* tumors, but not the mutant *ras* tumors, in MNU-treated rats (10). Recent data also suggested that the response to CLA is unlikely to be due to a displacement of linoleic acid in the mammary tissue (20). Collectively, the above information provides supportive evidence that these two fatty acids may have distinctive mechanisms in the modulation of mammary carcinogenesis.

Mutations of the *ras* gene have been reported to occur in a target organ- and chemical carcinogen-specific manner in a number of experimental models (21). Zarbl *et al.* (22) have previously described that in MNU-induced mammary tumors, GGA→GAA mutation in codon 12 of the *Ha-ras* proto-oncogene is a common event. The mutation probably results from methylation of guanine by diazomethane, a spontaneous decomposition product of MNU. In contrast, these same investigators found that only 21% (three out of 14) of DMBA-induced mammary tumors express a CAA→CTA mutation in codon 61 of the *Ha-ras* gene (22). It has been proposed that the A→T transversion is probably due to the affinity of the DMBA diol epoxide to the adenine residue as well as to the sequence selectivity in binding of the metabolite to the *Ha-ras* DNA (23,24). To our knowledge, there has been one other study examining *Ha-ras* codon 61 mutation in the DMBA model. Interestingly, Waldmann *et al.* (25) did not find such a mutation in a total of 50 tumors. The relatively low incidence of *Ha-ras* codon 61 mutation in our study is similar to that reported by Zarbl *et al.* (22). However, it should be noted that

our analysis was performed on a much larger sample size. In any case, the data in Table I clearly indicate that DMBA-initiated cells, with or without a *Ha-ras* codon 61 mutation, are equally sensitive to the inhibitory activity of CLA.

Recent studies by Banni *et al.* (26) have shown that in rats fed only 0.04% CLA in the diet for 1 week, conjugated diene-C18:3 and -C20:3 were recovered in the liver. Thus it appears that desaturation and elongation of CLA can occur *in vivo* while maintaining the conjugated diene structure. The presence of a conjugated diene-C20:4 metabolite could compete with arachidonic acid for the cyclo-oxygenase and lipo-oxygenase enzymes, thereby altering the biosynthesis of prostaglandins, thromboxanes and leucotrienes. These downstream products of arachadonic acid have been implicated by many investigators to be associated with promotion of carcinogenesis (27–32). By acting as a precursor to conjugated diene-C20:4, CLA could potentially play the role of a metabolic modulator in this process. Thus it becomes imperative to determine if conjugated diene-C20:4 is found in the mammary gland and if it is compartmentalized in a specific lipid fraction.

From our CLA analytical data, it is tempting to postulate that neutral lipid CLA may be a better indicator of protection than phospholipid CLA. Neutral lipid is far more plentiful than phospholipid in the mammary gland (see our previous work quoted in Reference 6). The larger pool of CLA in the former fraction may be more responsive to dietary intake because it serves as a depot for fatty acids that are not immediately utilized. Furthermore, the rate of decay of neutral lipid CLA following CLA withdrawal (Figure 3) seems to match more closely the rate of emergence of new tumors (refer to 4 week- or 8 week-CLA groups in Figure 1).

At first glance, the data on the changes in tissue concentration of CLA (Figures 3 and 4) appear to provide a reasonable explanation of why an uninterrupted supply of CLA is necessary to achieve tumor inhibition. As long as there is an abundant source of CLA present in the target organ, tumor appearance will be blocked or delayed. However, one must not lose sight of the possibility that CLA or a metabolite may induce an effect that is independent of its accumulation in mammary lipids. Future research will be aimed at delineating: (i) whether neutral lipid and phospholipid CLA levels simply represent indicators of CLA exposure; (ii) whether they serve as a local supply of CLA for further metabolism; and (iii) whether different cellular compartments of the mammary gland are involved in the accumulation and metabolism of CLA that ultimately leads to cancer prevention.

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Morphological and Biochemical Status of the Mammary Gland as Influenced by Conjugated Linoleic Acid: Implication for a Reduction in Mammary Cancer Risk[1]

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ABSTRACT

Previous research showed that treatment with conjugated linoleic acid (CLA) during the period of active mammary gland morphogenesis was sufficient to confer a lasting protection against subsequent mammary tumorigenesis induced by methylnitrosourea. The present study was designed to characterize certain morphological and biochemical changes of the mammary gland that might potentially render it less susceptible to cancer induction. Female Sprague Dawley rats were fed a 1% CLA diet from weaning until about 50 days of age. The mammary gland parameters under investigation included (*a*) the deposition of neutral lipid, (*b*) the identification and quantification of CLA and its metabolites, (*c*) the density of the epithelium, and (*d*) the proliferative activity of various structural components. Our results showed that CLA treatment did not affect total fat deposition in the mammary tissue nor the extent of epithelial invasion into the surrounding fat pad but was able to cause a 20% reduction in the density of the ductal-lobular tree as determined by digitized image analysis of the whole mounts. This was accompanied by a suppression of bromodeoxyuridine labeling in the terminal end buds and lobuloalveolar buds. The recovery of desaturation and elongation products of CLA in the mammary gland confirmed our prior suggestion that the metabolism of CLA might be critical to risk modulation. The significance of the above findings was investigated in a mammary carcinogenesis bioassay with the use of the dimethylbenz[*a*]anthracene model. When CLA was started at weaning and continued for 6 months until the end of the experiment, this schedule of supplementation produced essentially the same magnitude of mammary tumor inhibition in the dimethylbenz[*a*]anthracene model as that produced by 1 month of CLA feeding from weaning. The observation is consistent with the hypothesis that exposure to CLA during the time of mammary gland maturation may modify the developmental potential of a subset of target cells that are normally susceptible to carcinogen-induced transformation.

INTRODUCTION

Past research showed that CLA[3] has powerful cancer protective activity in a number of animal tumor models (1–6). With respect to mammary carcinogenesis in the rat, dietary supplementation of CLA has been reported to exert a unique inhibitory effect that is not commonly shared by many anticancer agents. The recent work of Ip and coworkers (7) demonstrated that CLA exposure limited to the period of active mammary gland development was sufficient to confer a lasting protection against subsequent chemically induced tumorigenesis in the target organ. In that experiment, CLA was fed to the animals between 21 days (weaning) and 55 days of age, and a single dose of MNU was administered for mammary tumor induction at day 56. No CLA was provided in the diet after carcinogen treatment. The above observation has a very significant implication for cancer prevention and is the subject of additional research reported herein.

The present study was designed to examine certain morphological and biochemical changes of the mammary gland after 1 month of CLA supplementation starting from weaning. Specifically, our objective was to define the degree of morphological development of the mammary gland as well as alteration in its biochemical constituents that might potentially render it less susceptible to cancer risk. The end points under investigation included (*a*) the total amount of lipid in the mammary gland, (*b*) the identification and quantification of CLA and its metabolites in the mammary tissue, (*c*) the density of the mammary epithelium and the area of the mammary fat pad occupied by the mammary tree, and (*d*) the proliferative activity of various mammary structural components.

Different carcinogens are known to cause specific mutations that may contribute to the process of oncogenesis (8). As indicated earlier, our initial study regarding the protective effect of CLA following short-term supplementation (instituted at an early age and prior to carcinogen treatment) was done using the MNU model. To rule out that this phenomenon of risk reduction is not an occurrence that is only characteristic of MNU-induced oncomutations, we repeated the tumor experiment with the DMBA model in the study reported here. Additionally, we also evaluated the relative efficacy of the timing of CLA supplementation by comparing the magnitude of mammary cancer inhibition in rats that were fed CLA from weaning to 50 days of age *versus* those that were given CLA from weaning to the end of the experiment, *i.e.*, including the entire period of tumor promotion and progression. This kind of information is important not only for formulating prevention strategies but also for targeting future research directions.

MATERIALS AND METHODS

Animals and CLA Supplementation. Pathogen-free female Sprague Dawley rats were purchased from Charles River Breeding Laboratories at weaning. They were fed the basal AIN-76A diet with or without supplementation with 1% CLA (Nu-Chek, Elysian, MN). For the studies that were designed to examine the morphological and biochemical changes of the mammary gland, the animals were sacrificed after 1 month on either the control or the CLA diet. The number of rats used in each type of analysis is indicated in the "Results" section. For the mammary carcinogenesis experiment, a total of 120 rats were divided equally into four groups according to the following dietary treatment: group A, control diet from weaning to termination of the experiment (see below); group B, 1% CLA diet from weaning to 50 days of age followed by a switch to the control diet; group C, 1% CLA diet from 55 days of age to termination of the experiment; and group D, 1% CLA diet from weaning to termination of the experiment.

Lipid Extraction. Total lipid was extracted from frozen pulverized mammary tissue by the method of Folch *et al.* (9). Neutral lipid and phospholipid were separated with the use of a Sep-Pak silica cartridge, as described in an earlier publication (5). The amount of lipid recovered in each of these fractions was measured.

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[3] The abbreviations used are: CLA, conjugated linoleic acid; MNU, methylnitrosourea; DMBA, dimethylbenz[*a*]anthracene; BrdUrd, bromodeoxyuridine.

Quantification of Conjugated Diene Polyunsaturated Fatty Acids. For the determination of conjugated diene polyunsaturated fatty acids, total lipid extracted from the abdominal-inguinal mammary glands (without separation into neutral lipid and phospholipid) was used as the starting material. Free fatty acids were obtained by a mild saponification procedure as described by Banni *et al.* (10) and collected in *n*-hexane. After solvent evaporation, the residue was redissolved in CH_3CN/0.14% CH_3COOH (v/v) for injection into the high-performance liquid chromatography system. Separation of unsaturated fatty acids was carried out with a Hewlett-Packard 1050 liquid chromatograph equipped with a diode array detector 1040M (Hewlett-Packard, Palo Alto, CA). A C-18 Alltech Adsorbosphere column, 5-μm particle size, 250 × 4.6 mm, was used with a mobile phase of $CH_3CN/H_2O/CH_3COOH$ (70/30/0.12, v/v/v) at a flow rate of 1.5 ml/min. Nonconjugated diene unsaturated fatty acids were detected at 200 nm, and conjugated diene unsaturated fatty acids were detected at 234 nm. Spectra (195–315 nm) of the eluate were obtained every 1.3 s and were stored electronically. Second-derivative UV spectra of the conjugated diene fatty acids were generated using the Phoenix 3D HP Chemstation software. These spectra were taken to confirm the identification of the high-performance liquid chromatography peaks. Details of the methodology regarding the characterization of conjugated diene fatty acids in both reference and biological samples have recently been published by Banni and coworkers (11).

Preparation of Mammary Gland Whole Mount and Analysis of Epithelial Density. The abdominal-inguinal mammary gland chain was excised in one piece and stretched onto a 75 × 50 mm microscope slide. The whole mount was fixed in 10% buffered formalin for 12–18 h and rinsed in distilled water. It was then dehydrated using a series of ethanol solutions (70, 95, and 100%) for 1 h each and cleared with two changes of toluene for 1 h each. The tissue was rehydrated with water and immersed in alum carmine stain for 5–7 days. Once staining was complete, the whole mount was dehydrated using ethanol as described above and cleared with one change of xylene for 2 h. Each whole mount was then placed in a 4 × 6-inch heat-sealable pouch and filled with 20 ml of methyl salicylate. Methyl salicylate was chosen as the clearing agent, because its refractory index is very close to that of tissue. This resulted in superior photographic resolution with a clean background. The pouch was left overnight, and on the next day, it was pressed flat to remove excess methyl salicylate and air. All whole mounts were photographed using a Nikon 55 mm camera equipped with a digital camera back. The digitized images were analyzed by scanning densitometry.

All images were presented as an array of pixels. The manipulation of images and all calculations of the parameters were performed on an MPC 200 workstation running the UN-SCAN-IT software (Silk Scientific, Inc., Orem, UT). For each mammary gland chain, the same anatomical region was assessed. This assessment was restricted to the abdominal inguinal mammary gland chain and specifically to the region cephalic to the anterior-most lymph node located in the fourth mammary gland. This lymph node served as an anatomical landmark from which a line perpendicular to the cephalocaudal axis of the animal was projected across the image of the mammary gland. The gland cephalic to this line was cut into image segments, including only the area occupied by the mammary epithelium. The absorbance of each segment and the total area of each segment were then quantified and saved to disk. The density and area of all segments were then summed and averaged. An interactive density thresholding technique was used to select the pixel intensity value (gray value) above which pixels were discriminated from the remainder of the gland as mammary epithelium. This was done for all images by one observer. Preliminary studies (data not shown) indicated that the use of absorbance per unit area is capable of detecting differences in the maturity of the mammary gland that occurs in young rats as well as the remarkable differences in gland development observed between the virgin and pregnant states. The above approach is based on previous work in rats and mice in which a scoring system for assessing mammary gland maturity was described (12) and on studies in humans in which digitized mammographic images were evaluated for breast density (13).

Assessment of Mammary Epithelial Proliferation by BrdUrd Labeling. Rats were injected i.p. twice a day for 3 consecutive days with 50 mg/kg of BrdUrd dissolved in saline. After excision, the abdominal-inguinal mammary glands were fixed in methacarn, processed in an autotechnicon, and embedded in paraffin blocks. Tissues were cut into 5-μm sections and placed onto 3-aminopropyl-triethoxysilane-prepared slides. The sections were then heat immobilized, deparaffinized in xylene, rehydrated in descending grades of ethanol, and rinsed in deionized water. The proportion of labeled cells was detected by immunohistochemical staining as described by Eldridge *et al.* (14). Endogenous peroxidase activity was quenched by incubating in 3% H_2O_2. Mouse anti-BrdUrd (Becton Dickinson) was then applied at a dilution of 1:40 for 60 min; this was followed by treatment with biotinylated rabbit antimouse antibody (DAKO Corp.) and streptavidin horseradish peroxidase. The slides were counterstained with hematoxylin, rinsed, dehydrated, and mounted with Permount. Cells that incorporated BrdUrd were identified by brown granules over the nuclei. One thousand nuclei in each of different mammary compartments (*i.e.,* duct, terminal end bud, and lobuloalveolar bud) were counted, and the number that stained positive was noted.

Mammary Tumor Induction and Determination of Proliferative Activity and Apoptosis in Mammary Tumors. Mammary tumors were induced by the intragastric intubation of 10 mg of DMBA at 50 days of age. Animals were palpated weekly, and the time of appearance and location of tumors in the mammary gland were recorded. The experiment was terminated 21 weeks after DMBA treatment. By that time, the development of palpable tumors had plateaued for several weeks across all groups. Only histologically confirmed adenocarcinomas are reported in "Results." Tumor incidences at the final time point were compared by χ^2 analysis, and the total tumor yield between the control and CLA-treated groups was compared by frequency distribution analysis, as described previously (15).

Proliferative activity in the mammary tumors was determined by BrdUrd labeling, as described in an earlier section. Apoptosis in paraffin-embedded tumor sections was evaluated following the procedure supplied with the ApopTag 228 kit, which was purchased from Oncor (Gaithersburg, MD). In brief, the principle of the assay is based on the addition of digoxigenin-labeled nucleotides to the 3′-hydroxyl ends of double- or single-stranded DNA, catalyzed by the terminal deoxynucleotidyl transferase enzyme. An antidigoxigenin antibody conjugated to peroxidase and a chromogenic substrate (diaminobenzidine) are then used to detect incorporated digoxigenin nucleotides in cells.

RESULTS

Effect of CLA Feeding on Lipid Content of the Mammary Gland. Recent studies indicated that mice and chickens fed a 0.5% CLA diet showed a 50% reduction in body fat (16). Because deposition of mammary fat is known to regulate epithelial growth in this tissue, we decided to first examine the effect of CLA on the concentration of fat in the mammary gland. Table 1 summarizes the lipid content in the mammary fat pad of rats fed either the control or 1% CLA diet. Both diets were given starting at weaning and continuing for 1 month before the animals were sacrificed. There was no difference in the amounts of total lipid, neutral lipid, and phospholipid that were extractable from the mammary tissue between the two groups. Neutral lipid represented a predominant constituent of total lipid. This is consistent with the fact that triglyceride-containing adipocytes are a major component of the mammary tissue. On the other hand, phospholipid is present at a concentration that is about 100 × less. Epithelial cells are the primary source of phospholipid in the mammary gland. The low phospholipid level is reflective of the incomplete differentiation state of the mammary epithelium seen in a nonpregnant and nonlactating animal. The method used for separating the neutral lipid and phospholipid fractions recovered approximately 90% of total lipid. It is possible that more polar lipids might be left on the column. Our immediate plan is to investigate this in greater detail and to

Table 1 *Lipid content in mammary fat pad of rats fed either control or 1% CLA diet*[a]

Diet[b]	Total lipid	Neutral lipid (mg/g of tissue wet wt)	Phospholipid
Control	266 ± 25	230 ± 20	2.2 ± 0.3
1% CLA	260 ± 16	227 ± 24	2.1 ± 0.3

[a] Values are expressed as mean ± SE (n = 6).
[b] Diets were fed from weaning to 50 days of age.

Table 2 *Conjugated diene polyunsaturated fatty acids (CD-PUFAs) in mammary tissue of rats fed control or 1% CLA diet*[a]

	CD-PUFA[b] (nmol/mg of lipid)		
Diet	CD 18:2	CD 18:3	CD 20:3
Control	2.7 ± 0.2	0.4 ± 0.1	0.4 ± 0.1
1% CLA	176.9 ± 6.0[c]	2.4 ± 0.3[c]	5.6 ± 0.3[c]

[a] Values are expressed as mean ± SE ($n = 6$).
[b] The type of polyunsaturated fatty acid is designated by the length of the carbon chain (18 or 20) and the number of double bonds (2 or 3).
[c] $P < 0.001$.

determine any potential changes in phospholipid composition due to CLA feeding.

Although our previous studies have hinted that the metabolism of CLA may represent a vital step in cancer protection, the characterization of various conjugated diene polyunsaturated fatty acids in the mammary gland of CLA-fed rats has not been delineated. Because the composition of individual saturated and unsaturated fatty acids in the mammary tissue of CLA-supplemented rats under various dietary conditions has been described in our earlier publication (5), the results shown here will focus only on CLA and its metabolites. Banni *et al.* (11) have recently published detailed analytical methodologies regarding the identification of various conjugated diene polyunsaturated fatty acids; the same method was used to quantify these derivatives in the mammary gland of control and CLA-fed rats. As shown in Table 2, in addition to CLA (CD 18:2), two other conjugated diene polyunsaturated fatty acids were detected, an 18-carbon triene fatty acid (CD 18:3) and a 20-carbon triene fatty acid (CD 20:3). All three conjugated diene polyunsaturated fatty acids were present at very low levels in the mammary tissue of control rats. Their concentrations were elevated to different degrees upon supplementation with 1% CLA in the diet: 65-fold with CD 18:2, 6-fold with CD 18:3, and 14-fold with CD 20:3. The above finding suggests that CLA could be desaturated further (from a diene to a triene) and elongated (from an 18-carbon to a 20-carbon fatty acid) while still maintaining the conjugated diene structure.

Effect of CLA on Morphology and Density of the Mammary Epithelium. It is possible that CLA may reduce mammary cancer risk by directly or indirectly inhibiting the expansion and proliferation of mammary epithelial cells. If this were the case, one might expect to observe a less complex mammary ductal-lobular tree and a reduction in proliferative index associated with the growing epithelium (terminal end buds and/or alveolar buds as opposed to the more stable subtending ducts). Fig. 1 shows representative mammary gland whole mounts from control rats or rats fed 1% CLA. A total of 10 mammary glands were examined from each group. Overall, we could not detect any significant differences in the area of the mammary fat pad occupied by the mammary epithelium as a result of dietary treatment. The density of the mammary epithelium was evaluated by densitometric analysis of the digitized images as described in "Materials and Methods." The data in Table 3 are expressed as absorbance per mm^2, giving the mean ± SE as well as the 95% confidence interval of the two groups. Our analysis indicated that there was a 21% reduction in the density of the mammary epithelium in the CLA-treated rats. This decrease was statistically significant ($P = 0.009$).

Table 4 reports the effect of CLA feeding on proliferative activity of the mammary epithelium as measured by BrdUrd labeling. No change was detected in the cells lining the ducts. However, DNA synthesis in the terminal end buds and lobuloalveolar buds was inhibited by about 30% ($P < 0.05$) as a result of CLA supplementation. The multiple BrdUrd dosing method over a 3-day period (see "Materials and Methods") increases the sensitivity of the technique by allowing more cells to be labeled and also provides the advantage of being able to mirror steady-state rather than snapshot information. Additionally, this method evens out the slight variations in proliferative activity of the mammary epithelial cells due to estrous cycle hormonal surges.

Effect of Timing of CLA Supplementation on DMBA-induced Mammary Carcinogenesis. What is the implication of the above morphological and biochemical changes in relation to mammary cancer risk reduction? This question was addressed by the carcinogenesis bioassay. Our primary objective was to determine whether continuous feeding with CLA for 6 months would be more effective in cancer prevention when compared to the 1-month CLA feeding protocol administered during the period of mammary gland morphogenesis. The results of this experiment are shown in Table 5. As indicated in "Materials and Methods," three different schedules of CLA supplementation were instituted: from weaning to 50 days of age (group B), from 55 days of age to the end of the experiment (group C), and from weaning to the end of the experiment (group D). In all three CLA-supplemented groups as well as in the control group, DMBA was administered to the animals at 50 days of age. At this point, it is important to clarify that our previous work has demonstrated that CLA intake did not affect DMBA binding to mammary DNA (3). Additionally, as will be discussed below, CLA has no effect on phase I and phase II enzymes that are involved in the metabolism of DMBA.

Regardless of whether CLA was given according to the protocol of group B or group C, it reduced the total number of tumors by about 50% ($P < 0.05$). These observations are consistent with that described in our previous reports (5, 7). Interestingly, when CLA was started at weaning and continued to the end of the experiment (Group D), this schedule of supplementation produced essentially a magnitude of tumor inhibition (57%) comparable to that seen in groups B or C. In terms of the timing and length of CLA exposure, group D was representative of the sum of groups B and C. Thus, it might have been predicted that the effects at each stage would have been additive; *i.e.,* the magnitude of tumor inhibition in group D would be in the range of 75% [50% inhibition from feeding CLA prior to DMBA, plus an additional 25% (50% of 50%) inhibition from feeding CLA after DMBA]. The fact that the additive effect was not observed suggests that different mechanisms may be operative depending on whether CLA exposure is coincidental with the period of active mammary gland morphogenesis and development (group B) or occurs during the period of tumor progression after the mature gland is exposed to a carcinogen (group C).

Given the somewhat unexpected result obtained in group D, the above mammary carcinogenesis study was repeated to confirm the reproducibility of the findings. When the data were evaluated at week 15 post-DMBA in the second experiment, the same pattern was found to emerge as that reported in Table 5. Specifically, the magnitude of tumor inhibition was again not statistically different between groups B and D; the total number of palpable tumors was reduced by 45% in group B and 50% in group D. Usually, this type of experiment is maintained for 20 weeks or longer after carcinogen treatment to achieve a plateauing of tumor appearance. In this duplicate experiment, we decided to terminate it at week 15 post-DMBA treatment, so that we could harvest a reasonable number of tumors that would be suitable for the BrdUrd labeling and apoptosis assays. A total of 10 tumors were obtained from group A (control), and 10 tumors were obtained from group D (continuous CLA supplementation). These tumors were chosen based on the criterion that they all showed a fairly uniform growth rate as determined by weekly caliper measurements. Our intention was to avoid the abnormally large tumors (which are frequently necrotic) and the very small tumors (which have a tendency to remain static).

Both the BrdUrd labeling and apoptosis results are reported as a

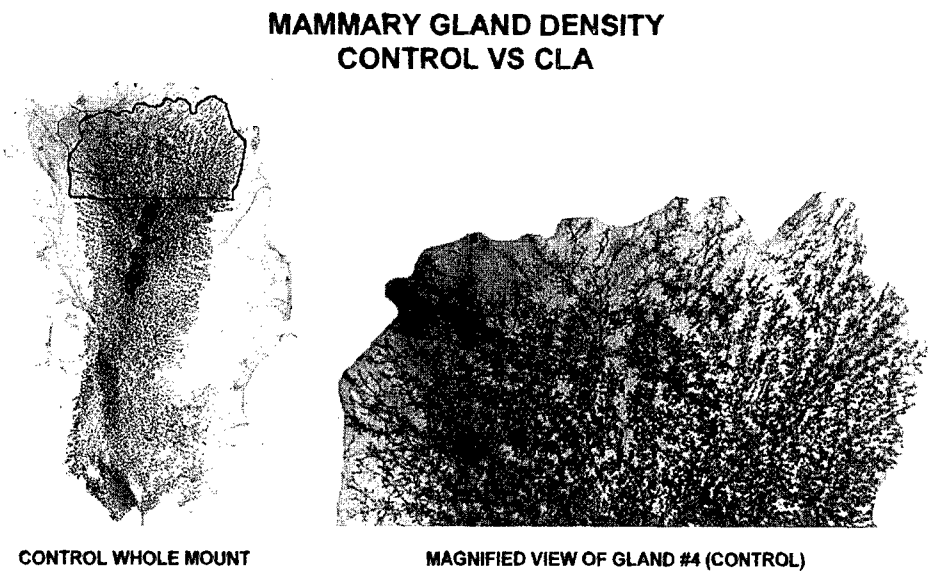

Fig. 1. Representative mammary gland whole mounts from a control rat and a rat fed 1% CLA.

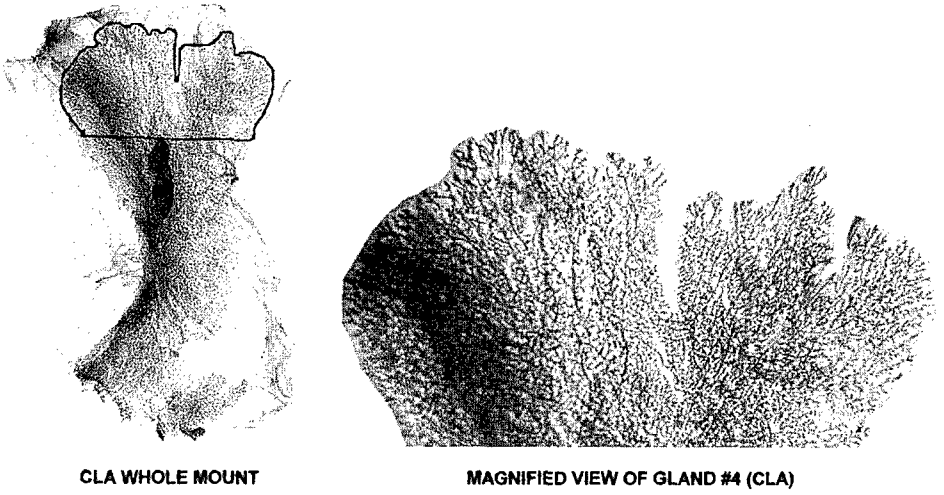

Table 3 *Quantitative analysis of mammary epithelial density in whole mounts of rats fed control or 1% CLA diet*[a]

	Optical density unit per mm^2	
Diet	Mean	95% confidence interval
Control	110 ± 6	96–125
1% CLA	87 ± 4[b]	77–97

[a] The digitized image was taken from photographs as represented in Fig. 1. The data are given as mean ± SE (n = 10) or 95% confidence interval.
[b] P = 0.009 according to Kruskal-Wallis one-way ANOVA.

percentage of cells that reacted positively to the respective immunohistochemical staining methods (Table 6). Although the number of proliferating cells and apoptotic cells appeared to be slightly higher in mammary tumors from CLA-fed rats when compared to those from control rats, the increase was not statistically significant. Whether a larger sample size will add new biological meaning to the above observation remains to be elucidated. Variabilities were generally greater with the apoptosis assay than with the BrdUrd labeling assay. No correlation was evident between the size of the tumor and the intensity of BrdUrd labeling or the extent of apoptosis in either group (data not shown). In general, the data support the hypothesis that tumors growing out in rats fed CLA are resistant to the effect of this fatty acid.

DISCUSSION

Adipocytes are a major and integral cellular component of the rodent mammary gland. They are known to have an important influence on the growth and development of the mammary epithelium. Mammary cells, when transplanted to the gland-free fat pad, will proliferate and expand up to the boundary of the fat pad but not

Table 4 *BrdUrd labeling in mammary epithelium of rats fed control or 1% CLA diet*

	Mammary compartment (% of cells labeled)[b]		
Diet[a]	Duct	Terminal end bud	Lobuloalveolar bud
Control	15.4 ± 1.5	21.7 ± 2.0	26.4 ± 2.1
1% CLA	14.6 ± 1.3	15.5 ± 1.3[c]	18.7 ± 1.9[c]

[a] There were 12 rats used in each group.
[b] A total of 1000 nuclei were counted per mammary compartment.
[c] $P < 0.05$.

Table 5 *DMBA-induced mammary carcinogenesis in rats fed control or 1% CLA diet*

Group[a]	Diet	Duration of CLA feeding	Tumor incidence	Total No. of tumors	% inhibition[b]
A	Control		26 of 30	82	
B	1% CLA	From weaning to 50 days of age	17 of 30[c]	42[c]	49%
C	1% CLA	From 55 days of age to end of experiment	14 of 30[c]	38[c]	54%
D	1% CLA	From weaning to end of experiment	14 of 30[c]	35[c]	57%

[a] DMBA was given to all groups at 50 days of age and the experiment was terminated at 21 weeks post-DMBA.
[b] Percentage inhibition was calculated using the total tumor number data.
[c] $P < 0.05$ compared to the corresponding control group without CLA

beyond (17, 18). Recent studies from Pariza's laboratory indicated that feeding 0.5% CLA in the diet to mice and chickens reduced their body fat by about 50% (16). The above finding prompted us to investigate whether the deposition of mammary fat in rats would be affected similarly. If CLA were to cause a modest reduction of fat deposition in the mammary gland, such an outcome might lead to less than full development of the mammary epithelium, which might explain the decrease in risk to carcinogenesis. This is clearly not the case, as evidenced by the lack of an effect of CLA on the amount of extractable mammary fat (Table 1). We did, however, find a small but significant reduction in the density of the branching mammary epithelium as a result of CLA feeding (Fig. 1; Table 3). The decrease in lateral branching, as best determined by digitized image analysis, should be distinguished from the unaffected ability of the subtending ducts to invade up to the edge of the surrounding fat pad. Thus, our study suggests that CLA has no apparent effect on fat deposition in the mammary tissue but is able to down-regulate the lateral proliferation of the mammary epithelium via either a direct or an indirect mechanism.

The data in Table 4 show that DNA synthesis (determined by BrdUrd labeling) in the terminal end buds and lobuloalveolar buds was inhibited by CLA. It was therefore gratifying to see that quantitative image analysis of reduced morphogenesis in the mammary gland of CLA-fed rats was confirmed by biochemical measurement of a lower rate of cell proliferation. Terminal end bud cells are the progenitors of lobuloalveolar bud cells. In a 50-day-old rat, most of the terminal end bud cells have already been differentiated to lobuloalveolar bud cells (19). Nonetheless, the former structures are the primary target sites for the induction of mammary carcinomas by chemicals. The lower rate of proliferation in the terminal end bud cells could explain in part the decreased risk to chemical initiation of carcinogenesis.

At this point, it is instructive to contrast the differential effects of CLA on DNA synthesis in normal mammary cells (Table 4) *versus* mammary tumor cells (Table 6). One interpretation is that CLA may decrease the turnover of normal cells (*e.g.,* terminal end bud cells and lobuloalveolar cells) and inhibit the clonal expansion of early transformed cells (Table 5, group C) but does not affect the proliferative rate of frank carcinoma cells (Table 6). The last part of the conclusion is logical, because the mere appearance of a tumor in a CLA-treated animal implies that the transformed cells in this tumor have already escaped the suppressive effect of CLA. On the other hand, it should be noted that CLA is able to suppress the growth of a human breast cancer line (MDA-MB-468) transplanted in SCID mice, as reported in a recent study (20). Whether this response is unique to the particular cell line grafted to a severely immunodeficient host remains to be investigated. The low frequency of apoptotic cells in the mammary tumors (Table 6) is not unexpected, because the methodology only provides a freeze-frame picture at a given point in time. Additional research is needed to elucidate whether early transformed cells are more sensitive than frank carcinoma cells to CLA-mediated changes in proliferation and apoptosis.

Table 6 *BrdUrd labeling and apoptosis in mammary tumors from rats fed either control or 1% CLA diet*

		% positive cells[a]	
Diet	Duration of CLA feeding	BrdUrd label	Apoptosis
Control		14.2 ± 1.8	0.6 ± 0.2
1% CLA	From weaning to end of experiment	19.2 ± 2.2	0.9 ± 0.2

[a] Values are expressed as mean ± SE ($n = 10$).

Previous studies have suggested that the metabolism of CLA may be critical in expressing its anticancer activity (21, 22). For example, the conversion of CLA to other related conjugated diene-polyunsaturated fatty acids has a number of potential implications that are relevant to modulation of carcinogenesis. This particular aspect has been discussed in our recent publications (21, 22). Thus, the recovery of desaturation and elongation products of CLA in the mammary gland represents a significant step in this direction. Although CD 18:3 and CD 20:3 were found to be much higher in CLA-fed rats, no CD 20:4 was detected. Suffice it to note that the above analysis was done by using total extractable lipid, which consisted predominantly of neutral lipid because of the contribution from adipocytes. Arachidonic acid is incorporated generally in phospholipid rather than neutral lipid. We are planning to isolate mammary epithelial cells so that a pure phospholipid fraction (free of neutral lipid) can be generated for the analysis of CD 20:4.

Future studies will also focus on the distribution of these conjugated diene polyunsaturated fatty acids in different tissues and blood, as well as in mammary tumors.

The precarcinogen protective effect of CLA (see group B in Table 5) was described initially in rats treated with MNU (7). By using DMBA in the present experiment, we were able to confirm the universality of this response, suggesting that the reduced risk conferred to the animals at an early age is not dependent on carcinogen-specific oncomutations. It is unlikely that CLA achieves this effect by modulating the metabolism of DMBA. As reported previously, CLA does not affect DMBA binding to mammary DNA (3). This observation is consistent with the lack of an effect of CLA on phase I P450 enzymes (1A1, 1A2, 2B1, 2E1, and 3A4),[4] and phase II detoxifying enzymes (23). Of particular interest is the finding that short-term supplementation with CLA prior to DMBA (Table 5, group B) is almost as efficacious as the continuous supplementation protocol (Table 5, group D). One possible reason for this is that exposure to CLA during the time of mammary gland maturation might modify the development of a subset of target cells, such that only CLA-resistant mammary epithelial cells were "available" for carcinogen targeting. This hypothesis could account for the reduced cancer risk of group B and the absence of additional protection seen in group D when CLA was continued after carcinogen treatment. In contrast, both the CLA-sensitive and -insensitive subsets of target cells were present in group C at the time of carcinogen administration. Consequently, the feeding of CLA after carcinogen administration was able to suppress the clonal expansion of those transformed cells that originated from the CLA-sensitive progenitors. This interpretation is supported by the

[4] C. Ip, unpublished data.

data in Table 6, which demonstrate that the proliferative rate of tumors from CLA-fed rats was not inhibited when compared to tumors from control rats. Although somewhat simplistic, the above concept offers a unifying hypothesis to interpret the collective results described in this paper, and is a reasonable starting point to investigate in depth the multiple mechanisms involved in the action of CLA in cancer prevention.

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MOLECULAR CARCINOGENESIS 20:204–215 (1997)

Gene Expression Changes Associated With Chemically Induced Rat Mammary Carcinogenesis

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Experimentally induced models of breast carcinogenesis in the rat are widely used for studying the biology of breast cancer and for developing and evaluating cancer prevention and control strategies. However, very little is known about gene expression changes that are associated with experimentally induced mammary carcinogenesis. This paper reports the identification, by differential display of mRNA and molecular cloning, of seven cDNA fragments of gene transcripts overexpressed in mammary carcinomas induced by 1-methyl-1-nitrosourea. These genes included the rat homologues of human galectin-7 gene, the human/mouse melanoma inhibitory activity/bovine chondrocyte-derived retinoic acid sensitive protein gene, the mouse stearoyl-CoA desaturase-2 gene, and the mouse endo B cytokeratin/human cytokeratin-18 gene. Although each of these genes has been implicated in some aspect of carcinogenesis in other organs, this paper is the first report of their overexpression in chemically induced mammary carcinomas. Two previously uncharacterized gene transcripts were also identified. A comparison of the expression levels of several genes in mammary carcinomas with those in the normal mammary gland tissue of virgin rats, mid-stage pregnant rats, and of day 1 postpartum lactating dams indicated that the overexpression of several genes observed in mammary carcinomas could not be accounted for by either a difference in the mammary epithelial content between mammary carcinoma and normal mammary tissue or by mammary epithelium–specific proliferation associated with pregnancy. Several genes were also overexpressed in rat mammary carcinomas induced by 7,12-dimethylbenz[*a*]anthracene but not in azoxymethane-induced rat colon adenocarcinomas. The genes identified in this study may therefore represent mammary carcinoma-specific molecular markers that may be helpful in investigations of mammary carcinogenesis and its prevention. *Mol. Carcinog. 20:204–215, 1997.*

Key words: mammary carcinogenesis; cancer markers; differential display; molecular cloning; gene expression

INTRODUCTION

The induction of mammary carcinogenesis in virgin female rats by administration of l-methyl-l-nitrosourea (MNU) or 7-12-dimethylbenz[*a*]anthracene (DMBA) is the most widely used model of investigating breast carcinogenesis in women [1–4]. Comparisons of the similarities and differences in mammary carcinogenesis in rats and humans have been reported, and the findings obtained by using these models have been extensively reviewed [3,4]. One aspect of carcinogenesis that has received limited attention, however, is the characterization of the pathogenetic changes associated with experimentally induced mammary carcinogenesis in the rat and comparison of these changes with those that occur during human carcinogenesis.

Cancer is the result of mutation or misregulation of normal cellular genes. The outcome of a gene mutation or misregulated gene expression, by and large, is not immediate; rather, the development of breast cancer and other types of cancer results from accumulation of pathogenetic events [5–7]. A pathogenetic event is a general term for the many different types of DNA damage that can lead to gene amplification, point mutation, rearrangement, and deletion. The complexity of the multistep pathogenetic process makes the task of understanding the development of specific cancers, and particularly the role of specific genes, quite difficult. Nevertheless, patterns of specific gene mutations are emerging and being correlated with the development of particular cancers [6–8]. The recent discovery and cloning of breast cancer susceptibility genes *BRCA-1* and *BRCA-2* [9–11] highlight the significant progress that has been made in understanding the role of specific genes in hereditary breast cancer, which accounts for only a minor fraction (<5%) of breast cancer cases. With respect to specific gene mutations in chemically induced mammary carcinomas in the rat, Ha-*ras* codon 12 mutations (in the MNU model) and codon 61 mutations (in the DMBA model) are the only well-characterized oncogene mutations in these models,

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Abbreviations: MNU, 1-methyl-1-nitrosourea; DMBA, 7,12-dimethylbenz[a]anthracene; DD, differential display; AOM, azoxymethane; PCR, polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; EST, expressed sequence tag; MIA, melanoma inhibitory activity; CD-RAP, chondrocyte-derived retinoic acid–sensitive protein; SCD-2, stearoyl-CoA desaturase-2; RT, reverse transcriptase; TPS, tissue polypeptide-specific.

and each is found in only some of the carcinomas induced in the model [12–15]. Little is known about other pathogenetic events in these models. One measurable consequence of these events is overexpression of particular genes. In fact, overexpression of specific genes such as *HER-2/neu* and *myc* has been associated with development of breast cancer [16–19].

As a step towards filling this information void, this study was undertaken to identify gene expression changes, by using differential display (DD) of mRNA [20] and molecular cloning, in mammary carcinomas induced in female rats by MNU injection. The underlying hypothesis was that changes in gene expression would be observed in mammary carcinomas relative to mammary tissues in three distinct physiological states: the virgin mammary gland, the fully differentiated day 1 postpartum lactating mammary gland, and the rapidly proliferating mammary gland during mid-stage pregnancy. Here, we report the identification and cloning of seven cDNA fragments of overexpressed genes in rat mammary carcinomas.

MATERIALS AND METHODS

Chemical Carcinogenesis and Tissue Sources

The rat mammary carcinomas used for the DD of mRNA were excised from MNU-injected rats treated with a short-term carcinogenesis protocol [21]. Briefly, female Sprague Dawley rats (Taconic Farms, Germantown, NY) were given an intraperitoneal injection of MNU (50 mg/kg body weight) at 21 d of age. One rat was found to have multiple mammary tumors. This animal was killed 2 mo after the injection, and three mammary adenocarcinomas, uninvolved mammary tissue, and a kidney were immediately excised and frozen in liquid nitrogen. Tumors, tissue samples, and organs were similarly obtained from other rats. Day 1 postpartum lactating mammary gland tissue was pooled from several dams. To obtain mammary tissues from mid-stage pregnant rats, female rats that had been with a male rat for 12 d were killed, and mammary tissues were immediately excised and frozen in liquid nitrogen. The uterus of each rat was inspected to confirm the approximate stage of pregnancy based on the size of the fetuses. Two females were determined to be in the mid-stage of pregnancy; thus, mammary tissues from these animals were used in this study.

To determine the relevance of the genes identified in this short-term model to the conventional MNU carcinogenesis model in which the carcinogen is administered at 50 d of age, mammary carcinomas were also obtained by intraperitoneal injection of MNU into rats at 50 d of age [22], and these carcinomas were analyzed for gene expression by Northern blot analysis. DMBA-induced rat mammary carcinomas were obtained from Dr. Clement Ip (Roswell Park Cancer Institute, Buffalo, NY) and were examined to determine the mRNA levels of several overexpressed genes identified in the MNU-induced mammary carcinomas. In addition, two rat colon adenocarcinomas induced by azoxymethane (AOM) and matching uninvolved colon tissue were obtained from Dr. Dennis Ahnen (University of Colorado Health Sciences Center, Denver, CO) and were analyzed for gene expression by Northern blotting.

RNA Isolation

Total RNA was extracted from carcinomas and tissues by acidic phenol extraction with RNAzolB reagent (Tel-Test, Inc., Friendswood, TX). For DD, the total RNA preparations were further digested with RNase-free DNase (GenHunter Corporation, Nashville, TN) to remove contaminating genomic DNA. For cDNA library construction, poly(A)+ mRNA was enriched by oligo (dT)-cellulose column chromatography.

DD

DD of mRNA [20] was performed with the RNAimage kit (GenHunter Corporation) according to the manufacturer's instructions with the following modification. In Denver, CO, where the altitude is 5280 ft, the optimal annealing temperature for polymerase chain reaction (PCR) was determined to be 42°C. The strategy used for DD is illustrated in Figure 1. The PCR products (labeled with [α-^{32}P]dCTP) from three mammary adenocarcinomas, the uninvolved mammary tissue, and the kidney were compared side by side on sequencing gels and detected by autoradiography. Only bands present in carcinoma lanes but absent in both the mammary tissue and kidney lanes were excised and reampli-

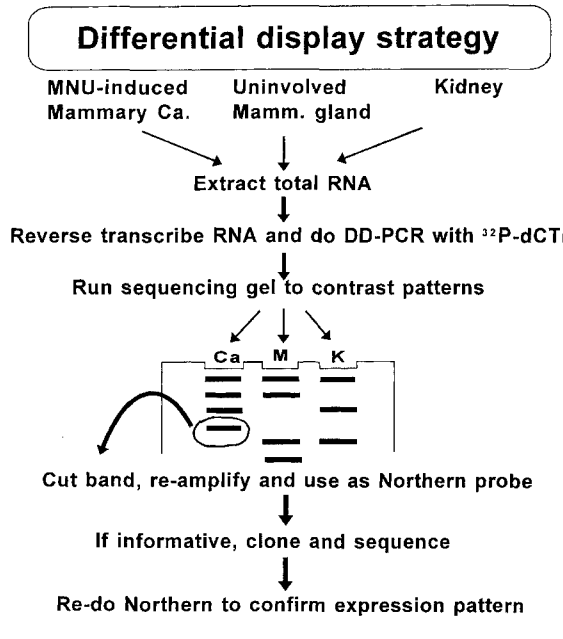

Figure 1. Schematic illustration of the DD strategy used in this study.

fied by PCR. The PCR products were separated on a low-melting-point agarose gel, and DNA bands of the expected size were eluted with a Wizard PCR DNA purification kit (Promega Corp., Madison, WI). The gel-purified PCR DNA products were used as templates to generate ^{32}P-labeled probes for Northern blot detection of gene expression on a screening panel of RNA preparations. This panel included the kidney, the three mammary carcinomas, and the uninvolved mammary tissue that were used for the original DD and two liver samples. In addition, the day 1 postpartum mammary tissue was included in this screening panel as a control for mammary epithelial cell number. The glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene was hybridized as an internal control for loading correction. Ethidium bromide staining of the agarose gels before the RNA was transferred to nitrocellulose membrane and hybridization for the β-actin gene also were performed in some cases to verify loading and RNA integrity.

Cloning and Sequencing

PCR bands that detected differential gene expression were cloned into pGEM-T (Promega Corp.). For each band, four clones were inoculated, and the plasmid DNA was isolated by an alkaline mini-prep procedure. At least two clones were sequenced on both strands by the dideoxy chain termination method of Sanger et al. [23] by using a kit from U.S. Biochemicals (St. Louis, MO). A commercial sequencing service (Cornell DNA Service, Ithaca, NY) also was used to confirm the sequence of a few of the clones. The cloned cDNA fragments were used as templates to generate randomly labeled probes for Northern blot analysis again to confirm that the cloned sequences corresponded to the gene transcripts originally detected by the PCR products eluted from the DD gels. A homology search was performed by using the BLASTN algorithm [24] with GenBank non-redundant (nr) databases and expressed sequence tag (EST) databases.

Cloning of Full-Length cDNA

A cDNA library was constructed with pooled poly(A)+ mRNA isolated from mammary carcinomas by using the Marathon cDNA construction kit (Clontech, Inc., Palo Alto, CA). The average length of the library inserts was 1.5 kb. Based on the sequence information obtained for each gene fragment, a gene-specific primer was synthesized (Integrated DNA Technology, Inc., Coralville, IA) as the downstream PCR primer. The gene-specific primer and a universal upstream primer that annealed to the adapter that had been ligated into the cDNA library were used for long-distance PCR with KlenTaq (a combination of Taq and Vent polymerases) (Clontech Inc.) to increase fidelity of cloning. The gel-purified PCR fragments were cloned into pGEM-T and sequenced as described above.

RESULTS

Differentially Expressed Gene Transcripts

A total of 15 primer-pair combinations were used to compare by DD the gene expression patterns of three MNU-induced mammary carcinomas with those of the uninvolved virgin mammary tissue and kidney from the same rat. In initial screening, 21 cDNA fragments were used as probes for Northern analysis, and 16 detected transcripts differentially expressed in the mammary carcinomas and uninvolved virgin or day 1 postpartum lactating mammary tissue. Seven cDNA clones were characterized in more detail and are reported here.

Representative Northern blot detection of steady-state gene transcripts in a screening panel of tissues by using cloned cDNA fragments as probes is shown in Figure 2. Several points are noteworthy. First, most of the clones detected overexpression in mammary carcinomas (lanes 2–4) in comparison with the mammary tissue (lanes 7 and 8), kidney (lane 1), and liver (lanes 5 and 6). The exceptions were clone 9, which detected low-level expression in one of two liver samples and clones 14 and 10, which detected weak expression in liver and mammary gland tissue. Second, some clones detected variable gene expression levels among mammary carcinomas (e.g., clones 1

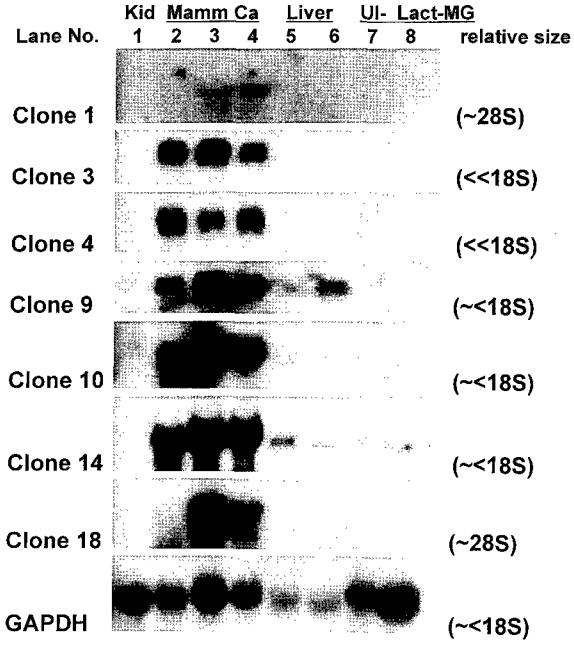

Figure 2. Northern blot analyses of gene expression detected by cloned cDNA fragment on a screening panel of RNA isolated from rat kidney (lane 1), three MNU-induced mammary carcinomas (lanes 2–4), livers (lanes 5 and 6), the uninvolved virgin mammary gland (UI, lane 7), and the day 1 postpartum lactating mammary gland (Lact-MG, lane 8). The sizes of the full transcripts relative to the 18S and 28S rRNA bands are indicated to the right of each blot. Approximately 30 μg of total RNA was loaded per lane. The *GAPDH* gene was hybridized as an internal control for loading correction.

and 18) from the same animal, whereas others detected gene expression in every carcinoma examined. Third, the overexpression of genes identified here could not be accounted for by a difference in the proportion of mammary epithelial cells between the mammary carcinoma and the mammary tissue. This was supported by the lack of or very weak gene expression in both uninvolved virgin mammary gland tissue, which is about 5% epithelial cells (lane 7), and the day 1 postpartum lactating mammary tissue, which consists primarily of secretory epithelium (lane 8).

Whether the overexpressed gene transcripts detected in the mammary carcinomas might be due to mammary epithelial-specific proliferation was addressed next. Because it is well established that during pregnancy, mammary epithelial cells undergo extensive proliferation, the expression levels of several genes (for which the full-length cDNA has been cloned, i.e., clones 3, 4, and 10) in mammary gland tissue from an early (Figure 3, lane 4) and two mid-stage (lanes 5 and 6) pregnant rats (judged by fetus size) were compared with the levels in mammary carcinomas by Northern blot analysis. Hybridization for β-casein expression confirmed the stages of pregnancy of the dams. To verify equal loading of RNA from the different tissues, the agarose gels were stained with ethidium bromide before RNA transfer and again after transfer to confirm complete transfer. *GAPDH* and β-actin were hybridized to verify RNA integrity. For clones 3 and 4, the mammary epithelial proliferation that is associated with pregnancy could not account for the overexpression observed in mammary carcinomas. However, for clone 10, mammary-specific proliferation may partially account for the overexpression observed.

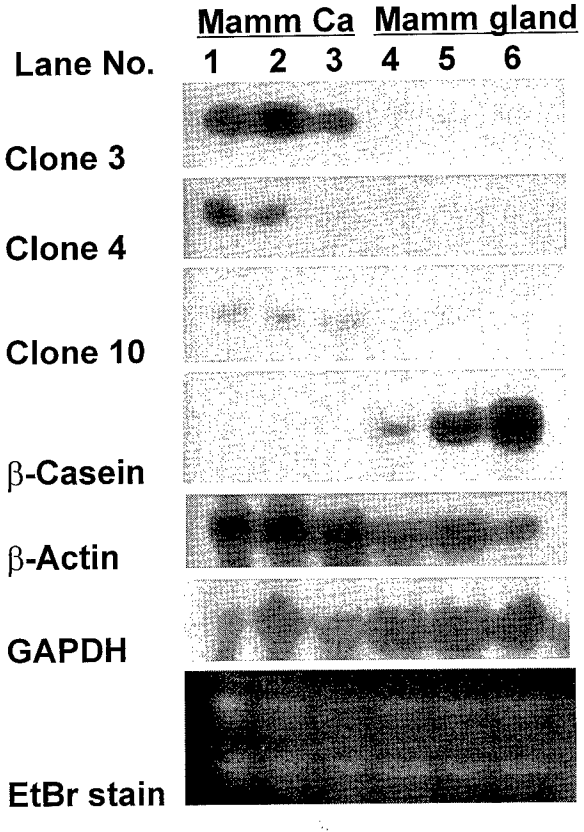

Figure 3. Northern blot analyses of expression of transcripts in mammary carcinomas (lanes 1–3) and early-stage pregnant (lane 4) and mid-stage pregnant mammary gland (lanes 5 and 6). The stage of pregnancy was determined by the size of the fetuses at necropsy and was confirmed by β-casein expression. Approximately 30 μg of total RNA was loaded per lane. Ethidium bromide (EtBr) was used to stain the agarose gel before transfer. The *GAPDH* and β-actin genes, along with the rRNA bands, served as loading and RNA integrity controls.

The sequences of the cloned gene fragments are shown in Figure 4. These sequences were submitted to GenBank in August 1996, and their accession numbers are given in Table 1. A homology search with BLASTN of the GenBank nr databases identified homologues of several known genes (Table 1). These included clone 3, human galectin-7 [25]; clone 4, human/mouse melanoma inhibitory activity (*MIA*) [26,27]/bovine chondrocyte-derived retinoic acid–sensitive protein (*CD-RAP*) [28]; clones 10 and 14 (which were identical except for five bases preceding the poly(A) tail), mouse endo B cytokeratin [29]/human cytokeratin-18 [30]; and clone 18, mouse stearoyl-CoA desaturase-2 (*SCD-2*) [31]. Comparison with the GenBank EST database revealed significant homology among clone 1, two mouse EST clones (gbAA014143 and gbW36666), and a human EST clone (gbN25349). Clone 9 had significant homology to human EST gbN35187 and to several mouse EST clones (i.e., gbW80253 and gbW82774).

Gene Expression in Rat Mammary Carcinomas Induced by Conventional MNU and DMBA Protocols

Because the mammary carcinomas used for the DD were obtained by injecting MNU into sexually immature rats at 21 d of age [21], the expression patterns of several genes were next examined in mammary carcinomas induced by MNU injection or gastric gavage of DMBA into female Sprague Dawley rats at 50 d of age (the conventional protocols). Figure 5 shows the expression patterns detected by clones 3 (galectin-7), 4 (MIA/CD-RAP), 9, and 10/14 (cytokeratin-18). Despite some variability in the expression levels among the carcinomas, these data provided strong evidence of expression of all four genes in the rat mammary carcinomas regardless of the induction protocol and the chemical carcinogen used.

Because many proteins present in carcinomas reflect derepressed expression of embryonic genes, the levels of expression of the four expressed genes were examined in RNA extracted from rat fetuses from mid-stage pregnant rats (Figure 5, lane 3). The expression levels were below the Northern blot detection limit

CLONE 1

AAGCTTCGACTGT

1 CAAGGAACTA AAACCTTTAA AAGCATAGGC ATGCTGGCCT GAGGTAAACA CTGGTACAGT TAGAGCGGAG GCAGCAGCCC CGGTGTTCAG
91 CACAGCTTGT CTGATGTGTT TATGGCCAGA GTGCAGTATT CGGCACTGGC TAGTACCGCT GCCTGACCGA ACTCCACTGG GAAGGTTTTG
181 CTTAAACGCA CATGTTTCTT TGTTACTCCT GACCATGCTT GGATGCAGTG CCTANATTGT GGCTGCTATT TTTAGTTCGC ATCATGACCT
271 TGACTGCGGA GTCCAGCCTT ACCCTCTTAC TTGATAACAG TAGCCCTTAA ACTGCAGTGG GAAGAAAGAA GAATTTGGTA TGAAAATTGG
361 TGAGCTCTGG CACTTGAGAT AAACAAGAAG AAAGTTCCAA CTTGATGCCT TAAGCCGCTC CTGCCCGAAG CTCTGAAGGA ACTAAGTTGG
451 CAGAGATCCT TACTTTGATA CTACTGCATT TTTTGTAGAA TTGTTACATT GAT**AATAAA**A CTTGCCTGCT TAACCT CAAAAAAAAAAAGCTT

CLONE 3

AAGCTTCGACTGT

1 GATCGGGGAT GACGAGTATC TCCACTTCCA CCACCGGATG CCATCCTCTA ACGTGCGCTC AGTGGAGGTG GGCGGAGACG TGCAGCTGCA
91 TTCTGTGAAG ATCTTCTGAG CAAGGACCCA GGGGCTTGGC GAGTGGGGGT GGGGTTTCGT CAGATCGTAG AGGAGGGTTG TGGATGGCG**A**
181 **ATAAA**CTGTA GCTGTAGTTC CAAAAAAAAAAAGCTT

CLONE 4

AAGCTTCGACTGT

1 CAATGAGCTC AACCCACCGA TGTTATCCCT GCAGTTACCC TTCCGGTTTG GGCAAATACA GCGGCCAACT GCAAAGTGTT TTGTCCCTTT
91 GGTTTTTGGG GTGGGCATGT ACAAAGAATG TTTCACGGGT TCCTGAACCT AGCC**AATTAA** **A**GCCCTGAAT GTTGTAACGT
CAAAAAAAAAAAGCTT

CLONE 9

AAGCTTTGGTCAG

1 AGGTGCCAGA AGTGTTTTGG GTCTCAATTT GAGAGCCCTG AGTTCTCCAC AGAGAGCATT GAAAGGATGC TGAATAACCT GGTTAATTAT
91 ATTCTGCAAA GATACTACGG ACATAGGAAG ATAGCATCGA TCTTGAATGC ATCTTTGGAT GAAAAGGTGC TTTTGGGATG GGCCCCAAGA
181 ACAACATAAA ATGGGGAGGC ATGCAGTTCT TGCACCCCCA TGGAAGTGTG CCGTTGCACA GAGAGTGAGG CCACCCAGAT CCCCATCTCC
271 ATCTCCATCG CCATCACCAA AGAGCCACAG TTCCTCTCCA TCAAGGAGCC ACCCTCCTTT ACTTCAGACT **GR**GATTACAG GTTTTAGGAA
361 CAGGCCTTTT CAGGAAGTCA GAGAGCCCGG TGAAGCAGCA TCACCACTAT TAGTGATTTT GTCTGTCGCT GCGCTTGCCC TTATTGGTTT
451 CTTCATTAGA TAATAGGTTT ATCATCTTGT TTTAATCCCG TGGTGATC**AA** **TAAA**ATGGAT GTCATTTTAA CGT
GAAAAAAAAAAGCTT **R**=A OR G (polymorphic in 2 clones)

CLONE 10

AAGCTTCTCAACG

1 ACGCCCTGGA CTCCAGCAAC TCCTAGCAAA CTGCCAGAGG ACAACTACCC GTAAGGTCGT GGATGGCAAA GTGGTGTCCG AGACCAATGA
91 TACCAGAGTT CTGAGGCACT AAGGCTCAGA AGAAGGGAAC CCTTGGGGAC TGAGGGTCC**A** **ATAAA**AGTTT AGAATCCACT G
GAAAAAAAAAAAGCTT

CLONE 14

AAGCTTCTCAACG

1 ACGCCCTGGA CTCCAGCAAC TCCATGCAAA CTGCCAGAGG ACAACTACCC GTAAGGTCGT GGATGGNAAA GTGGTGTCCG AGACCAATGA
91 TACCAGAGTT CTGAGGCACT AAGGCTCAGA AGAAGGGAAC CCTTGGGGAC TGAGGGACC**A** **ATAAA**AGTTT AGAATC CAAAAAAAAAAAGCTT

CLONE 18

AAGCTTTGGTCAG

1 GGTAGGAGAT CTGAGATGCC GTGGACTTTG CAGAA**R**AGAT TTTGTGCAGC AACGCAGAAG CCTGGCTGTG TTAAGATTGG CTGTACAGGA
91 TAGCGAACTG TGGTTGGAGG GCACAGTCTT CCCGTGTTAG TTAAATAGAG AGGCTTTAGG ACGTCTCTGC GCCCACTTGA AGGATGCCTT
181 ATCCTTTAGC TGTTCGCTAA AATAGAATCC TTGAGAGATG ACAATATATT TGCTGCCTGT CGCCCTATTC CTTGGAGAGG CCTTTTGAAT
361 GGTTTTAATT CCACTGGTTA TCCGGTGCCC ACTTAATCAA ATGT GAAAAAAAAAAAGCTT **R**=A OR G (polymorphic in 2 clones)

Figure 4. Nucleotide sequences of cloned cDNA fragments. The lightly-underlined sequences are the primers used for DD of mRNA. Translation termination codons for known genes are heavily underlined. Canonical polyadenylation signals are in bold.

Table 1. Gene Fragments Cloned by DD of mRNA from MNU-induced Rat Mammary Carcinomas

Clone no.	Fragment size (bp)*	GenBank accession no.	Homologues identified by BLASTN search	% homology†
1	526	U67990	Mouse EST AA014143	234/250 = 94%
			Mouse EST W36666	131/173 = 76%
			Human EST N25349	138/178 = 78%
3	200	U67883	Human galectin-7 U06643&L07769	91/124 = 73%
4	170	U67884	Human melanoma inhibitory activity, MIA, X75450	61/105 = 58%
			Mouse MIA X94322	96/106 = 90%
9	523	U67991	Mouse EST W82774	260/318 = 82%
			Mouse EST W80253	143/166 = 86%
10	171	U67992	Mouse endo B cytokeratin, M11686	102/116 = 87%
			Human cytokeratin-18, X12876, M26325	64/82 = 78%
14	166	U67992	Same as clone 10, except missing 5 bp proceeding poly (A)	
18	314	U67995	Mouse stearoyl-CoA desaturase-2, U67995	194/239 = 81%

*Excluding DD-PCR primer sequences.
†Homology was calculated based on the sum of stretches of DNA sequences matched by BLASTN with GenBank nr or EST databases.

for all four genes. However, a low level of expression in the fetuses was likely because the full-length rat *MIA/CD-RAP* cDNA (clone 4) was successfully cloned by reverse transcriptase (RT)-PCR from this RNA extract (see "Cloning of Full-length cDNA" below).

Expression Patterns in Non-mammary Tissues and Colon Adenocarcinomas

The expression patterns detected by clones 3 (galectin-7), 4 (*MIA/CD-RAP*), 10/14 (cytokeratin-18), and 18 (*SCD-2*) in nonmammary tissues are shown in Figure 6A. Clear-cut mammary carcinoma–specific expression was detected by clone 4 when compared with 11 other non-mammary tissues. Modest expression was detected by clone 3 in the stomach (about one-tenth the expression level in carcinomas). Strong expression was detected by clone 18 in the brain. Clones 10/14 (cytokeratin-18) detected weak expression in liver and in organs with epithelial linings, such as stomach, intestine, colon, and lung.

The expression levels of clones 3 (galectin-7) and 4 (*MIA/CD-RAP*) in two rat colon adenocarcinomas induced by AOM and the matching uninvolved colon tissues were determined by Northern blot analysis (Figure 6B). There was no detectable signal for either gene in either the cancerous or uninvolved

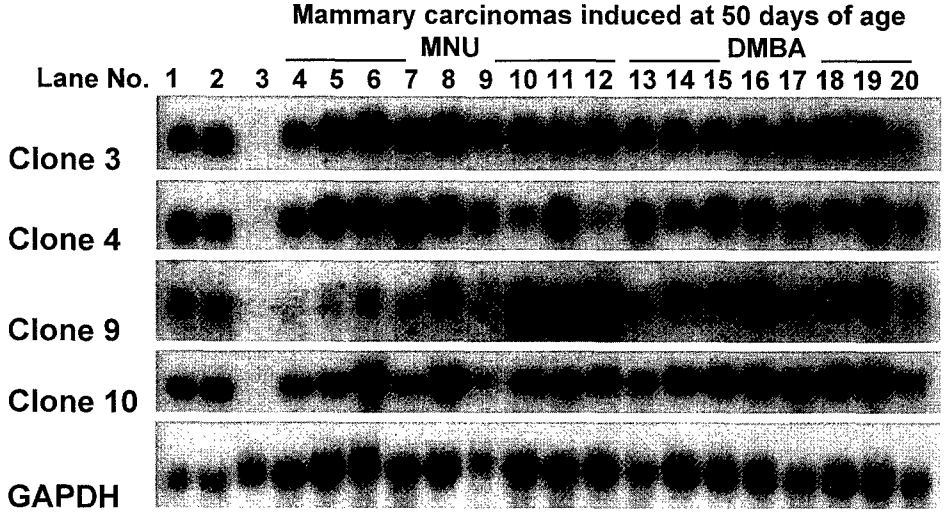

Figure 5. Northern blot analyses of expression of galectin-7 (clone 3), *MIA/CD-RAP* (clone 4), cytokeratin-18 (clones 10/14), and clone 9 in rat mammary carcinomas induced by MNU or DMBA administered when the rats were 50 d of age. Lanes 1 and 2, mammary carcinomas used for DD; lane 3, mid-term rat fetuses; lanes 4–12, mammary carcinomas induced by an intraperitoneal injection of MNU at 50 d of age; lanes 13–20, mammary carcinomas induced by gastric gavage of DMBA at 50 d of age. Approximately 30 μg of total RNA was loaded per lane. The *GAPDH* gene was hybridized as an internal control for loading correction.

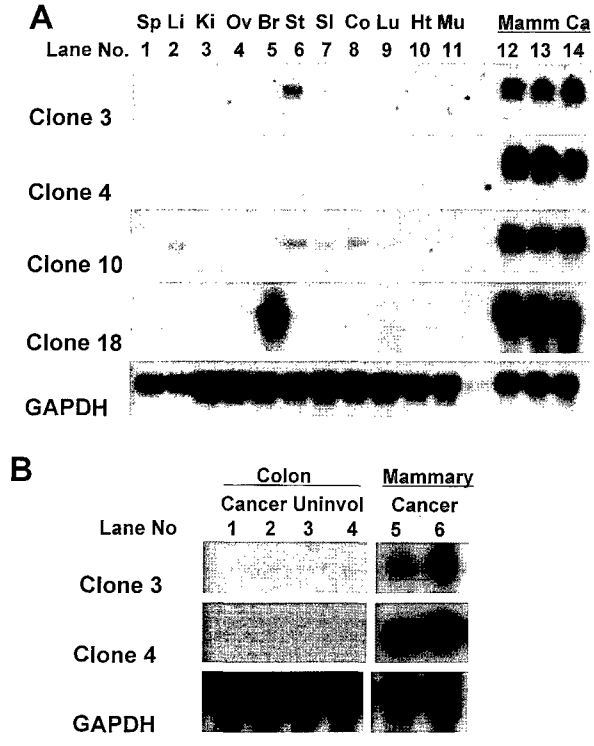

Figure 6. (A) Northern blot analyses of expression of galectin-7 (clone 3), *MIA/CD-RAP* (clone 4), cytokeratin-18 (clones 10/14), and *SCD-2* (clone 18) in rat non-mammary tissues. The tissues examined were spleen (lane 1), liver (lane 2), kidney (lane 3), ovary (lane 4), brain (lane 5), stomach (lane 6), small intestine (lane 7), colon (lane 8), lung (lane 9), heart (lane 10), and leg muscle (lane 11). Lanes 12–14, mammary carcinomas induced by MNU by using the short-term protocol. (B) Northern blot analyses of expression of galectin-7 (clone 3) and *MIA/CD-RAP* (clone 4) in AOM-induced rat colon adenocarcinomas (lanes 1 and 2) and matching uninvolved colon tissue (lanes 3 and 4). Two MNU-induced rat mammary adenocarcinomas (lanes 5 and 6) were analyzed on the same blots for comparison. Approximately 30 μg of total RNA was loaded per lane. The *GAPDH* gene was hybridized as an internal control for loading correction.

colon tissue, whereas the mammary carcinomas on the same blots showed the expected bands.

Cloning of Full-length cDNA

The full-length sequences were obtained for rat galectin-7 (Figure 7, GenBank Accession No. U67883) and *MIA/CD-RAP* (Figure 8, Accession No. U67884). Full-length rat galectin-7 was 76% homologous to human galectin-7 at the nucleotide level and shared 72% identity and 84% positivity (i.e., similar charge characteristics) at the predicted amino-acid level (Figure 7).

The rat *MIA/CD-RAP* cDNA was also cloned from both a mid-term and a full-term fetal rat RNA preparation by RT-PCR, and the sequences were compared with that derived from the mammary carcinomas. No mutation was detected in the coding region of the cDNA cloned from the carcinomas. The deduced rat MIA/CD-RAP protein sequence was aligned with the human sequence and those of other species in Figure 8. Despite some variability in the signal peptide region (the first 22–24 amino acids) among species, the predicted mature MIA proteins (i.e., minus the secretory peptide) were highly conserved (94% identity with both human and mouse MIA and 90% identity with bovine CD-RAP). The effect of expression of full-length cDNAs of galectin-7, *MIA/CD-RAP*, and other genes on mammary epithelial cells is currently being evaluated.

DISCUSSION

This paper reports the identification and cloning of seven cDNA fragments of genes whose overexpression appeared to be specifically associated with chemically induced rat mammary carcinomas. A comparison of the expression patterns of several of these genes in mammary carcinomas with genes in uninvolved virgin mammary tissue, day 1 postpartum lactating mammary tissue, and mammary tissue of mid-stage pregnant dams indicated that the overexpression observed in mammary carcinomas could not be accounted for by a difference in the epithelial content of the mammary carcinoma and the mammary tissue or by mammary epithelial–specific proliferation associated with pregnancy (Figures 2 and 3). That the overexpressed gene transcripts identified in MNU-induced rat mammary carcinomas were also detected in DMBA-induced carcinomas (Figure 5) indicates a commonality of these models with respect to the overexpression of these genes and may imply that the products of these genes play a role in mammary carcinogenesis in both chemically induced breast cancer models. Among 11 organs and tissues examined by Northern blot analysis, the expression of most of the genes was restricted to the mammary carcinomas (Figure 6A). This was further supported by the lack of expression of galectin-7 and *MIA/CD-RAP* in AOM-induced rat colon adenocarcinomas (Figure 6B). Taken together, these results support the hypothesis that at least some of these genes may serve as specific markers of mammary carcinogenesis. Whether the altered expression of these genes plays a causal role in mammary carcinogenesis is currently under investigation.

None of the known homologues of the genes identified in this study has previously been examined in chemically induced rat mammary carcinomas. However, each of these known genes or gene families has been implicated in some aspect of carcinogenesis in other organ sites. The relevant information is discussed below.

Whereas little is known about the role of galectin-7 in normal physiology or carcinogenesis, other members of the galectin family of proteins, which are characterized by the common property of binding to galactosyl moieties with conserved primary structural features [32,33], have been examined as cancer-specific markers in several organ sites [34–39]. Galectin-4 was recently been shown to be expressed

CLONE 3 RAT GALECTIN-7

```
                                                    5'-TTGC CGTGCCAGCC

15    ATG TCT GCC ACC CAT CAC AAG ACC CCT CTG CCT CAG GGT GTC CGC
1     Met Ser Ala Thr His His Lys Thr Pro Leu Pro Gln Gly Val Arg

60    CTG GGC ACC GTC ATG AGA ATT CGA GGC GTG GTC CCT GAC CAG GCT
16    Leu Gly Thr Val Met Arg Ile Arg Gly Val Val Pro Asp Gln Ala

105   GGC AGG TTC CAT GTA AAC CTG CTA TGC GGC GAG GAG CAA GAG GCA
31    Gly Arg Phe His Val Asn Leu Leu Cys Gly Glu Glu Gln Glu Ala

150   GAC TGC GCC CTG CAC TTT AAC CCG AGG CTG GAC ACA TCC GAG GTT
46    Asp Cys Ala Leu His Phe Asn Pro Arg Leu Asp Thr Ser Glu Val

195   GTC TTC AAC ACC AAA CAG CAA GGC AAA TGG GGC CGT GAG GAG CGG
61    Val Phe Asn Thr Lys Gln Gln Gly Lys Trp Gly Arg Glu Glu Arg

240   GGC ACC GGC ATC CCC TTC CAG CGT GGG CAG CCC TTT GAA GTG CTC
76    Gly Thr Gly Ile Pro Phe Gln Arg Arg Gln Pro Phe Glu Val Leu

285   ATC ATC ACC ACA GAG GAA GGC TTC AAG ACT GTG ATC GGG GAT GAC
91    Ile Ile Thr Thr Glu Glu Gly Phe Lys Thr Val Ile Gly Asp Asp

330   GAG TAT CTC CAC TTC CAC CAC CGG ATG CCA TCC TCT AAC GTG CGC
106   Glu Tyr Leu His Phe His His Arg Met Pro Ser Ser Asn Val Arg

375   TCA GTG GAG GTG GGC GGA GAC GTG CAG CTG CAT TCT GTG AAG ATC
121   Ser Val Glu Val Gly Gly Asp Val Gln Leu His Ser Val Lys Ile

420   TTC TGA GCAAGGACCC AGGGGCTTGG CGAGTGGGGG TGGGGTTTCG TCAGATCGTA
136   Phe Stop

476   GAGGAGGGTT GTGGATGGCG AATAAACTGT AGCTGTAGTTC C poly (A) -3'
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Rat           1  MSATHHKTPL PQGVRLGTVM RIRGVVPDQA GRFHVNLLCG EEQEADCALH
                 ||   ||+ | |+|+| |||+ ||||+|| +|  ||||||||| ||| +| |||
Human [25]    1  MSNVPHKSSL PEGIRPGTVL RIRGLVPPNA SRFHVNLLCG EEQGSDAALH

Rat          51  FNPRLDTSEV VFNTKQQGKW GREERGTGIP FQRGQPFEVL IITTEEGFKT
                 |||||||||| |||+|+|| | |||||| |+| |||||||||| || +++|||
Human        51  FNPRLDTSEV VFNSKEQGSW GREERGPGVP FQRGQPFEVL IIASDDGFKA

Rat         101  VIGDDEYLHF HHRMPSSNVR SVEVGGDVQL HSVKIF
                 |+|| +| ||  ||+| + ||  |||||||||  ||+||
Human       101  VVGDAQYHHF RHRLPLARVR LVEVGGDVQL DSVRIF
```

Figure 7. Sequence of full-length cDNA of rat galectin-7 and alignment of its deduced amino-acid sequence with the human homologue [25]. Translation initiation and termination codons are in bold type. Vertical lines indicate amino-acid identity, and plus signs indicate similar charge characteristics for the encoded amino acids. The GenBank accession number is U67883.

CLONE 4 RAT MIA/CD-RAP

```
                                                    5'-TT GAAGTCCATG

 13    ATG GTG TGC TCC CCA GTG CTC CTT GGT ATT GTC ATC TTG TCT GTT
  1    Met Val Cys Ser Pro Val Leu Leu Gly Ile Val Ile Leu Ser Val

 58    TTT TCA GGC CTC AGC AGG GCT GAT CGA GCC ATG CCC AAG CTG GCT
 16    Phe Ser Gly Leu Ser Arg Ala Asp Arg Ala Met Pro Lys Leu Ala

103    GAC CGG AAG CTG TGT GCA GAT GAG GAG TGT AGC CAT CCT ATC TCC
 31    Asp Arg Lys Leu Cys Ala Asp Glu Glu Cys Ser His Pro Ile Ser

148    ATG GCT GTG GCC CTT CAG GAC TAC GTG GCC CCT GAT TGC CGC TTC
 46    Met Ala Val Ala Leu Gln Asp Tyr Val Ala Pro Asp Cys Arg Phe

193    TTG ACT ATA TAC AGG GGC CAA GTG GTA TAT GTC TTC TCC AAG TTG
 61    Leu Thr Ile Tyr Arg Gly Gln Val Val Tyr Val Phe Ser Lys Leu

238    AAA GGC CGT GGA CGG CTT TTC TGG GGA GGC AGT GTG CAG GGA GAT
 76    Lys Gly Arg Gly Arg Leu Phe Trp Gly Gly Ser Val Gln Gly Asp

283    TAC TAT GGA GAC CTG GCA GCC CAC CTG GGC TAT TTC CCC AGT AGC
 91    Tyr Tyr Gly Asp Leu Ala Ala His Leu Gly Tyr Phe Pro Ser Ser

328    ATT GTC CGG GAG GAC CTG ACT CTG AAA CCT GGC AAA GTC GAT ATG
106    Ile Val Arg Glu Asp Leu Thr Leu Lys Pro Gly Lys Val Asp Met

373    AAG ACA GAT GAA TGG GAT TTC TAC TGT CAA TGA           GCTCAACCCA
121    Lys Thr Asp Glu Trp Asp Phe Tyr Cys Gln Stop

416    CCGATGTTAT CCCTGCAGTT ACCCTTCCGG TTTGGGCAAA TACAGCGGCC AACTGCAAAG
476    TGTTTTGTCC CTTTGGTTTT TGGGGTGGGC ATGTACAAAG AATGTTTCAC GGGTTCCTGA
536    ACCTAGCCAA TTAAAGCCCT GAATGTTGTA ACGTC poly (A)-3'
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This study           MVCSPVLLGI VILSVFSGLS RADRAMPKLA DRKLCADEEC SHPISMAVAL
Rat CD-RAP [28]                    (partial)                LCADEEC SHPISVTVAL
Mouse MIA  [26]      MVWSPVLLGI VVLSVFSGPS RADRAMPKLA DWKLCADEEC SHPISMAVAL
Cow CD-RAP [28]      MAWSLVFLGV VLLSAFPGPS AGGRPMPKLA DRKMCADEEC SHPISVAVAL
Human MIA  [26,27]   MARSLVCLGVIILLSAFSGPG VRGGPMPKLA DRKLCADQEC SHPISMAVAL

This study           QDYVAPDCRF LTIYRGQVVY VFSKLKGRGR LFWGGSVQGD YYGDLAAHLG
Rat CD-RAP           QDYVAPDCRF LTIYRGQVVY VFSKLKGRGR LFWGGSVQGD YYGDLAAHLG
Mouse MIA            QDYVAPDCRF LTIYRGQVVY VFSKLKGRGR LFWGGSVQGG YYGDLAARLG
Cow CD-RAP           QDYVAPDCRF LTIHQGQVVY IFSKLKGRGR LFWGGSVQGD YYGDGAARLG
Human MIA            QDYMAPDCRF LTIHRGQVVY VFSKLKGRGR LFWGGSVQGD YYGDLAARLG

This study           YFPSSIVRED LTLKPGKVDM KTDEWDFYCQ
Rat CD-RAP           YFPSSIVRED LTLKPGKVDM KTDE
Mouse MIA            YFPSSIVRED LNSKPGKIDM KTDQWDFYCQ
Cow CD-RAP           YFPSSIVRED QTLKPAKTDV KTDIWDFYCQ
Human MIA            YFPSSIVRED QTLKPGKVDV KTDKWDFYCQ
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Figure 8. Sequence of full-length cDNA of rat *MIA/CD-RAP* and alignment of its deduced amino-acid sequence with homologues from mouse [26], human [26,27], and cow [28] and the reported rat partial sequence [28]. The translation initiation and termination codons are in bold type. The predicted cleavage site for the signal peptide is between amino-acid residues 22 and 23. The GenBank accession number is U67884.

in ductal carcinoma in situ and invasive breast carcinomas but was not expressed in morphologically normal mammary epithelium [40]. Galectin-3 has been reported to protect cells against apoptosis [41], whereas galectin-1 has been implicated as a mediator of T-cell apoptosis [42]. Although each type of galectin may have specific biological functions, their common galactosyl-binding property [32,33] suggests that they may be involved in mediating cell-cell recognition and cell-matrix interactions, processes that are being increasingly implicated in the regulation of cell fates and in the maintenance of tissue-size homeostasis. Indeed, many publications support this possibility [43–48].

The human MIA protein was purified from the conditioned medium of a slow-growing melanoma cell line [26]. Molecular cloning confirmed that the gene product is a secretory protein with inhibitory activity against melanoma cell growth in culture [26]. The expression of this gene appears to be restricted to malignant melanomas [26,27] but was recently detected in cultured bovine chondrocytes as coding for a retinoic acid–sensitive protein (CD-RAP) and in fetal rodent skeletal cartilage tissues [28]. Recent data showed that MIA/CD-RAP binds to fibronectin, laminin, and tenascin [49]. In that study, it was hypothesized that such an activity could interfere with cell attachment to these matrix proteins through specific integrins and thus be involved in tumor invasion and metastasis. It is conceivable that in chemically induced mammary carcinogenesis, MIA/CD-RAP may play a similar role in tumor cell/extracellular matrix interactions. The fact that retinoic acid is a morphogen in embryogenesis and that retinoic acid represses the expression of MIA/CD-RAP in chondrocytes [28] suggests that this gene has a role in morphogenesis, tissue remodeling, and differentiation. Our sequence comparison between *MIA/CD-RAP* cloned from mammary carcinomas and from rat fetuses did not reveal any mutation in the coding region, supporting a role for gene overexpression rather than specific gene mutation in chemically induced rat mammary carcinogenesis.

Endo B cytokeratin/cytokeratin-18 and its partner endo A cytokeratin/cytokeratin-8 are extremely early embryonic genes detectable at the four- or eight-cell stage of embryonic development in the mouse [50,51]. In adult tissue, low-level gene expression, if any, is restricted to simple epithelium [52]. The low level of expression in stomach, small intestine, colon, and lung detected in this study (Figure 6A) is consistent with these findings. Clinically, overexpression of cytokeratin-18 has been observed in many types of cancer arising from simple epithelium and has been used as a diagnostic for metastatic disease [53–59]. In fact, tissue polypeptide–specific (TPS) antigen has been advocated over the past two decades as a serum tumor marker, but it was a long time before it was proven that these proteins in the serum are related to cytokeratin fragments [58]. For instance, the TPS test was based on detection of fragments of cytokeratin-18, and the TPA(cyk) test was based on detection of fragments of both cytokeratin-18 and its partner cytokeratin-8 [58]. Although a recent study showed that at the time of diagnosis of metastatic breast disease 86% of the serum values for TPS were above the upper reference value [59], the clinical experience described above suggests that cytokeratin-18 is probably not a mammary-specific cancer marker. Nevertheless, overexpression of cytokeratin-18 may play an important role in some aspect of carcinogenesis, as indicated by the observation that enforced overexpression of both cytokeratin-8 and cytokeratin-18, which are partners for assembly into intermediate filaments, confers multiple drug resistance to mouse fibroblasts in culture [60].

The *SCD-2* (clone 18) gene was initially cloned from mouse 3T3-Li cells induced to differentiate into adipocytes [31]. Under normal feeding conditions (e.g., a diet containing unsaturated triacylglycerol), *SCD-2* is expressed very strongly in mouse brain; weakly in lung, kidney, and adipose tissue; and below the detection limit in heart, spleen, and liver [31]. In contrast, the related *SCD-1* gene is expressed very strongly in the adipose tissue and was below the detection limit in other tissues under the same feeding conditions [31]. The *SCD-2* transcript is primarily localized in oligodendrocytes in the brain [61]. The size of the transcript and the tissue distribution profile of the gene detected by clone 18 in our study (Figures 2 and 6A) are consistent with this clone being the rat homolog of *SCD-2*. The products of SCDs are thought to play a key regulatory role in unsaturated fatty acid biosynthesis. The presence of *SCD-2* in the brain oligodendrocytes [31,61] and its resistance to induction by starvation and refeeding of a triacylglycerol-free diet [31] support a role for this gene product in myelination of critical brain cells. The overexpression of *SCD-2* we observed in mammary carcinomas may indicate altered lipid metabolism in mammary carcinoma cells.

In summary, seven cDNA fragments were identified that detected gene overexpression in chemically induced rat mammary carcinomas. This study provides important leads in an area that has received limited attention despite the extensive use of the MNU- and DMBA-induced mammary carcinogenesis model systems. However, many questions need to be addressed to validate these genes as specific markers of mammary carcinogenesis. These questions include (i) What specific cell type or types within mammary carcinomas express these gene transcripts? (ii) Are the protein products of these genes also overexpressed? (iii) Is the gene expression observed specific to chemically induced mammary carcinogenesis? and (iv) At what stage of mammary carcinogenesis can the expression be detected? In situ detection approaches as well as the more conven-

tional methodologies described in this paper will be used to address these issues. We note that the short-term model system developed by Thompson et al. [21] permits the investigation of these questions in intraductal hyperplastic lesions and ductal carcinomas in situ as well as frank mammary carcinomas. Carcinomas in this model system have also been observed to be locally invasive and to metastasize to the lung. Work is in progress to evaluate the role or roles of these genes in the multistep process of mammary carcinogenesis.

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SHORT COMMUNICATION

Pathogenetic characterization of 1-methyl-1-nitrosourea-induced mammary carcinomas in the rat

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The induction of mammary carcinogenesis in the rat by 1-methyl-1-nitrosourea (MNU) is widely used in experimental breast cancer research. In the experiments reported, the Ha-*ras* codon 12 (*ras*12) mutation (GGA→GAA) was used as a molecular marker to address issues of the clonality of carcinomas induced, pathogenetic independence among multiple carcinomas within the same animal and topographic distribution of mutant *ras*12 carcinomas in different mammary gland chains. In order to determine whether the frequently observed morphologically distinguishable lobules within carcinomas originate from the coalescence of independent lesions or whether cancerous cells within a carcinoma share a common origin, 44 randomly selected MNU-induced mammary carcinomas were genotyped for two to four lobules each for the *ras*12 mutation. A total of 43 carcinomas out of 44 (97.7%) had concordant *ras*12 genotypes among the multiple sites within each tumor, which is consistent with the latter possibility. Next, it was observed that as carcinoma multiplicity increased, the discordance rate of *ras*12 genotypes among multiple carcinomas within the same animal increased in a manner that was in excellent agreement with the expected discordance rate based on an assumption of no pathogenetic association among carcinomas. Furthermore, a significant difference was observed in the occurrence of mutant *ras*12 carcinomas between the cervical-thoracic and the abdominal-inguinal mammary glands in that three times as many carcinomas were mutant in the former as in the latter glands, whereas the occurrence of wild-type carcinomas was approximately the same in both regions. Taken together, the data are consistent with (i) carcinomas induced by MNU and detected by palpation are monoclonal in origin, (ii) independently-initiated cells emerge as distinct mammary carcinomas in the same animal, and (iii) the anatomical location of the gland may affect the prevalence of mammary carcinomas that harbor a mutant *ras*12.

The 1-methyl-1-nitrosourea (MNU*)-induced rat mammary carcinogenesis model (1) has contributed significantly to the current understanding of the biology of breast cancer and to potential approaches for its prevention. Major attributes of this model include that the proportion of mammary carcinomas that are ovarian-hormone dependent is similar to that observed in the human disease; that the carcinomas induced are aggressive and locally invasive; and that there is a clear operational distinction between the initiation and promotion stages of the disease process based on the action of MNU as a direct methylating agent (1–4). This latter feature of the model is often exploited to study effects of cancer preventive agents or risk factors on the promotion and progression stages of mammary carcinogenesis. Technical improvements since its original publication have made this model easier to implement and more reproducible (2,5,6). For example, Thompson and coworkers (5,6) have examined this model with respect to the route of carcinogen administration and have found that a single dose of MNU given intraperitoneally (i.p.) or subcutaneously (s.c.) was as effective as when it was given by intravenous (i.v.) injection, the method of administration originally reported (1). When MNU was administered by i.p. injection, smaller coefficients of variation in the number of carcinomas per rat were observed, an improvement the authors attributed to the consistent manner and the ease with which the MNU was delivered (6). The work reported here was based on the induction of mammary carcinogenesis by i.p. administration of MNU to female Sprague–Dawley rats at 50 days of age.

The pathogenetic characteristics of this experimental model of breast cancer are being defined with the use of molecular techniques. One of the identifiable somatic genetic changes is a GGA→GAA transition in Ha-*ras* proto-oncogene codon 12 (*ras*12) in a percentage of the carcinomas (7–9). Numerous studies have indicated that this mutation is an early initiating event (9–11) probably as a result of methylation of the guanine nucleosides (12,13), although some data have suggested that there might be a low frequency of spontaneous mutation of this codon in mammary epithelial cells (14). The early nature of the *ras*12 mutation in MNU-induced mammary carcinogenesis could therefore mark the initiated cells and their resultant carcinomas into two pathogenetic subpopulations, i.e. those with a mutant *ras*12 and those with a wild-type *ras*12 gene. Taking advantage of this mutation as a molecular marker, we addressed the following questions in order to gain further insights concerning the biology of the disease process in this model system:

1. Do the different morphologically discernible lobules that are frequently observed within mammary carcinomas (see examples in Figure 1A) indicate either that they arise from a coalescence of independent lesions (Figure 1B, Scheme 1) or that these lobules result from morphological diversification of clonally derived cells during tumor progression (Figure 1B, Scheme 2)?
2. Do multiple carcinomas within the same animal share the same pathogenetic characteristics such as *ras*12 mutation or do independently initiated foci of cells develop into distinct carcinomas?
3. Is the prevalence of mutant *ras*12 in carcinomas within an animal best modeled as a stochastic process or is there a bias based on the topographic location of the mammary gland from which a carcinoma arises?

Two animal experiments were conducted to provide the

*Abbreviations: MNU, 1-methyl-1-nitrosourea; BW, body weight; H&E, hematoxylin and eosin; C-T, cervical-thoracic; A-I, abdominal-inguinal.

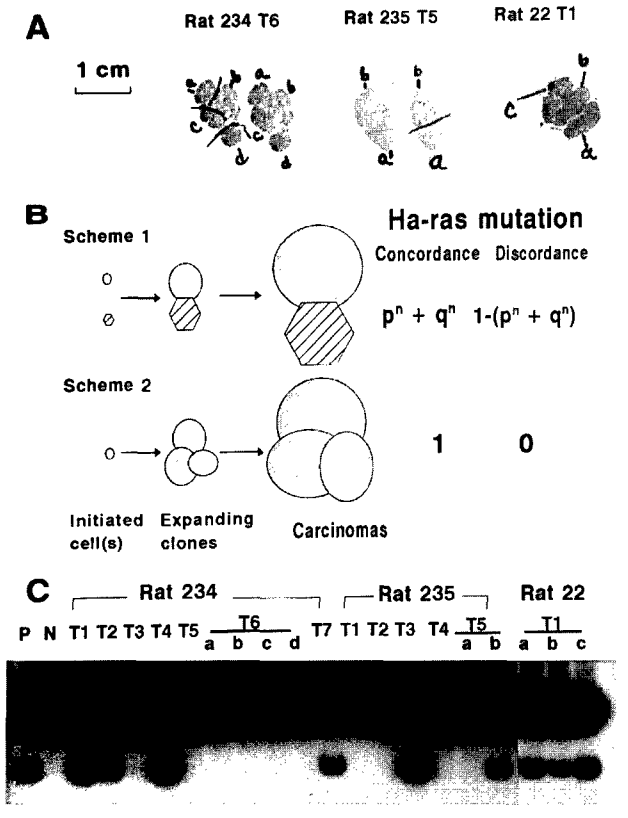

Fig. 1. (**A**) Examples of the gross appearance of tumor sections (H&E) on thin plastic slides. Tumors, especially large ones, were often observed to be made up of morphologically discernible lobules. The horizontal bar represents 1 cm in length. Sample code key: Rat234T6a, Rat #234, tumor #6, sampled site a. Lower case letters indicate sites from which tissue was sampled for *ras*12 genotyping. (**B**) Schematic illustration of multiple independent origins (polyclonality, Scheme 1) and a common origin of cancerous cells within a carcinoma (monoclonality, Scheme 2). (**C**) PCR-RFLP analysis of *ras*12 genotype in carcinomas. P, positive control for *ras*12 mutation. N, negative control for *ras*12 mutation, i.e. non-carcinogen treated rat mammary gland DNA. The presence of the shorter band is diagnostic of the *ras*12 mutation.

tissue samples for this study. Female Sprague–Dawley rats were purchased from Taconic Farms (Germantown, NY) at 21 days of age and fed a modified AIN76A diet. At 50 days they were given an i.p. injection of MNU (Ash Stevens Inc., Detroit, MI) by the method reported by Thompson and Adlakha (6). The dose level was 37.5 and 25 mg MNU per kg body weight (BW) for experiments 1 and 2 respectively. The rats were palpated for mammary tumors twice per week. When a tumor was first palpated, the date and the tumor location were recorded. The experiments were terminated at 22 and 25 weeks post-carcinogen for experiments 1 and 2 respectively. At necropsy, tumors and suspicious lesions were excised, fixed in 10% neutral buffered formalin (12 h) and later embedded in paraffin and sectioned for histological evaluation. The pathological criteria were as described by Young and Hallowes (15). Only tumors that were classified as carcinomas were used for genotyping the *ras*12 status.

The paraffin-embedded tumor blocks were serially cut into 5-μm sections and were mounted on thin transparent plastic slides coated with polylysine (Sigma Chemical Company, St Louis, MO) and stained with hematoxylin and eosin (H&E). Each section was viewed without a cover slip under light microscopy and marked into distinct lobules for tissue retrieval (see examples in Figure 1A). A small piece (~2×2 mm) was carefully cut with flame-sterilized scissors from each marked area. Each piece was incubated with 10 μg proteinase K in 50 μl of 100 mM Tris–HCl, 2 mM EDTA at 50°C for 3 h. After the proteinase K was inactivated by heating at 95°C for 8 min, 2–5 μl of the extract was used as the source of DNA for 40 cycles of PCR amplification.

The mutational status of *ras*12 was determined by a modified polymerase chain reaction-generated restriction fragment length polymorphism (PCR-RFLP) method (16,17). The upstream primer (5′AGTGTGATTCTCATTGGCAG-3′) was placed in intron-1 to avoid amplifying the Ha-*ras* pseudogene (17). The G→A mutation and two introduced mismatches in the downstream primer (5′-AGGGCACTCTTTCgaACGCC-3′, mismatches denoted by low case letters) generated an XmnI site in the PCR product (116 bp). Upon digestion of the product with XmnI (New England Biolabs, Beverly, MA), a fragment of 98 bp would be generated that was diagnostic for the mutation. A tracer amount of α-^{32}P-dCTP was used to label the PCR products. The digested products were separated by electrophoresis on a 6% polyacrylamide gel and detected by autoradiography as shown in Figure 1C.

Statistical methods used in the analyses of these experiments included descriptive statistics and χ^2-tests including Mantel–Haenszel tests for homogeneity of the association stratified by number of carcinomas per animal.

To address the first issue, 44 randomly selected carcinomas were analyzed. Of these carcinomas, 25 were sampled with two sites each, five with three sites each, and 14 with four sites each. Each site was genotyped for *ras*12 status (see examples in Figure 1C, rat234T6a-d, rat235T5a,b and rat22T1a-c). A total of 43 of 44 mammary carcinomas analyzed showed concordant *ras*12 (i.e. either all sites were mutant or all sites were wild type) among the multiple sites sampled (Table I). The exception was rat235 T5 in which the two sites were discordant for *ras*12. In fact, this observation initially prompted us to examine the issue of the origin of morphologically discernible lobules, which were often observed within carcinomas, especially in large ones.

It is of interest to note that the intensity of the diagnostic band varied considerably from carcinoma to carcinoma. Because the level of the mutant *ras*12 fraction in a sample can be influenced by the time frame of the occurrence of the mutation in relationship to carcinoma development, i.e. a mutation that occurred very late in the carcinogenesis process would be expected to result in a small mutant fraction in a tumor, the following factors were considered in the interpretation of these data. First, due to the stochastic nature of the carcinogenic initiation, the probability of mutating both *ras*12 alleles in the same initiated epithelial cell would be much lower than that of mutating only one allele. It was therefore expected that most of the mutant *ras*12 carcinomas would be heterozygous yielding at most a 50% mutant signal. In fact, out of >3000 MNU-induced mammary carcinomas analyzed so far in our laboratories, only two were observed to show a mutant *ras*12 signal that was >50% (unpublished data). Second, the percentage of non-epithelial cells in a tumor, which are less likely to harbor *ras*12 mutation, is quite variable among different carcinomas. Since the cancerous epithelial cells were not microdissected in this work, the inclusion of the non-epithelial cells would result in a varying degree of dilution of the mutant *ras*12 signal. Third, the carcinomas were fixed in formalin and DNA was extracted by proteinase K digestion and boiling. A varying degree of DNA damage could result from these

Table I. Ha-*ras* codon 12 genotyping of multiple lobules of randomly selected mammary carcinomas from experiment 1

Number of sites analyzed per carcinoma (n)	Predicted *ras* concordance rate among sampled sites		Observed number of carcinomas with		Observed *ras*12 concordance rate among sampled sites
	Assuming polyclonality[a]	Assuming monoclonality[b]	concordant *ras* among sampled sites	discordant *ras* among sampled sites	
2	0.505	1	24	1[c]	0.96
3	0.258	1	5	0	1
4	0.132	1	14	0	1
Total		1	43		0.977

[a]Predicted *ras*12 concordance rate based on multiple, independent origins for cells in different lobules within a carcinoma (see Figure 1B, Scheme 1). The probability by chance for say 3 sites to show the same mutant *ras*12 genotype is $p \times p \times p$ and to show wild-type *ras*12 genotype is $q \times q \times q$, where p = probability for mutant *ras*12 and $q = 1 - p$ = probability for wild-type *ras*12 at a given site. Thus the overall concordance probability = $p^3 + q^3$. For n sites sampled, the predicted concordance is calculated by formula $p^n + q^n$. p was estimated by the overall frequency of mutant *ras*12 carcinomas and in this experiment, $p = 0.45$.

[b]Predicted based on monoclonal origin. The discordant *ras*12 genotypes among different sites is 0 because all sites will be either wild type or mutant at codon 12. The concordant rate is independent of the number of sites (n) sampled.

[c]This section (Rat 235 Tumor 5) displayed two distinctly H&E-stained regions. The discordant *ras*12 genotypes of the two portions sampled indicated that this section represented two independently initiated carcinomas growing together side-by-side.

treatments and lead to less than perfect templates for PCR. Fourth, the Taq polymerase used for PCR has a low but detectable level of amplification error per base incorporated (~0.02% with 20 cycles), which involves predominantly A→G transitions (21). Because the detection of the diagnostic mutant signal relies on the XmnI enzyme to recognize a six-base restriction sequence (...GAA*nnnn*TTC...), any amplification error in that sequence as a result of these latter two factors would lead to resistance to enzyme digestion of the PCR products, further reducing the mutant signal intensity. It was therefore reasoned that mutant *ras*12 signal ranging from 5% to 50% would be consistent with this mutation being an early marker in MNU induced mammary carcinogenesis. The diagnostic band intensity observed in both experiments was within this range.

With these factors taken into consideration, the high degree of *ras*12 concordance among multiple sites within a carcinoma (97.7%) strongly support Scheme 2 (Figure 1B), i.e. morphological heterogeneity, often manifesting as distinct lobes within a carcinoma, is likely the result of diversification of progeny cells of the original initiated cell during clonal expansion and subsequent progression as a carcinoma develops. A practical implication of this information is that tissue sampling for genotyping purposes, at least as far as the *ras*12 mutation is concerned, can be achieved by a single sample per tumor with good accuracy.

Do multiple carcinomas within the same animal share the same pathogenetic characteristics? If the answer to this question is yes, it should follow that multiple carcinomas within an animal will display concordant *ras*12 genotype because all carcinomas are either all mutant or all wild type. As shown in Figure 1C (rat234, T1-T7 and rat235, T1-T5) this was not the case. Table II tabulates the observed *ras*12 discordance rate as a function of the number of carcinomas borne by a rat. The data are consistent with the probabilities predicted based on independent origins among multiple carcinomas within the same animal (as illustrated in Figure 1B, Scheme 1). The result was observed in two independent experiments in which different amounts of carcinogen were used to induce mammary carcinogenesis. The independent nature of individual carcinomas within an animal supports the use of carcinoma multiplicity as a parameter for assessing the effects of preventive agents as well as risk factors. It should be noted, however, that the independent nature of initiation inferred here is true only at the molecular marker level. Our data do not rule out physiological (i.e. epigenetic) interdependence among carcinomas within the same animal. Such an epigenetic interaction among carcinomas or initiated cells can potentially result from changes in the endocrine factors and metabolic milieu brought about by a preexisting carcinoma and could influence the emergence of additional carcinomas in the same animal and/or the latency of their appearance. In an early study with this model, the kinetics of appearance of additional carcinomas was observed to slow down significantly after the appearance of the first carcinoma (2). The implication of a secreted inhibitory factor from a primary tumor in suppressing the emergence of secondary tumors (18) might account for this observation.

To address the issue of topographic location of mutant *ras*12 carcinomas with respect to the mammary gland chains, Table III summarizes the prevalence of wild-type and mutant carcinomas arising in the cervical-thoracic (C-T) and the abdominal-inguinal (A-I) glands. A significant regional difference in total carcinoma occurrence was observed between the C-T and the A-I glands in that there were approximately twice as many carcinomas in the former as in the latter glands, which is consistent with previous reports (1,5,6,19). But surprisingly, more than three times as many mutant *ras*12 carcinomas were located in the C-T glands as in the A-I glands, whereas the wild-type *ras*12 carcinomas were almost equally distributed between the two regions (Table III). The disproportional distribution pattern held true upon secondary analyses stratifying by the number of carcinomas per animal and by experiment. In fact, the previously observed 2:1 C-T to A-I ratio of carcinoma occurrence (1,5,6,19) could be almost entirely attributed to this preferential localization of mutant *ras*12 carcinomas in the C-T mammary gland chains. Whether this difference is related to the asynchronous post-natal development of the C-T versus and A-I glands (19) remains to be determined. Nonetheless, the practical implication of the observed regional differences should not be overlooked. Until the cause and the biological significance of the regional differences observed in this study are clearly understood, it is advisable to follow a consistent tissue collection protocol with respect to carcinoma location in the mammary gland chains so that this source of bias is minimized when carcinoma tissues

Table II. Ha-*ras* genotype profile of multiple mammary carcinomas within the same animals

Number of carcinomas per rat (*N*)	Predicted *ras* discordance rate among multiple carcinomas assuming independent origin[a]	Number of rats with concordant *ras* genotypes among carcinomas	Number of rats with discordant *ras* genotypes among carcinomas	Total number of rats in category	Observed *ras* discordance rate among multiple carcinomas
Experiment 1 (37.5 mg MNU per kg)					
2	0.495	11	10	21	0.476
3	0.742	3	8	11	0.727
4	0.868	0	8	8	1
5	0.931	0	11	11	1
6	0.964	1	9	10	0.9
7 or greater	>0.981	0	17	17	1
Experiment 2 (25 mg MNU per kg)					
2	0.442	22	22	44	0.5
3	0.663	8	12	20	0.6
4	0.787	4	11	15	0.73
5	0.861	2	4	6	0.67
6 or greater	>0.908	0	3	3	1

[a]Predicted discordance rate among multiple carcinomas borne by the same animal assuming pathogenetic independence. Calculated by formula $1 - (p^N + q^N)$, where p was estimated by the overall *ras*12 mutation frequency in carcinomas. $p = 0.45$, $q = 1 - p = 0.55$ in experiment 1 and $p = 0.67$, $q = 0.33$ in experiment 2 respectively. N = number of carcinomas per rat.

Table III. Distribution of mutant and wild-type *ras*12 mammary carcinomas by anatomical regions

Location of glands	Number of carcinomas with		Total	% Ha-*ras* mutation	χ^2, *P*-value[a]
	mutant *ras*12	wild type *ras*12			
Experiment 1 (37.5 mg MNU per kg)					
Cervical-thoracic chains	132	115	247	53	
Abdominal-inguinal chains	41	91	132	31	
Total	173	206	379	45	17.4 ($P < 0.005$)
Experiment 2 (25 mg MNU per kg)					
Cervical-thoracic chains	171	61	232	74	
Abdominal-iguinal chains	54	51	105	51	
Total	225	112	337	67	16.1 ($P < 0.005$)

[a]2×2 contingency table analysis, degree of freedom = 1. The strong association between the anatomical region and occurrence of mutant *ras*12 carcinomas observed in both experiments were further examined by stratifying over the total number of carcinomas per animal and by experiment. The overall Cochran–Mantel–Haenszel $\chi^2 = 33$, $P < 0.001$. The disproportional pattern of mutant *ras*12 carcinoma occurrence was observed for each of the 10 strata in experiment 1, and 7 out of 8 strata in experiment 2. The probability for such observed disproportional distribution occurring by chance is $P < 0.01$. This secondary analyses did not support the existence of bias of the distribution pattern due to carcinoma multiplicity per animal.

are collected for biochemical and cytological assessment. The sampling issue is especially significant when 'gene-specific' prevention of subpopulations of pathogenetically identifiable neoplasia is concerned. For such applications of molecular markers, it is imperative that identifiable cancerous lesions from every gland be genotyped.

The overall *ras*12 mutation frequency in mammary carcinomas was 45% at a MNU dose of 37.5 mg/kg and 67% at 25 mg/kg (Table III). These results confirmed an earlier report that the percentage of mutant *ras*12 carcinomas was inversely related to the dose of MNU (10). That study also reported the disproportional increase in wild-type *ras*12 carcinomas in rats with experimental hyperprolactinemia (10). We have reported that the incidence of wild-type *ras*12 carcinomas could be increased preferentially by dietary risk factors over those with the mutation (17,20). These studies highlight the potential importance of risk assessment based on a knowledge of the pathogenetic characteristics of the disease.

In summary, experimental data presented in this study were consistent with the clonal evolution of multiple, independently-initiated cells giving rise to distinct mammary carcinomas in the same animal, and pointed to a significant topographic difference in the occurrence of mutant *ras*12 carcinomas between the C-T and the A-I mammary glands. These observations support the validity of statistical tests based on the assumption of independent emergence of lesions for the evaluation of the carcinogenesis data in this model and they further stress the need of representative sampling with gland location to be taken into consideration.

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