Inhibition of Human Cancer Cell Growth and Metastasis in Nude Mice by Oral Intake of Modified Citrus Pectin

Pratima Nangia-Makker, Victor Hogan, Yuichiro Honjo, Sara Baccarini, Larry Tait, Robert Bresalier, Avraham Raz

Background: **The role of dietary components in cancer progression and metastasis is an emerging field of clinical importance. Many stages of cancer progression involve carbohydrate-mediated recognition processes. We therefore studied the effects of high pH- and temperature-modified citrus pectin (MCP), a nondigestible, water-soluble polysaccharide fiber derived from citrus fruit that specifically inhibits the carbohydrate-binding protein galectin-3, on tumor growth and metastasis *in vivo* and on galectin-3-mediated functions *in vitro*. *Methods: In vivo* tumor growth, angiogenesis, and metastasis were studied in athymic mice that had been fed with MCP in their drinking water and then injected orthotopically with human breast carcinoma cells (MDA-MB-435) into the mammary fat pad region or with human colon carcinoma cells (LSLiM6) into the cecum. Galectin-3-mediated functions during tumor angiogenesis *in vitro* were studied by assessing the effect of MCP on capillary tube formation by human umbilical vein endothelial cells (HUVECs) in Matrigel. The effects of MCP on galectin-3-induced HUVEC chemotaxis and on HUVEC binding to MDA-MB-435 cells *in vitro* were studied using Boyden chamber and labeling assays, respectively. The data were analyzed by two-sided Student's *t* test or Fisher's protected least-significant-difference test. *Results:* Tumor growth, angiogenesis, and spontaneous metastasis *in vivo* were statistically significantly reduced in mice fed MCP. *In vitro,* MCP inhibited HUVEC morphogenesis (capillary tube formation) in a dose-dependent manner. *In vitro*, MCP inhibited the binding of galectin-3 to HUVECs: At concentrations of 0.1% and 0.25%, MCP inhibited the binding of galectin-3 (10 μg/mL) to HUVECs by 72.1% ($P = .038$) and 95.8% ($P = .025$), respectively, and at a concentration of 0.25% it inhibited the binding of galectin-3 (1 μg/mL) to HUVECs by 100% ($P = .032$). MCP blocked chemotaxis of HUVECs toward galectin-3 in a dose-dependent manner, reducing it by 68% at 0.005% ($P<.001$) and inhibiting it completely at 0.1% ($P<.001$). Finally, MCP also inhibited adhesion of MDA-MB-435 cells, which express galectin-3, to HUVECs in a dose-dependent manner. *Conclusions:* MCP, given orally, inhibits carbohydrate-mediated tumor growth, angiogenesis, and metastasis *in vivo,* presumably via its effects on galectin-3 function. These data stress the importance of dietary carbohydrate compounds as agents for the prevention and/or treatment of cancer. [J Natl Cancer Inst 2002;94:1854–62]**

Carbohydrates have an enormous potential for encoding biologic information. All cells express carbohydrates on their surfaces in the form of glycoproteins, glycolipids, and polysaccharides. Lectins, the carbohydrate-binding proteins, not only distinguish different monosaccharides but also bind specifically to oligosaccharides, detecting subtle differences in complex carbohydrate structures *(1).* The continuous growth and subsequent metastasis of cancers are dependent on tumor vasculature, and carbohydrate-mediated recognition interactions play a role in angiogenesis *(2).* Soluble forms of lectins (e.g., E-selectin, vascular cell adhesion molecule-1 [VCAM-1], and P-selectin) can promote endothelial cell migration and morphogenesis after binding to their respective glycoconjugate ligands *(3).*

The clinical manifestation of an elevated concentration of E-selectin in the sera of cancer patients provides *in vivo* evidence of the importance of these molecules in cancer progression *(4–7).* However, this premise was challenged by a report *(8)* showing that E- and P-selectin-deficient mice were able to induce normal angiogenesis. Therefore, we previously investigated whether another soluble carbohydrate-binding lectin, i.e., galectin-3, could provide an alternative angiogenic pathway and showed that carbohydrate-dependent galectin-3 binding to endothelial cells induces endothelial cell morphogenesis *in vitro* and angiogenesis *in vivo (9).* Galectin-3 belongs to the galectin superfamily of proteins, defined by a shared conserved sequence of the carbohydrate-binding domain and affinity to β-galactosides *(10).*

A notable feature of galectin-3 is its implication in neoplastic transformation and cancer progression. A direct relationship has been shown between galectin-3 levels and the stage of progression of some tumors [for review, *see (11,12)*]. Moreover, experimentally, a monoclonal antibody against galectin-3 strongly inhibits experimental lung metastasis of B16 melanoma and UV-2237 fibrosarcoma cells *(13).* Synthetic glycoamines Fru-D-Leu and Lac-L-Leu were used as effective inhibitors of spontaneous human breast cancer metastasis in nude mice *(14),* and D-galactose and arabinogalactan substantially inhibited the formation of experimental liver metastasis of L-1 sarcoma cells *(15).* More recently, it was reported that anti-galectin-3 antibody and lactose inhibit liver metastasis by adenocarcinoma cell lines XK4A3 and RPMI4788 *(16).* These studies suggest the potential for carbohydrate-mediated cancer therapy.

Pectin is a highly complex branched polysaccharide fiber rich in galactoside residues and present in all plant cell walls. Initially it was reported to bind to the carcinogen 1,2-dimethylhydrazine (DMH) with an increasing efficiency as pH was raised

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See "Notes" following "References."

from acidic to alkaline *(17).* In its native form, citrus pectin (CP) has a limited solubility in water and is unable to interact with galectin-3, but in its modified form (MCP) after hydrolysis to form a smaller linear water-soluble fiber, it acts as a ligand for galectin-3 *(18–20).* Injection of MCP-treated mice with melanoma B16-F1 cells resulted in a statistically significant reduction in lung colonization *(19).* Furthermore, oral administration of MCP to male Copenhagen rats injected with the prostate cancer cell line MAT-LyLu reduced spontaneous lung colonization in a dose-dependent fashion *(18),* suggesting that MCP interferes with galectin-3-dependent tumor embolization in the circulation, leading to reduced metastasis *(18,19).* A reduction in the growth of colon tumors implanted in mice after oral administration of MCP has been demonstrated *(21).* Hsieh and Wu *(22)* have recently reported that MCP treatment of human prostatic JCA-1 cells reduced cell growth and DNA synthesis, which was associated with reduced expression of cyclin B, nm23, p34, and cdc2.

The role of dietary nondigestible, water-insoluble carbohydrate fibers in the etiology of various human cancers is of considerable interest, because their use as chemopreventive agents has important implications for cancer prevention. Data from our laboratories *(18–20)* and others *(22–26)* have shown that carbohydrate-supplemented diets can inhibit tumor growth and metastasis in experimental murine tumor systems. Here, we studied the effect of MCP on galectin-3-mediated functions *in vitro* and on angiogenesis, tumor growth, and metastasis *in vivo* in athymic mice.

Materials and Methods

Cell Lines and Culture

Human umbilical vein endothelial cells (HUVECs) were purchased from the American Type Culture Collection (ATCC, Manassas, VA). The metastatic human breast cancer cell line MDA-MB-435 was a gift from Dr. Eric W. Thompson (St. Vincent's Institute of Medical Research and University of Melbourne, Melbourne, Australia). LSLiM6 is a well-characterized metastatic colonic adenocarcinoma cell line derived from low metastatic LS174T *(27,28).* HUVECs were cultured in Ham's F12K medium (Irvine Scientific, Irvine, CA) supplemented with 100 μg/mL heparin (Sigma Chemical Co., St. Louis, MO), 50 μg/mL endothelial cell growth supplement (Collaborative Biomedical Products, Bedford, MA), and 10% fetal bovine serum (FBS; Summit Biotechnology, Fort Collins, CO). MDA-MB-435 and LSLiM6 cells were maintained in Dulbecco's Minimal Essential Medium (Invitrogen Corporation, Carlsbad, CA) containing 10% heat-inactivated fetal calf serum (FCS), essential and nonessential amino acids (Invitrogen Corporation), vitamins, and antibiotics (Mediatech, Inc., Herndon, VA). The cells were maintained in a humidified chamber with 95% air and 5% CO_2 at 37 °C. The cells were grown to confluence and detached from the monolayer with 0.25% trypsin with 2 m*M* EDTA. The use of the cell lines was approved by the Human Investigations Committee, Wayne State University (Detroit, MI).

For collection of conditioned media, the cells were plated to confluence in a 60-mm dish. After 24 hours the medium was removed, and the cells were washed thoroughly with phosphate-buffered saline (PBS) and allowed to grow in serum-free medium. The medium was collected after 24 hours, concentrated 20-fold by centrifugation through Ultrafree-MC centrifugal filter units (Millipore, Bedford, MA) with a 10 K molecular weight cut-off, and analyzed for the presence of galectin-3 by Western blot analysis.

Preparation of Recombinant Galectin-3 and Modified CP

Recombinant human galectin-3 was expressed in *Escherichia coli* and isolated by affinity chromatography using an asialofetuin column as previously described *(29).* CP was purchased from Sigma Chemical Co.; pH and temperature modification of pectin was performed as described *(19).* Briefly, CP was solubilized as a 1.5% solution in distilled water, and its pH was increased to 10.0 with NaOH (3 N) for 1 hour at 50–60 °C. The solution was then cooled to room temperature while its pH was adjusted to 3.0 with 3 N HCl and stored overnight. Samples were precipitated the next day with 95% ethanol and incubated at –20 °C for 2 hours, filtered, washed with acetone, and dried on Whatman filters. For oral feeding of the nude mice, a 1% solution of MCP was prepared in autoclaved water, its pH was adjusted to approximately 7.0, and the solution (500 mL) was sterilized by γ-irradiation using a Mark 1–68 irradiator (J. L. Shepherd & Associates, Glendale, CA) at 552 Rads/minute for 45 minutes.

Composition Analysis of CP and MCP

Composition analysis was performed at the Complex Carbohydrate Research Center, University of Georgia (Athens). The samples were hydrolyzed using freshly prepared 1 *M* HCl in 3% methanol for 16 hours at 80 °C. The released sugars were dried down and N-acetylated using methanol and acetic anhydride for 15 minutes at 45 °C. The acetylated sample was trimethyl sialylated with Tri-Sil reagent (Pierce, Rockford, IL) and resolved on a 30-m DB-1 column (J&W Scientific, Folsom, CA) on a 5985 GC-MS system (Hewlett-Packard, Palo Alto, CA) using myoinositol as an internal standard.

Tumor Growth and Metastasis

NCR nu/nu mice were injected in the mammary fat pad region with 7.5×10^5 MDA-MB-435 cells. The site, time of inoculation, and autopsy were as described by Price et al. *(30).* Two groups of 10 mice each were given 1% (w/v) MCP (pH ≈7.0) in drinking water starting 1 week prior to the injections of tumor cells. Two control groups of 10 mice each were maintained on regular autoclaved water. For one group each of MCP-fed and control mice, the tumors were measured twice a week for 7 weeks, and the volumes were calculated by formula (length × width_1 × width_2 × 0.5). After 51 days, the mice were anesthetized (with ketamine [70 mg/kg body weight] and xylazine [7.5 mg/kg body weight]), and the primary tumors were surgically removed because some of them were larger than 1.5 cm. The mice continued to receive water or MCP solution for 8 more weeks, after which they were killed by cervical dislocation. The lungs were removed, fixed with Bouin's fixative, and examined visually as well as microscopically for the formation of tumor cell colonies.

The other groups of control and MCP-fed mice were killed at day 33. The tumors were removed, weighed, fixed with 10% formalin in PBS, and processed for immunohistochemical staining of blood vessels. The tumors were removed at 33 days, because some tumors became necrotic at later stages.

The ability of MCP to inhibit spontaneous metastasis of human colon cancer cells from the cecum to the liver was tested as previously described *(28)*. We selected the colon adenocarcinoma cell line LSLiM6, because these cells exhibit a high liver metastasis during cecal growth and high liver-colonizing ability after splenic portal injection in nude mice *(27)*. These cells also produce high levels of intracellular and cell surface galectin-3 *(28)*. A group of 10 pathogen-free nude mice was fed 1% MCP in their drinking water for 1 week, after which they were anesthetized with methoxyflurane by inhalation, the cecum was exteriorized, and 5×10^6 viable LSLiM6 cells in 0.1 mL were injected into the cecal wall. The cecum was replaced *in situ,* and the abdomen was closed with stainless steel clips. After 6 weeks, the animals were killed, and the cecum, abdominal tumor mass, and liver were removed. The number of animals with macroscopic liver nodules was determined and compared with that of control animals given plain water. All procedures were carried out in accordance with the guidelines provided by the Animal Investigation Committee at Wayne State University. All mice were checked daily, and no variations in body weight or behavior among the control and treated mice groups were observed.

Immunohistochemical Analysis to Visualize Blood Capillary Vessels in Primary Tumors

To visualize the blood vessels in the primary tumors removed from control and MCP-fed mice, the sections were stained with alpha smooth muscle actin, which stains the smooth muscle cells of the vessels. Immunohistochemistry was performed using a modification of the avidin–biotin–peroxidase complex technique. Briefly, 4-μm tissue sections were deparaffinized, rehydrated, and placed in a 3% hydrogen peroxide solution to inhibit endogenous peroxidase. The tissue sections were treated with 0.1% trypsin and 0.1% $CaCl_2$ for 30 minutes at 37 °C to expose the antigenic sites masked by formalin fixation, blocked for 1 hour with 3% normal goat serum (Sigma Chemical Co.), and subsequently incubated overnight with monoclonal mouse anti-human alpha smooth muscle actin (DAKO, Carpinteria, CA) at a dilution of 1 : 100. The sections were then treated with biotinylated secondary antibody (Vectastain Elite ABC kit; Vector Laboratories, Burlingame, CA) for 30 minutes at room temperature, followed by avidin-biotinylated horseradish peroxidase (HRP) complex reagent (according to the manufacturer's instructions) for 30 minutes and diaminobenzidine (Sigma Chemical Co.) for 1 minute. Counterstaining was performed with hematoxylin.

Capillary Tube Formation by HUVECs

Capillary tube formation by HUVECs was assayed on Matrigel (Collaborative Biomedical Products, Bedford, MA) as described earlier *(9)*. To prepare a gel, 200 μL of Matrigel thawed on ice was added to each chamber of an eight-chamber slide. The air bubbles were carefully removed, and the slide was transferred to a 37 °C incubator for 15 minutes. After gelation, 5×10^4 endothelial cells, separated from monolayers with trypsin treatment, were plated onto the gel in 200 μL of medium. In some chambers, MCP or CP was added to the cells at the time of incubation. The tube formation was observed after 16 hours.

Chemotaxis of HUVECs in Response to Galectin-3

Galectin-3-induced chemotactic response of endothelial cells was analyzed using a Boyden chamber. Briefly, 30 μL of serum-free F12K medium containing 10 μg/mL galectin-3 in the presence or absence of various concentrations of MCP was added to the lower chamber as a chemoattractant. HUVECs (5×10^4) were added to the upper chamber. The two chambers were separated by polycarbonate filters (8-μm pore size) and incubated at 37 °C. After 5 hours, the cells attached to the lower surface of the filter were fixed and stained using the Protocol Hema 3 Stain set (Fisher Scientific Company, Pittsburgh, PA). Cells in a total of 10 fields from each chamber were counted under a microscope, and the average number of cells per field was plotted. Each assay was carried out in quadruplicate. To investigate the specificity of chemotaxis inhibition by MCP, a comparative evaluation was performed using fibronectin and basic fibroblast growth factor (bFGF)-induced chemotaxis.

Biotinylation of Recombinant Galectin-3

Recombinant galectin-3 was isolated from transformed bacteria and purified as described previously *(29)*. The protein was biotinylated using an EZ-Link Sulfo-NHS-Biotinylation Kit (Pierce) according to the manufacturer's instructions. Briefly, the protein solution was concentrated to 2 mg/mL in PBS and mixed into 30 μL of sulfo-NHS-biotin (2 mg/100 μL H_2O) to get a molar ratio of 1 : 20. Biotinylation was achieved by incubating the mixture on ice for 2 hours. Excess salt was removed by passing the protein solution through the desalting column. The fractions were collected, and the protein content of each fraction was determined.

Galectin-3-HUVEC Binding Assay

HUVECs were seeded at a density of 1×10^4 cells/well in a 96-well plate. After 24 hours, the cells were washed four times with PBS and incubated with various concentrations of MCP in 100 μL of serum-free F12K medium for 15 minutes at 37 °C. After incubation, various concentrations of biotinylated recombinant galectin-3 were added to the wells and incubated for 2 hours at 37 °C. The wells were washed carefully three times with PBS. Next, 100 μL of a 1 : 1000 dilution of HRP-conjugated streptavidin was added to the wells and incubated at room temperature for 30 minutes. Unbound proteins were removed by washing three times with PBS. Color development was obtained by using 100 μL of citrate buffer mixed with ABTS (2,2 azino-di[3-ethylbenzthiazoline]sulfonic acid) and H_2O_2 and measured by an enzyme-linked immunosorbent assay (ELISA) plate reader (Molecular Devices, Sunnyvale, CA) at a wavelength of 405 nm.

Indirect Immunofluorescence Assay to Detect Galectin-3 on Tumor Cells

MDA-MB-435 cells were trypsinized and seeded in a four-chamber slide at a density of 50 000 cells/chamber. After 24 hours, the cells were fixed with 3.4% paraformaldehyde for 15 minutes at room temperature and washed four times with PBS containing 1% bovine serum albumin (BSA). The cells were then blocked for 30 minutes with 1% BSA in PBS followed by incubation with primary antibody (TIB-166 anti-galectin-3 monoclonal antibody made in rat; ATCC) at a 1 : 1 dilution with 1% BSA in PBS at 4 °C for 1 hour. Subsequently, the cells were washed three times with 0.1% BSA in PBS and incubated with fluorescein isothiocyanate (FITC)-labeled goat anti-rat immunoglobulin G (IgG; Zymed, San Francisco, CA) at a 1 : 50 dilution for 30 minutes. The primary antibody was omitted

in controls. The chambers were peeled off the slide, and the cells were mounted in gelvatol (13% w/v polyvinyl alcohol-2000, 0.6× PBS, and 30% glycerol) and observed under a fluorescence microscope (Olympus, Tokyo, Japan) for the presence of galectin-3.

Western Blot Analysis for Galectin-3

To study the expression of galectin-3, HUVECs or MDA-MB-435 cells were trypsinized and mixed with trypan blue. The viable cells were counted by a hemacytometer, and cells were suspended in a sample buffer (0.76% Tris, 10% glycerol, 1% sodium dodecyl sulfate [SDS], 1% 2-mercaptoethanol, 1% bromophenol blue) at 5000 cells/μL. To study the secretion of galectin-3, the conditioned media were collected and concentrated as described in the "Cell Lines and Culture" section. Equal amounts of protein (50 μg) or lysed cells (1×10^5) were loaded in each lane. The proteins were separated on a 12.5% polyacrylamide separating gel and a 3.5% stacking gel and electroblotted to polyvinylpyrrolidine fluoride (PVDF) membranes (MSI, Westborough, MA). Nonspecific binding was blocked in 5% nonfat dry milk in PBS for 1 hour, followed by incubation with the first antibody (rat monoclonal anti-galectin-3 or rabbit polyclonal anti-galectin-3 antibody) for 1 hour at room temperature. Subsequently, the membranes were washed five times with a blocking solution containing 0.1% Tween 20 and incubated with secondary antibody (HRP-conjugated rabbit anti-rat IgG or goat anti-rabbit IgG, respectively; Zymed) for 1 hour. After washing as before, they were processed for enhanced chemiluminescence using ECL western blotting detection reagents (Amersham, Piscataway, NJ) to locate the galectin-3 protein, according to the manufacturer's instructions.

Tumor Cell–HUVEC Adhesion Assay

MDA-MB-435 cells were suspended at a concentration of 3×10^6 cells/mL in serum-free medium containing 1% BSA and radiolabeled with 5 μCi of $Na^{51}CrO_4$ (Dupont NEN Research Products, Boston, MA) for 2 hours at 37 °C. At the end of the incubation, the cell suspensions were washed extensively and plated in quadruplicate in 16-mm Costar culture dishes (Corning Costar, Cambridge, MA) containing HUVEC monolayers in the presence or absence of different final concentrations (0.01%, 0.05%, 0.1%, or 0.25%) of MCP. After 2 hours, the cells were washed gently and thoroughly with PBS, and the attached cells were lysed with 0.1 N NaOH (30 minutes, 37 °C). To determine the percentage of adhesion of MDA-MB-435 cells to HUVECs, the cell-associated radioactivity was determined in a Packard Auto Gamma Counter (model 5650; Packard Biosciences Co./Perkin Elmer, Downer's Grove, IL). The adhesion of tumor cells to HUVECs in control experiments without MCP was given the value of 100%; percent adhesion in the presence of MCP was calculated accordingly.

Statistical Analysis

Tumor growth, chemotaxis, angiogenesis *in vivo,* and binding were the primary outcomes measured. The data were provided as means of either two or three experiments with 95% confidence intervals (CIs). (The experiments conducted to measure growth of the tumors were repeated twice with multiple animals.) We used Student's *t* test or Fisher's protected least-significant-difference (PLSD) test from StatView software (Abacus Concepts, Inc., Berkeley, CA) to analyze the statistical significance of the results. All statistical tests were two-sided, and *P* values less than .05 were considered statistically significant.

Results

To test the ability of MCP to inhibit primary tumor growth and metastasis, 7.5×10^5 MDA-MB-435 human breast carcinoma cells were injected into the mammary fat pad of NCR nu/nu mice previously fed for a week with 1% MCP in their drinking water. The pH of the solution was adjusted to 7.0 to neutralize the acidic taste of MCP. The mice were fed continuously with MCP throughout the duration of the experiments. The addition of MCP to the drinking water did not affect the overall tumorigenic efficiency. However, a statistically significant reduction in tumor growth rate was observed in mice fed with MCP compared with that in mice from the control group ($P = .050$, Student's *t* test; Fig. 1). The tumors in control mice reached 1.5 cm at 7 weeks after tumor inoculation, forcing us to terminate the analysis in all mice. The average tumor volume for control mice was 552 ± 14 mm^3 (95% CI = 540 to 564 mm^3) versus 165 ± 48 mm^3 (95% CI = 128 to 201 mm^3) for MCP-fed mice. The difference in tumor volumes between the control group and the treated group was 387 mm^3 (95% CI = 363 to 412 mm^3). At the termination of the experiments, after 15 weeks, the experimental mice were killed, autopsied, and examined for tumor metastases. Three mice (one control mouse and two MCP-fed mice) died during or immediately after surgery; therefore, the metastasis was not analyzed in all 10 mice in each group. The number of mice with lung metastases was statistically significantly smaller in the MCP-fed group than in the control group (zero of eight mice versus six of nine mice, respectively). Representative lungs from each group are depicted in Fig. 2. Daily water intake was similar in all groups. The mice did not show any dislike for MCP. Animal body weight and overall behavior were similar in the control group and the treated group.

To analyze whether MCP would inhibit the growth of other tumor types, we also studied the colonic growth of human colon carcinoma cells and spontaneous metastasis in MCP-fed nude mice. Previously, we had shown that galectin-3 plays a role in

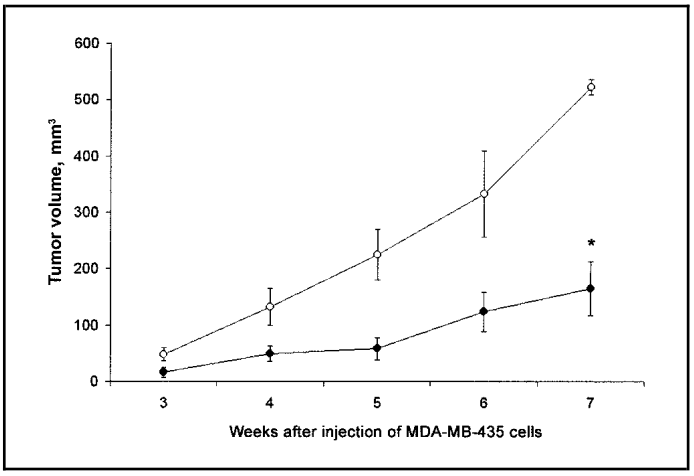

Fig. 1. Tumor growth in modified citrus pectin (MCP)-fed mice. MDA-MB-435 cells were injected into the mammary fat pad region of nude mice, and tumor volumes were measured twice a week. At the end of 51 days, the tumor volumes were calculated from 10 mice. Larger tumors were observed in the control mice (**open circles**) than in the MCP-fed mice (**closed circles**). The **error bars** represent 95% confidence intervals. *, $P = .050$.

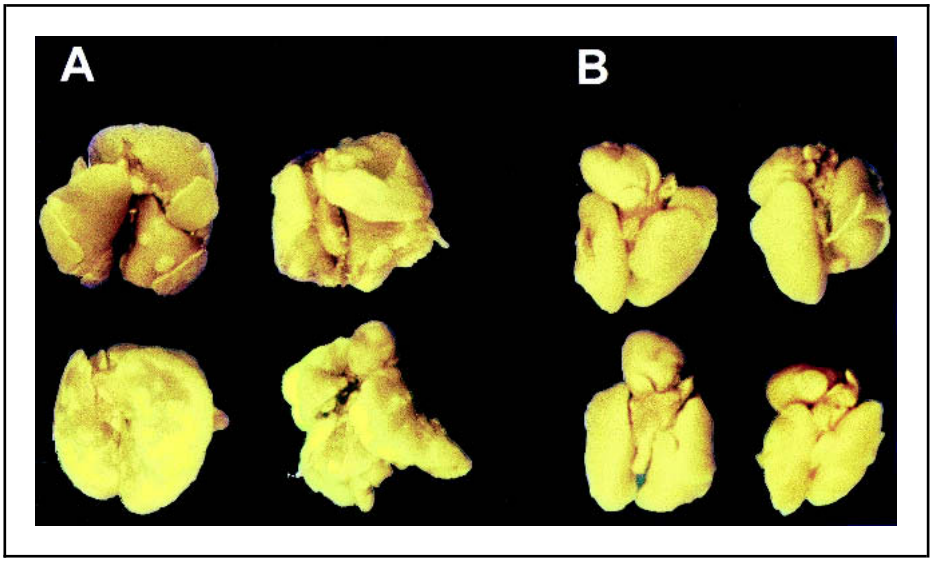

Fig. 2. Lung metastasis of MDA-MB-435 cells in modified citrus pectin (MCP)-fed mice. Mice were injected with 0.75×10^6 cells in the mammary fat pad region. Some mice were fed water alone (**A**) and others received 1% MCP in their drinking water (**B**). Tumors were removed after 7 weeks, and after 8 more weeks, the mice were killed and the presence or absence of lung metastasis (nodules on lung surface) was recorded.

liver colonization of human colon carcinoma cells after splenic-portal or cecal growth *(27,28).* We therefore examined whether MCP would affect the dissemination of these cells from the cecum to the liver. Five million LSLiM6 cells were surgically implanted into the cecum of nude mice (10 each of control and MCP-fed mice), and 6 weeks later, after continuous MCP feeding, the mice were killed, tumors were excised and weighed, and the incidence of metastasis was recorded. The average weights of the primary tumors in the control and 1% (w/v) MCP-fed mice were 1.16 g (95% CI = 1.13 to 1.19 g) and 0.65 g (95% CI = 0.37 to 0.93 g), respectively. The intra-abdominal tumor weights in the control and the MCP-fed mice were 2.0 g (95% CI = 1.94 to 2.06 g) and 0.88 g (95% CI = 0.37 to 0.93 g), respectively. The difference in primary tumor weight between the control group and the treated group was 0.51 g (95% CI = 0.40 to 0.82 g). The difference in intra-abdominal tumor weights between the control group and the MCP-fed group was 1.12 g (95% CI = 1.01 to 1.69 g). Metastases to lymph nodes and to the liver were present in 100% (nine of nine) and 60% (six of 10) in control mice, respectively, versus in 25% (two of eight) and 0% (zero of nine) in the MCP-fed mice. Similar results were observed in repeat experiments. Daily water intake was similar in all groups. Animal body weight and overall behavior were unchanged between the control group and the treated group.

We have shown previously that galectin-3 mediates endothelial cell morphogenesis *in vitro* and angiogenesis *in vivo* in a carbohydrate-dependent manner *(9).* Thus, we investigated whether the inhibitory effects of MCP on tumor growth were associated with reduced angiogenesis. Primary MDA-MB-435 tumors growing in the mammary fat pad of water- or MCP-fed nude mice (five mice per group) were excised, fixed, and stained for the presence of blood vessels. The tumors from MCP-fed mice had one-third the number of vessels per unit area as the tumors in the control mice (Fig. 3). Three tumors from each group were sectioned, and one field per slide was counted from three slides per tumor for the blood vessels. The average number of blood vessels (and 95% CIs) for control and MCP-fed mice, respectively, were 15.0 (95% CI = 12.9 to 17.2) and 4.9 (95% CI = 3.0 to 6.7). To directly test the effect of MCP on endothelial cell morphogenesis, an *in vitro* capillary tube formation assay was performed. A thin layer of Matrigel was formed in each chamber of an eight-chamber slide by incubation at 37 °C for 15 minutes. Fifty thousand HUVECs were then plated in each chamber, along with varying concentrations of MCP or CP. A dose-dependent inhibition of the ability of the cell to form a capillary network on Matrigel in the presence of MCP (Fig. 4, B–D) was observed as compared with control PBS (Fig. 4, A) and intact CP controls (Fig. 4, E and F).

Chemotaxis is an integral part of angiogenesis, invasion, and metastasis *(31).* Like bFGF, galectin-3 induces a chemotactic response in HUVECs *(9).* To determine whether MCP would inhibit galectin-3-induced chemotaxis of endothelial cells, we performed Boyden chamber chemotaxis assays. As a chemoattractant, galectin-3 (10 μg/mL) in serum-free medium containing various concentrations of MCP was placed in the lower chamber, and HUVECs (5×10^4 cells) were loaded into the upper chambers. After 5 hours of incubation at 37 °C, the cells that migrated toward the chemoattractant were fixed, stained, and counted under a phase-contrast microscope (Fig. 5, A). MCP statistically significantly inhibited the chemotactic response of galectin-3 in a dose-dependent manner. At a concentration of 0.005% (w/v), chemotaxis was reduced by 68%. At a concentration of 0.1% (w/v), there was a complete inhibition of chemotaxis, with the same number of migratory cells as in the negative control. Next, we investigated whether the effect of MCP is specific to the chemotactic response to galectin-3 by analyzing fibronectin- and bFGF-induced chemotaxis in the presence of MCP. MCP had a small inhibitory effect (at a concentration of 0.05%) on fibronectin-induced migration (26% inhibition) and strongly reduced bFGF-induced chemotaxis (by 34% and 86% at concentrations of 0.01% and 0.05%, respectively) (Fig. 5, B). Calculated *P* values using Fisher's PLSD test for galectin-3 were .016 with 0.001% MCP and less than .001 with 0.005%, 0.01%, and 0.1% MCP; for fibronectin, the *P* values were .122 and .007 at 0.01% MCP and 0.05% MCP, respectively; for bFGF, the *P* values were .015 and less than .001 at 0.01% MCP and 0.05% MCP, respectively.

We have previously demonstrated that galectin-3 binds to endothelial cell surface high- and low-affinity receptors *(9)* and that this binding specifically initiates endothelial cell capillary tube formation. To establish whether MCP inhibits this binding, 1×10^4 HUVECs were plated in a 96-well plate and incubated

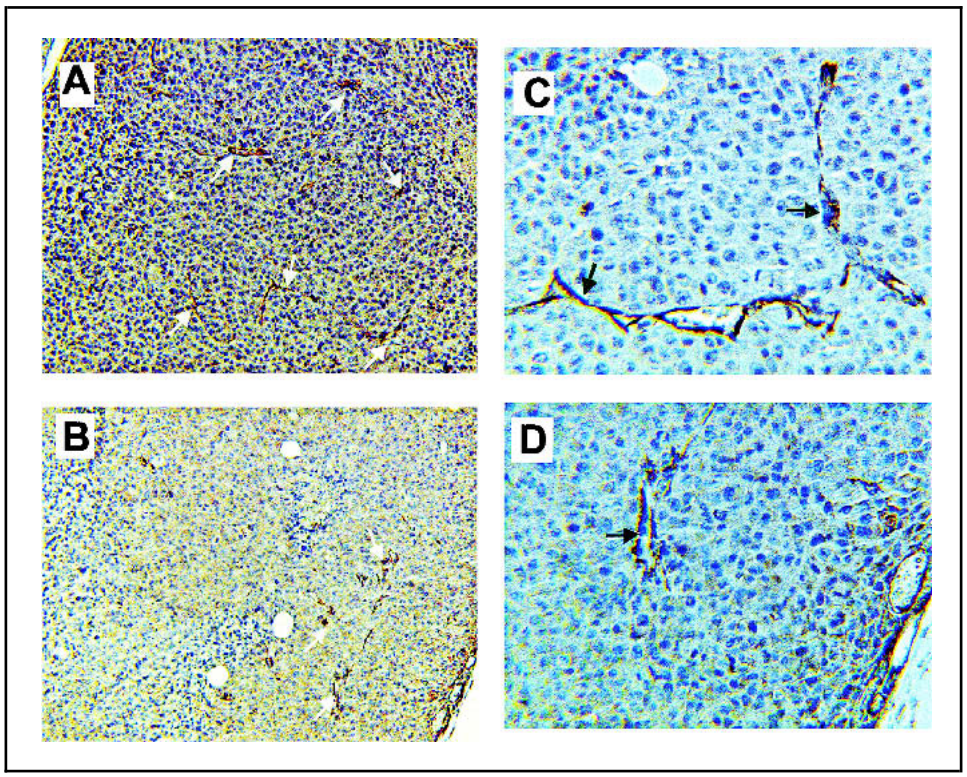

Fig. 3. *In vivo* angiogenesis in modified citrus pectin (MCP)-fed mice. Nude mice were injected with breast cancer cells. Tumors were removed after 33 days, fixed, embedded in paraffin, sectioned, and stained for the presence of blood vessels using antibody against smooth muscle actin. Three tumors from each group were sectioned, and three slides per tumor were studied. **A**) Control, ×100; **B**) MCP-fed, ×100; **C**) control, ×250; **D**) MCP-fed, ×250.

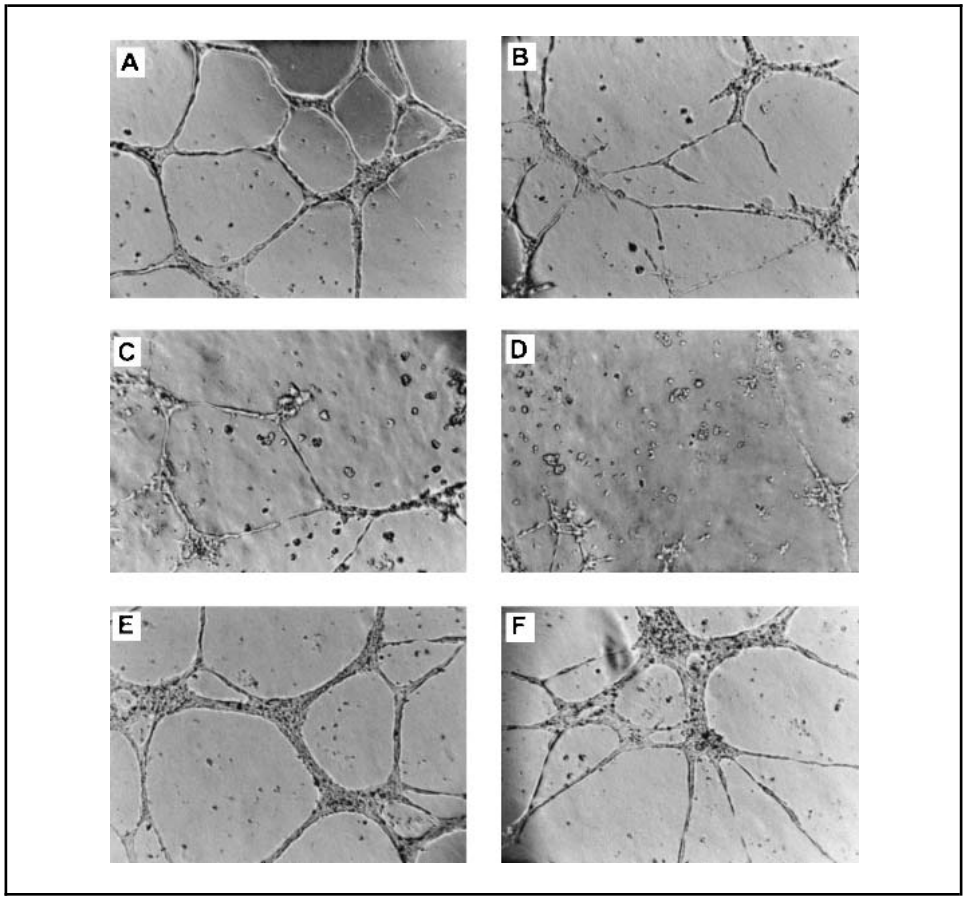

Fig. 4. Inhibition of *in vitro* capillary tube formation. Human umbilical vein endothelial cells were plated on gelled Matrigel (200 μL/chamber) at a density of 50 000 cells per chamber in the absence (**A**) and presence of 0.01% (**B**), 0.05% (**C**), or 0.1% (**D**) modified citrus pectin (MCP) or 0.05% (**E**) or 0.1% (**F**) citrus pectin (CP). The ability to form tubes was inhibited in the presence of MCP compared with control (**A**) or CP (**E** and **F**). The experiment was repeated three times, and representative pictures are shown.

for 15 minutes at 37 °C with various concentrations of MCP. Biotinylated galectin-3 (1 μg/mL and 10 μg/mL) was then added, and after 2 hours of incubation at 37 °C, the cells were thoroughly washed and binding efficiency was determined by color development with ABTS and H_2O_2. The results (Fig. 6) show that galectin-3 bound to HUVECs and that MCP specifically inhibited this binding. Similar experiments were performed with CP, lactose, and sucrose; inhibition was seen with lactose

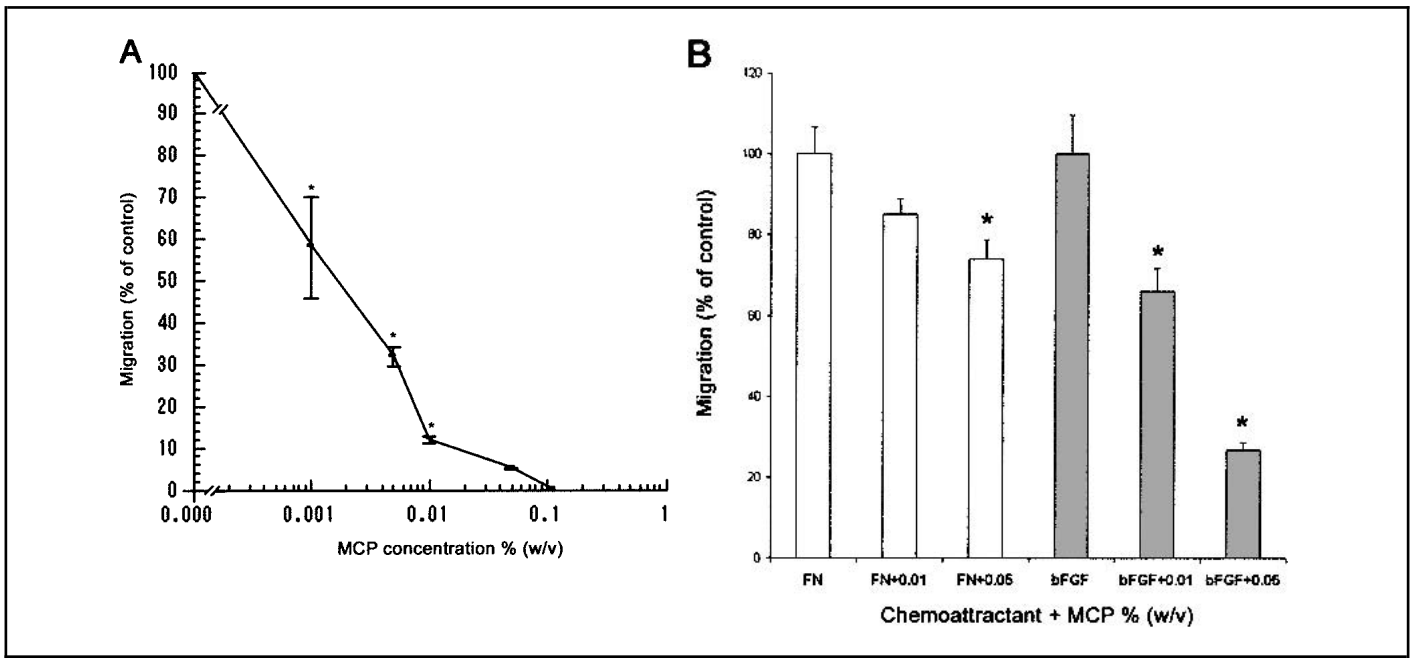

Fig. 5. Inhibition of chemotaxis. **A**) Galectin-3 was added in the lower chamber with different concentrations of modified citrus pectin (MCP). In the upper chamber, 5×10^4 human umbilical vein endothelial cells were loaded. The two chambers were separated by a polycarbonate filter of 8-μm pore size and incubated at 37 °C for 5 hours. The number of cells migrating to the lower side of the filter was calculated. Each point represents an average of eight readings. **B**) Same experiment performed using fibronectin (FN) with or without MCP (**open bars**) and basic fibroblast growth factor (bFGF) with or without MCP (**gray bars**). **Error bars** represent 95% confidence intervals. *, *P* values using Fisher's protected least-significant-difference test in **A:** .016 with 0.001% MCP and less than .001 with both 0.005% and 0.01% MCP; in **B:** .007 and .122 at 0.05% and 0.01% MCP, respectively, when used with fibronectin; and .015 and less than .001 at 0.01% and 0.05% MCP, respectively, when used with bFGF.

and MCP only and not with sucrose or CP (data not shown). The binding of 10 μg/mL galectin-3 to HUVECs was inhibited by 72.1% and 95.8%, respectively, with MCP concentrations of 0.1% and 0.25%, and binding of 1 μg/mL galectin-3 to HUVECs was inhibited by 100% in the presence of 0.25% MCP. (*P* values using Fisher's PLSD test were .045 and .032 at MCP concentrations of 0.1% and 0.25% for a 1 μg/mL galectin-3 concentration and .038 and .025 at MCP concentrations of 0.1% and 0.25% for a 10 μg/mL galectin-3 concentration.) Western blot and direct immunofluorescence analyses showed that MDA-MB-435 cells express galectin-3 on the cell surface and in the cytoplasm and secrete it (Fig. 7). There was a progressive inhibition (33%, 58.4%, 66.5%, and 83.4%) of the binding ability (adhesion) of these tumor cells to the HUVECs by increasing doses of MCP, i.e., 0.01%, 0.05%, 0.1%, and 0.25%, respectively (Fig. 8). *P* values, as calculated by Fisher's PLSD test, were less than .001 at 0.05%, 0.1%, and 0.25% MCP and .003 at 0.01% MCP. Thus, inhibition of tumor cell–endothelial cell interaction by MCP may affect adhesive interactions that play a role in invasion and metastasis.

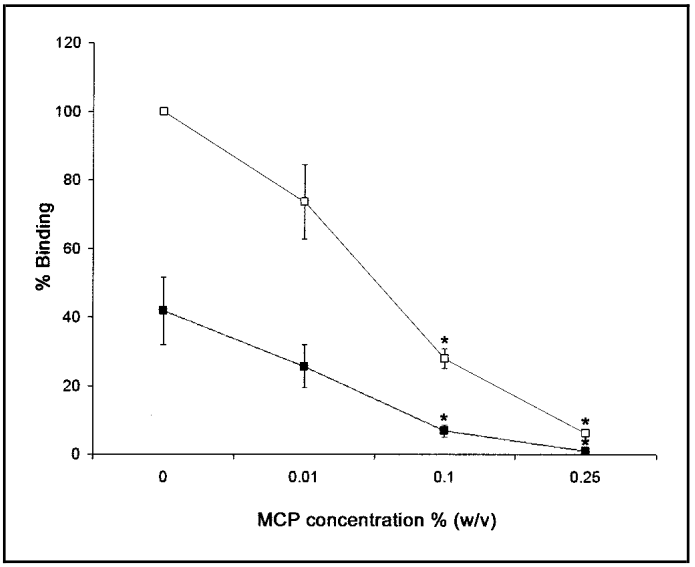

Fig. 6. Binding of recombinant galectin-3 to human umbilical vein endothelial cells (HUVECs) in the presence of modified citrus pectin (MCP). Recombinant galectin-3 was biotinylated using the EZ-Link Sulfo-NHS-Biotinylation kit (Pierce). Endothelial cells were incubated with 1 μg (**closed squares**) or 10 μg/mL (**open squares**) galectin-3 in the presence of 0.01%, 0.1%, or 0.25% MCP. Binding was determined by color development as described in the "Materials and Methods" section. Optical density of cells incubated with 10 μg of galectin-3 was arbitrarily given a value of 100% binding. Other values were calculated accordingly. Each point represents a mean of three readings. **Error bars** represent 95% confidence intervals. *, *P* values using Fisher's protected least-significant-difference test were .045 and .032 at MCP concentrations of 0.1% and 0.25%, respectively, for 1 μg of galectin-3 and .038 and .025 at MCP concentrations of 0.1% and 0.25%, respectively, for 10 μg/mL galectin-3.

DISCUSSION

The use of dietary components having protective and/or preventive effects on cancer progression and metastasis is an important emerging field of research. Identifying new food supplements and understanding their mechanisms of action are some of the main challenges in using functional foods as a cancer therapeutics modality.

It has been previously shown that pectin hydrolysate, whether administered orally *(18)* or intravenously *(19),* reduces both the spontaneous and experimental lung colonization of tumor cells. CP in the form of water-insoluble fibers also may reduce the

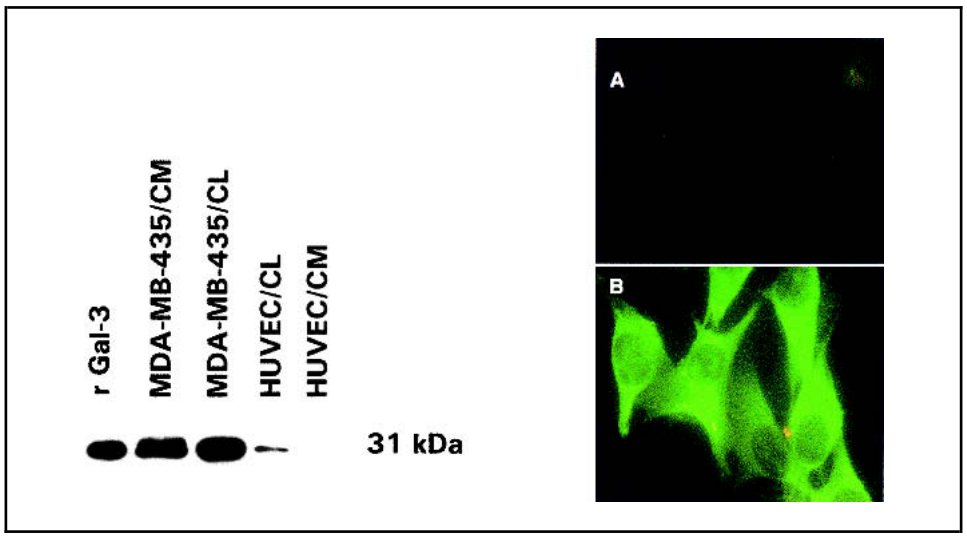

Fig. 7. Left panel: Western blot analysis of conditioned media (CM) and total cell lysates (CL) of MDA-MB-435 and human umbilical vein endothelial cells (HUVECs). For comparison, 100 ng of recombinant galectin-3 (r-gal-3) was loaded into one lane. **Right panel:** Indirect immunofluorescence of MDA-MB-435 cells for surface expression of galectin-3. **A**) Negative control, in which the primary antibody was omitted. **B**) Stained cells expressing galectin-3.

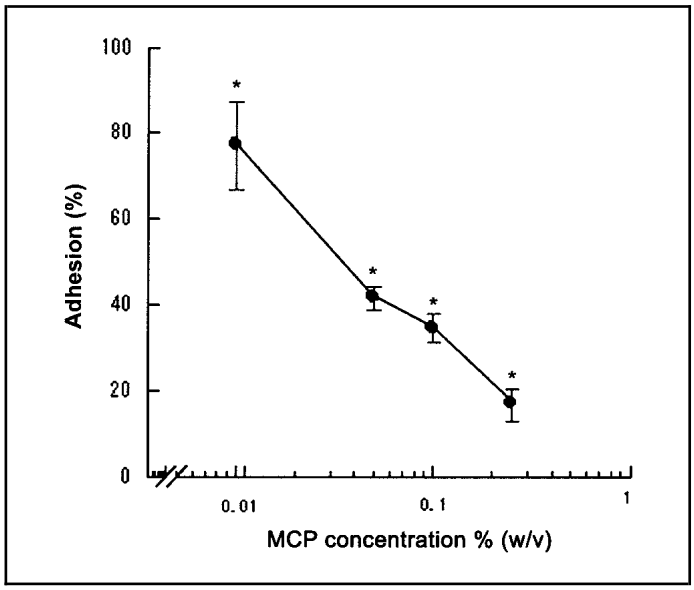

Fig. 8. Adhesion of tumor cells to human umbilical vein endothelial cells (HUVECs). MDA-MB-435 breast cancer cells were labeled with $Na^{51}CrO_4$ and incubated with HUVECs in the presence of various concentrations of modified citrus pectin (MCP). After 2 hours, the cells were washed, lysed, and counted by scintillation counter. Controls were given a value of 100%, and the other values were calculated accordingly. Each value represents a mean of three readings. **Error bars** represent 95% confidence intervals. *, $P<.001$.

incidence of chemically induced colon cancer *(17),* presumably by promoting *Bifidobacteria (26).* Rats fed on a 15% CP-enriched diet showed a higher apoptotic index in their colon *(23–25).* Reduced cell growth and corresponding [^{3}H]thymidine incorporation into DNA was reported when human prostatic JCA-1 cells were grown in media containing MCP *(22).* Pectins have also been found to exhibit anti-mutagenic activity against nitroaromatic compounds *(32).* Daily oral administration of MCP reduced the growth of implanted colon-25 tumors in BALB/c mice, and dietary pectin reduced the growth of intramuscularly transplanted mouse tumors from tumor cell lines TLT and EMT6 *(33).* The results presented in the current study are the first report showing an inhibition of tumor growth and metastasis of orthotopically grown breast and colon cancer cells by a soluble, orally ingested dietary carbohydrate fiber.

The data indicate that MCP might reduce mammary and colonic tumor growth and metastasis by inhibiting angiogenesis. In mammary carcinoma cells growing in the mammary fat pad of nude mice, we observed a 70.2% reduction in the mean tumor volume by 7 weeks following the oral intake of MCP (Fig. 1). This was associated with a 66% reduction in blood vessels and a complete inhibition of metastasis to the lungs (Fig. 3). Similarly, there was less tumor burden and metastasis in the MCP-fed nude mice into which human colon carcinoma cells (LSLiM6) were implanted than in the control mice. Metastases to lymph nodes and the liver were present in 100% and 66% of control mice versus 25% and 0% of mice fed with MCP.

Pectin consists of "smooth" and "hairy" regions. The smooth region is composed of partially esterified galacturonic acid residues, whereas hairy regions contain galacturonic acid residues with irregularly inserted rhamnose residues with side chains composed of neutral sugars such as arabinose, galactose, glucose, mannose, and xylose. The modification of CP to MCP by pH involves degradation of the main galacturonic acid chain by β elimination (high pH) followed by partial degradation of the natural carbohydrates (low pH), resulting in simpler carbohydrate chains of basically the same sugar composition as the unmodified CP. Composition analysis of CP and MCP showed that MCP is richer in galactose, rhamnose, and xylose (data not shown). MCP effectively binds to recombinant galectin-3 and inhibits galectin-3-mediated functions, such as homotypic tumor cell aggregation, binding of tumor endothelial cells, anchorage-independent growth, and binding to the laminin ligand by blocking its carbohydrate-binding domain *(18–20).* Our results show that MCP also acts as an angiogenesis inhibitor. In an *in vitro* assay, HUVECs migrated and differentiated into capillary-like structures, and MCP prevented this migration and capillary tube formation, either by binding to the galectin-3 present in the matrix and/or on the endothelial cells or interfering with its binding to the receptor. *In vivo* this leads to a marked reduction in the density of tumor-associated blood vessels. Similarly, MCP specifically inhibited the binding of the galectin-3-expressing breast cancer cell line MDA-MB-435 to endothelial cells, explaining in part its inhibition of invasion and metastasis. Recent findings demonstrate that hematogenous cancer metastases originate from intravascular growth of cancer cells attached to the endothelium rather than from extravasated ones *(34).* This

suggests a key role of tumor–endothelial cell interactions in cancer metastasis. Here we have shown that MCP inhibited galectin-3-induced and bFGF-induced chemotactic migration, and others have reported that CP inhibits the binding of bFGF to its receptor FGFR in the presence of heparin, also a complex carbohydrate *(35)*.

In summary, our results demonstrate that MCP inhibits *in vitro* and *in vivo* carbohydrate-mediated angiogenesis by blocking the association of galectin-3 to its receptors. These data stress the importance of dietary carbohydrate compounds as cancer-preventive and/or -therapeutic agents. The complex nature of carbohydrate specificities will require the development of new antagonists for the recognition of angiogenic factors and glycoconjugate receptors.

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NOTES

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