[CANCER RESEARCH 57, 2823–2826, July 15, 1997]

Advances in Brief

Filament Disassembly and Loss of Mammary Myoepithelial Cells after Exposure to λ-Carrageenan

Joanne K. Tobacman[1]

Department of Internal Medicine, College of Medicine, The University of Iowa, Iowa City, Iowa 52242-1081

Abstract

Carrageenans are naturally occurring sulfated polysaccharides, widely used in commercial food preparation to improve the texture of processed foods. Because of their ubiquity in the diet and their observed preneoplastic effects in intestinal cells, their impact on human mammary myoepithelial cells in tissue culture was studied. At concentrations as low as 0.00014%, λ-carrageenan was associated with disassembly of filaments with reduced immunostaining for vimentin, α-smooth muscle-specific actin, and gelsolin; increased staining for cytokeratin 14; and cell death. The absence of mammary myoepithelial cells is associated with invasive mammary malignancy; hence, the destruction of these cells in tissue culture by a low concentration of a widely used food additive suggests a dietary mechanism for mammary carcinogenesis not considered previously.

Introduction

Mammary myoepithelial cells possess characteristics of both muscle and epithelial cells. These features include arrays of myofibrillar proteins with positive staining for α-smooth muscle-specific actin, myosin, gelsolin, and vimentin. In addition, they produce proteins such as laminin and type IV collagen, which contribute to the basement membrane in the mammary gland, and also various cytokeratins. The myoepithelial cells are located between the epithelial cells that surround the lumen of the ducts, ductules, alveoli, and capillaries, and their absence in invasive mammary malignancy has been recognized and used as a criterion for the diagnosis of invasive malignancy (1–5). Commercially available since 1937, carrageenans are widely used as food additives to improve texture by acting as a thickener, stabilizer, and emulsifier. Characteristically, they bind to milk proteins, and κ- and ι-carrageenans form gels, unlike λ-carrageenan, which does not form gels. λ-Carrageenan is used predominantly in cold milk products to improve solubility properties in foods such as puddings, chocolate milk, and whipped cream, and the concentration in the food product is as much as 2% by weight. The carrageenans are present as the potassium, sodium, magnesium, calcium, and ammonium sulfate esters of D-galactose and 3,6-anhydrous-D-galactose copolymers with the hexose residues alternatively linked by α-1,3 and β-1,4 bonds. The designations of λ, κ, or ι depend on the number and location of the sulfate groups on the hexose structure; λ-carrageenan has the most sulfate residues, and they are located at the 2 and 2,6 sites. Previous work has demonstrated the inflammatory potential of carrageenan in models of arthritis, and uptake by intestinal macrophages and associated systemic effects have been reported. Their propensity to cause premalignant changes of intestinal cells has been demonstrated. The degraded form of carrageenan, poligeenan, produced by acid hydrolysis of carrageenan, induces neoplastic changes in animal models of intestinal carcinogenesis. In addition, changes in c-*fos* have been seen in a neuron model of inflammation after exposure to carrageenan, when used as an inflammatory agent (6–14).

The present study was undertaken to determine the effect of λ-carrageenan on human mammary myoepithelial cells grown in tissue culture.

Materials and Methods

Mammary cells were obtained from discarded reduction mammoplasty specimens, and primary cultures were established using modifications of previously reported methods (15, 16). Portions of tissue that appeared white and gelatinous were carefully excised from the surrounding connective tissue and fat and placed in a 50:50 mixture of Ham's F-12 medium and DMEM with 10% glucose, each with 10% fetal bovine serum, and maintained at room temperature. Tissue was carefully minced into 5-mm^2 pieces and digested overnight in a collagenase-containing digestion mixture composed of 3 mM glucose, 4% BSA, and 1 mM calcium with 2 mg/ml collagenase (Sigma) in PBS and combined 1:1 with media. Subsequently, the top layer of fat was aspirated, and fractions of cells were collected and resuspended in Ham's F-12 medium with 10% fetal bovine serum and antibiotics. The cells were grown to confluence in tissue culture flasks in 5% CO_2 and 90% humidity at 37°C, and subcultures were obtained after mechanical disruption by repeated tapping of the primary culture flasks and grown to confluence. Some variation in the proportion of epithelial:myoepithelial cells was evident in the primary cultures; subcultures of some of the fractions led to highly pure cultures of mammary myoepithelial cells, based on immunostaining and morphology. Cultures that had <1% of cells that did not stain for α-smooth muscle-specific actin were used in these experiments.

Immunostaining for α-smooth muscle-specific actin, gelsolin, vimentin, laminin, and cytokeratin 14 was done with commercial products (Sigma). Additional staining for S-100, muscle-specific actin, cytokeratin 18, epithelial membrane antigen, and broader-spectrum cytokeratin antibodies was performed. All reactions were performed with positive and negative tissue controls, using avidin-biotinylated reagents and peroxidase-conjugated stains. λ-Carrageenan was obtained from Sigma in its purest form. λ-Carrageenan was added at a concentration of 1.4 g/1000 cc to Ham's F-12 media and subsequently diluted to concentrations of 0.14, 0.07, 0.014, 0.0014, and 0.00014%. Cells grown on four-compartment plastic slides using standard media or carrageenan-containing media at varying concentrations were air-dried and subsequently immunostained using standard techniques and commercially available products.

Cells obtained by mechanical disruption from primary cultures were plated at low density in T-25 flasks and grown with standard or carrageenan-containing media. Cell counts were performed in demarcated equivalent regions at various time points. Phase-contrast images from tissue culture flasks were made using a Nikon phase-contrast microscope fitted for photography with a camera attachment. Electron microscopy was performed after fixation in a mixture of 2.5% glutaraldehyde in 0.1 M cacodylate after 30 min in a 1:1 mixture with Ham's F-12 media. The cells were examined with a Hitachi 6000 transmission electron microscope, after

Received 1/6/97; accepted 5/29/97.

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[1] To whom requests for reprints should be addressed, at Department of Internal Medicine, College of Medicine, The University of Iowa, 200 Hawkins Drive, Iowa City, IA 52242-1081.

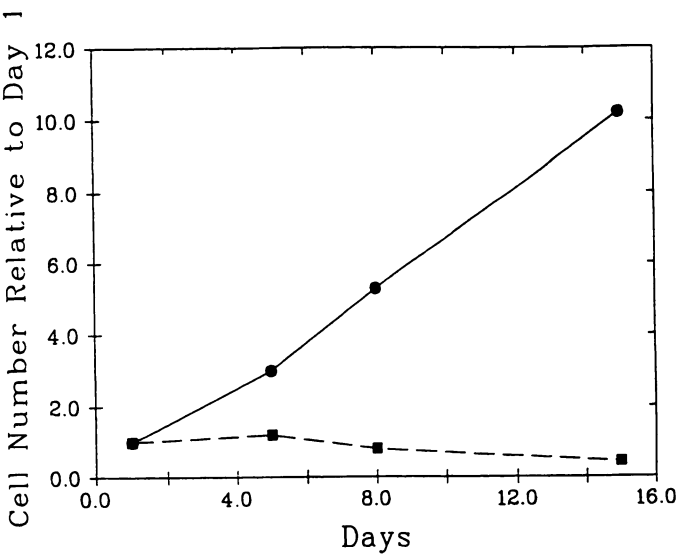

Fig. 1. The marked reduction in cell growth of mammary myoepithelial cells in tissue culture due to the effect of λ-carrageenan at a concentration of 0.07% is evident. ○, standard media; ■, media with carrageenan. Cell counts are the mean of three measurements with a variation of <2%.

preparation of grids, using cells grown in standard media and at varying concentrations of carrageenan.

Results and Discussion

The effect of carrageenan on the growth of mammary myoepithelial cells was determined by cell counts of the living cells in tissue culture over several weeks. The data are presented in Fig. 1. A marked decline in cell numbers with carrageenan treatment is evident. Fig. 2 demonstrates the marked destruction of the carrageenan-treated cells as seen by phase-contrast microscopy. Variation in cell shape and development of intracellular vacuoles are depicted. Long-term observation of the λ-carrageenan-treated cultures demonstrates that the remaining cells do not grow to confluence or regain their initial morphology when returned to normal media after exposure.

After carrageenan treatment, disruption of the internal cellular architecture is apparent. Cells stained for gelsolin, α-smooth muscle-specific actin, vimentin, and cytokeratin 14 are pictured in Fig. 3. After exposure to carrageenan, marked destruction of cells is seen. The immunostaining for gelsolin (Fig. 3, *A* and *B*) indicates disruption of the previous regular pattern of staining and a marked reduction of gelsolin. The α-smooth muscle-specific actin staining (Fig. 3, *C* and *D*) demonstrates vacuolation in cytoplasmic regions that had previously stained uniformly for α-smooth muscle-specific actin. In addition, the intensity of stain is remarkably diminished. Change in cell shape and alteration in the distribution of vimentin with loss of the original elongated morphology and extensive cell processes are evident after carrageenan exposure (Fig. 3, *E* and *F*). Fig. 3*F* reveals an increase in the relative population of more cuboidal cells, suggesting an epithelialization of the surviving myoepithelial cells. The overall reduction in cell density is not evident from Fig. 3*F*, a region in which a high density of cells was still present. Cytokeratin 14 immunostaining (Fig. 3, *G* and *H*) increased in intensity, and its distribution was more extensive. Due to the relative purity (>99%) of the cultures for myoepithelial cells, the effect of λ-carrageenan on mammary epithelial cells or fibroblasts is not evident.

Electron micrographs (Fig. 4) demonstrate severe disruption of the internal architecture, with an increase in the apparent disassembly of microfilaments as the concentration of the carrageenan increased from 0.00014 to 0.14%. In addition, the increased abundance of lysosomes with prominent internal inclusions is evident. Clumping is seen near the nuclear membrane, as well as enlarged mitochondria.

As evident, the widely used food additive λ-carrageenan has marked effects on the growth and characteristics of human mammary myoepithelial cells in tissue culture at concentrations much less than those frequently used in food products to improve solubility. Because carrageenan has been demonstrated to be present in intestinal epithelial cells after oral intake (12) and to be taken up by macrophages (11), transport to other tissues, perhaps by macrophages, may occur *in vivo*. Hence, it is possible that carrageenan may adversely affect the structure and function of the normal mammary gland and possibly even that of other tissues that possess myoepithelial cells, such as the prostate gland. Because carrageenan is known to affect the solubility of milk proteins, it may interfere with the normal solubility of intracellular proteins in mammary cells.

The marked diminution in staining for α-smooth muscle-specific actin, gelsolin, and vimentin and the apparent disassembly of microfilaments suggest that carrageenan may interfere with the normal process of actin assembly. This effect in mammary myoepithelial cells is consistent with the possible role of down-regulation of gelsolin and α-smooth muscle-specific actin in malignancy (17–20) and the observed preneoplastic effects of carrageenans on intestinal cells (14, 21, 22).

Because mammary myoepithelial cells are absent in invasive mammary malignancy, identification of a food additive in widespread use that at a concentration of 0.00014% leads to loss of human myoepithelial cells in tissue culture may have significant implications for mammary carcinogenesis. In addition, the marked destruction of the cytoskeletal components in this experimental model suggests new approaches to consider with regard to the etiology of invasive mammary malignancy.

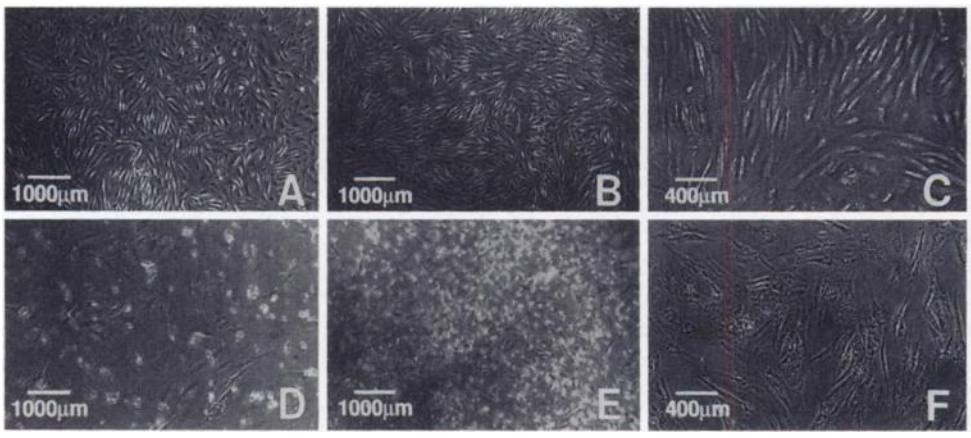

Fig. 2. Phase-contrast images reveal the changes in mammary myoepithelial cells that occur after exposure to carrageenan. *A*, day 1, no exposure; *B*, day 8, no exposure; *C*, day 23, no exposure. *D*, concentration of 0.014% at day 8; *E*, concentration of 0.0014% at day 23; *F*, concentration of 0.00014% at day 23. From the normal elongated morphology with prominent cellular processes and swirling pattern, the cells change before death, with the prominence of intracellular vacuoles.

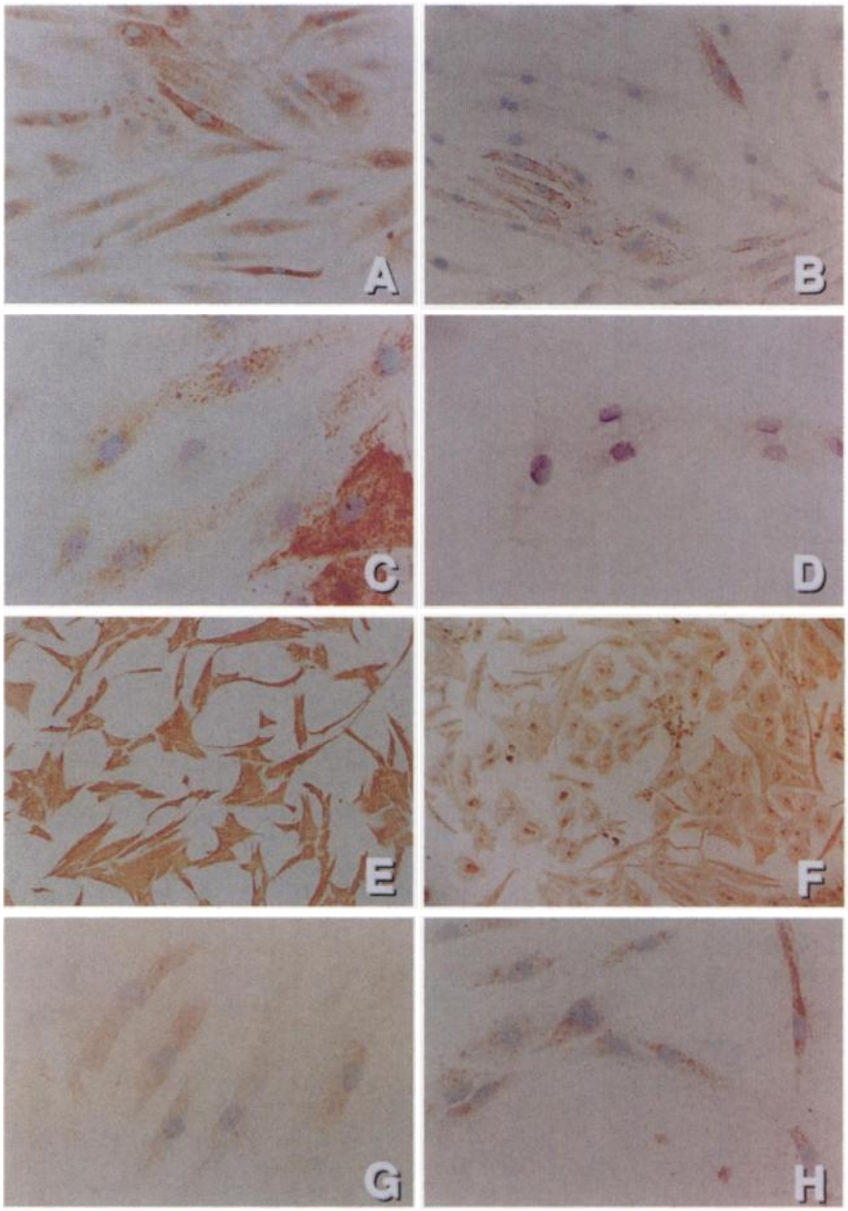

Fig. 3. Immunostaining of mammary myoepithelial cells after carrageenan exposure (concentration, 0.0014%); *A, C, E,* and *G,* no carrageenan exposure. *A* and *B,* gelsolin staining, ×50; *C* and *D,* α-smooth muscle-specific actin, ×100; *E* and *F,* vimentin staining, ×25, the area of the slide with higher density is seen for post-carrageenan-treated cells, although overall cell density was diminished; *G* and *H,* CK14 staining, ×100. Diminished staining intensity and altered distribution of stain are evident for α-smooth muscle-specific actin, gelsolin, and vimentin. Cells stained for CK-14 show an increased intensity of staining.

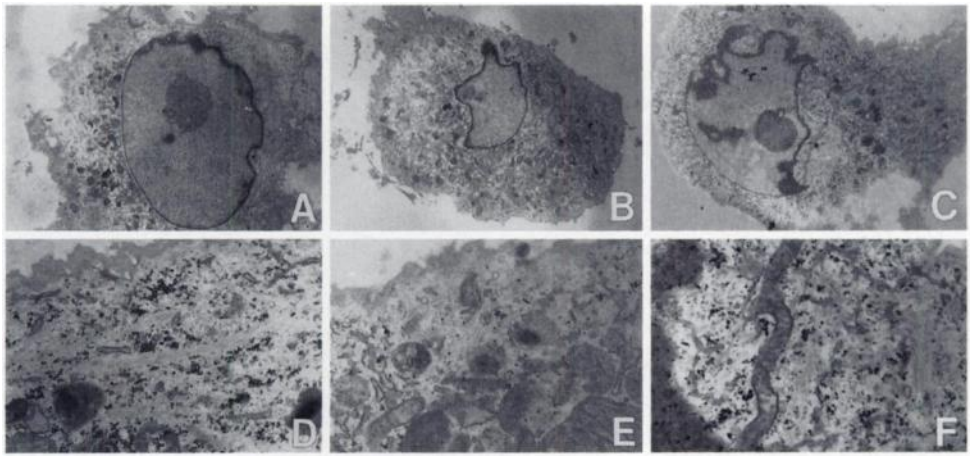

Fig. 4. Electron microscopy showing the impact of carrageenan exposure on the intracellular characteristics of mammary myoepithelial cells. *A* and *D,* cells grown in standard media, ×5,000 (*A*) and ×30,000 (*D*). *B* and *E,* cells after exposure to λ-carrageenan at a concentration of 0.00014%, ×5,000 (*B*) and ×40,000 (*D*). *C* and *F,* cells after exposure to λ-carrageenan at a concentration of 0.14% ×5,000 (*C*) and ×40,000 (*F*). The disruption of the intracellular filaments is evident, as seen in an extended array (*D*), but is disrupted in *E* and further disrupted in *F*. Prominence of intracellular lysosomes, clumping of nuclear chromatin, and elongation of mitochondria are seen after exposure to λ-carrageenan.

Acknowledgments

I acknowledge the technical assistance of Kathy Walters, Paula Sufficool, and Joe Reardon and support from the Cancer Center, the University of Iowa.

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