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Growth-inhibitory effects of a mineralized extract from the red marine algae, *Lithothamnion calcareum*, on Ca^{2+}-sensitive and Ca^{2+}-resistant human colon carcinoma cells

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ABSTRACT

Proliferation and differentiation were assessed in a series of human colon carcinoma cell lines in response to a mineral-rich extract derived from the red marine algae, *Lithothamnion calcareum*. The extract contains 12% Ca^{2+}, 1% Mg^{2+}, and detectable amounts of 72 trace elements, but essentially no organic material. The red algae extract was as effective as inorganic Ca^{2+} alone in suppressing growth and inducing differentiation of colon carcinoma cells that are responsive to a physiological level of extracellular Ca^{2+} (1.4 mM). However, with cells that are resistant to Ca^{2+} alone, the extract was still able to reduce proliferation and stimulate differentiation.

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1. Introduction

Several epidemiological studies have demonstrated a role for Ca^{2+} in colon cancer chemoprevention [1–5]. Dietary studies in animals are supportive. Several studies have shown that dietary Ca^{2+}-supplementation, alone or in conjunction with vitamin D, reduces colonic epithelial cell abnormalities including areas of hyperplasia and formation of aberrant crypts and raised tubular polyps in healthy animals on a high-fat diet or in carcinogen-exposed animals [6–12]. Cell culture studies provide insight into how Ca^{2+} may exert its chemopreventive activity. In the presence of Ca^{2+} there is a modulation of several proteins that are associated with the proliferation response. Among these are cyclin D1, P27 (Kip1), P21 (WAF1), c-fos, c-myc, c-jun, and members of the TGF-β family [13,14]. In addition, Ca^{2+} is critical for E-cadherin production and membrane localization in epithelial cells, and fosters sequestration of β-catenin at the cell surface along with E-cadherin [15–17]. Since nuclear β-catenin is a transcriptional activator for Wnt pathway (growth-stimulating) signaling [18–21], sequestration at the cell surface could be expected to down-regulate signaling events that lead to proliferation.

Although dietary calcium is an important contributor to health of the colonic mucosa, the degree of Ca^{2+}-induced protection against colon cancer can be described as "modest." Some studies, in fact, have failed to demonstrate any statistically significant protection [22,23]. Identifying additional dietary materials that could be used for colon cancer chemoprevention would have obvious value. In the present study we show that a mineral-rich extract of the red marine algae, *Lithothamnion calcareum*, [24] is effective in suppressing proliferation of human colon cancer cell lines *in vitro*, including cells that are resistant to physiological levels of extracellular Ca^{2+}. The effectiveness of the mineral-rich extract in suppressing growth of Ca^{2+}-resistant as well as Ca^{2+}-sensitive colon carcinoma cells provides a rationale for assessing the effectiveness of this GRAS

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(generally-regarded as safe) natural product as a colon cancer chemopreventative.

2. Materials and methods

2.1. Red marine algae extract

A mineral-rich extract, derived from the red marine algae, *L. calcareum*, was obtained as a gift from Marigot, Limited (Cork, IR). The red algae is harvested from the Atlantic waters off the southwest coast of Ireland and northwest coast of Iceland [24]. The mineralized fronds are separated from extraneous materials, sterilized, dried and milled under ISO and HACCP certification. The mineral extract contains 12% calcium, 1% magnesium, and measurable levels of 72 other trace minerals, including manganese, selenium, copper and zinc. Because the elements accumulated in the algal fronds represent minerals in seawater, there is little variation from batch to batch. The mineral extract is sold as a food supplement under the name Aquamin® (GRAS 000028) and is used in various products for human consumption in Europe, Asia, Australia and North America.

At 12% elemental calcium, the concentration of ionized Ca^{2+} in a solution of 2.5 mg/ml (highest amount used), was calculated to be approximately 6.7 mM. However, a small amount of precipitate was always seen when the extract was solubilized in culture medium. Taking this into account, we estimated the level of available Ca^{2+} in the algae extract to be 5.6 mM at 2.5 mg/ml. Low passage human dermal (neonatal foreskin) fibroblasts were used in a Ca^{2+}-dependent survival assay to validate the Ca^{2+} concentration in the red algae extract. These cells do not survive in culture medium with an extracellular Ca^{2+} concentration below 0.15 mM [25]. Consistent with these past results, cells did not survive in a Ca^{2+}-free, spinner-modified version of Dulbecco's minimal essential medium supplemented with 5% dialyzed fetal bovine serum (SMEM-dFBS) alone, but survived when the Ca^{2+} level (calcium chloride) was raised to a concentration of 0.15 mM. When the algae extract was used in place of calcium chloride, survival was seen at concentrations of approximately 60–70 μg/ml.

2.2. Colon carcinoma cells

Human colon carcinoma cell lines derived from five different tumors (CBS, Moser, Fet, HCT-116 and SW480) were used in the present investigation. A cloned subpopulation of the CBS line referred to as Ca^{2+}-non-responsive variant-1 (NR-1) was also used. All of the cell lines were available from a previous study [26]. Cells were routinely maintained in monolayer culture using SMEM-dFBS and various amounts of the algal extract or calcium chloride as indicated in Section 3. Growth was at 37 °C in an atmosphere of 95% air and 5% CO_2. Cells were subcultured by brief exposure to trypsin/ethylenediamine tetraacetic acid (EDTA) as needed.

2.3. Proliferation assay

Colon carcinoma cells (4×10^4 per well) were added to wells of a 24-well culture dish using SMEM-dFBS and allowed to attach overnight. The next day, cell counts were made to provide precise time-zero cell counts. Varying amounts of the algae extract or calcium chloride were added as indicated in Section 3. After incubation for three days, the cells were harvested by exposure to trypsin/EDTA and counted. Counting was done with an automated particle counter after verifying that the cells were in single cell suspension.

2.4. Cytotoxicity and apoptosis assays

To assess viability after treatment, the cells were incubated under the desired conditions for one day as above and then harvested and counted. Immediately following this, the cells were resuspended and again added to wells of a 24-well dish. All cells were added in the same culture medium, which consisted of DMEM with 10% non-dialyzed FBS. Cells that reattached were counted after 6 h and compared to the number plated in each well. In parallel wells, cells were incubated for an additional 24 h and then counted. Those cells that were able to reattach and proliferate were assumed to be viable.

Apoptosis was assessed by staining the cells with Annexin V-FITC and propidium iodide and analyzing stained cells via flow cytometry [27]. Briefly, cells were grown in SMEM-dFBS alone or in the same medium supplemented with either the algae extract or calcium chloride. After 48 h, cells were washed twice with ice cold PBS and then resuspended in 1× binding buffer (BD Pharmingen, San Diego, CA) at a concentration of 1×10^6 cells/ml. 200 μl of the cell suspension was transferred to wells of a 96 well V bottom plate. Ten μl of Annexin V-FITC (BD Pharmingen, San Diego, CA) and five μl of propidium iodide (Invitrogen Molecular Probes, Carsbad, CA) were added to each well and incubated for 15 min in the dark. Samples were then analyzed by flow cytometry (LSR II, BD Biosciences, San Diego, CA). Data acquisition and analysis were done using BD FACSDiva software.

2.5. Preparation of cell lysates and immunoblot analysis

Cells were plated at 3×10^5 cells per well in 6-well tissue culture dishes and treated as described in Section 3. After three days of incubation, cells were washed and then lysed in 1× cell lysis buffer consisting of 20 mM Tris–HCl (pH 7.4), 2 mM sodium vanadate, 1.0 mM sodium fluoride, 100 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 25 μg/ml each of aprotinin, leupeptin and pepstatin, and 2 mM EDTA and EGTA. Lysis was performed by adding 200 μl of lysis buffer to each well and incubating on ice for 5 min. After incubation, cells were scraped and samples sonicated. Then the extracts were cleared by microcentrifugation at 14,000*g* for 15 min. Supernatants were collected and protein concentrations estimated using the BioRad DC protein assay kit (BioRad, Hercules, CA).

Western blotting for E-cadherin was carried out as described previously [28]. Briefly, samples were separated in SDS–PAGE under denaturing and reducing conditions and transferred to nitrocellulose membranes. After blocking with a 5% nonfat milk solution in Tris-buffered saline with 0.1% Tween (TTBS) at 4 °C overnight, membranes were incubated for 1 h at room temperature with the

desired antibody, diluted 1:1000 in 5% nonfat milk/0.1% TTBS. Thereafter, the membranes were washed with TTBS and bound antibody detected using the Phototope-HRP Western blot detection kit (Cell Signaling Technology, Inc., Danvers, MA). A Kodak – 1000 X-OMAT processor was used to capture the positive images of the Western blots and these positive images were scanned and digitized. The digitized images were quantitated using NIH image analysis software. Membranes were also reprobed with Actin for normalization. The antibody to E-cadherin was from BD Biosciences (San Jose, CA) and antibody to actin was from Santa Cruz Biotech (Santa Cruz, CA).

2.6. Confocal immunofluorescence microscopy

Immunostaining for E-cadherin was done as follows: briefly, cells were grown on uncoated Lab Tek II chamber slides in SMEM containing 5% dialyzed FBS or in the same medium supplemented with either the red algae extract or calcium chloride. After three days, cells were fixed with 4% formaldehyde for 20 min. After fixation, cells were washed 2× with wash buffer (0.05% Tween-20 in Dulbecco's Phosphate Buffered Saline [DPBS]), followed by permeabilization with 0.1% Triton X-100 for 10 min. Cells were again washed and then exposed to a blocking solution consisting of 1% BSA in DPBS for 30 min. Next, cells were treated with antibody to E-cadherin in blocking solution for 1 h. After three subsequent washing steps with DPBS (5 min each), cells were treated with Alexa Fluor 488-conjugated secondary antibody in blocking solution and incubated for 45 min (Invitrogen, Carslbad, CA). Following three additional washing steps, the cells were rinsed one time with water. Coverslips were mounted onto microscope slides with Prolong Anti-fade (Invitrogen). Stained cells were examined with a Zeiss LSM 510 confocal microscope using a 63× (C-Apochr) NA = 1.2 water immersion objective lens. Laser excitation wavelengths included 364, 488 and 543 nm scanned in sequence by the line method.

2.7. Statistical evaluation

Data for most experiments were based on duplicate or triplicate samples. Data from several experiments were pooled and presented as means and standard errors. Since there were multiple groups in each experiment, data were analyzed by ANOVA followed by paired-group comparisons. Differences with either treatment (red algae extract or calcium chloride) were compared to values from controls (absence of either treatment) and to each other. Differences were accepted as significant at $p < 0.05$.

3. Results

3.1. Effects of the mineral-rich red algae extract on proliferation of Ca^{2+}-sensitive and Ca^{2+}-resistant human colon carcinoma (CBS) cells

The upper-left panel of Fig. 1 compares proliferation of parental CBS cells and Ca^{2+}-resistant (NR-1) variant cells in SMEM-dFBS alone and in the same medium supplemented with the algae extract or with calcium chloride. The parental cells responded to a physiological level of calcium chloride (1.4 mM Ca^{2+}) with growth reduction. Consistent with our past reports [28,29], the NR-1 variant cells did not respond to this level of Ca^{2+}. That is, there was no reduction in growth. In contrast to these results, both the parental CBS cells and the NR-1 variant cells responded to the red algae extract. As part of our analysis, we assessed cytotoxicity and apoptosis under the same conditions. Growth suppression in neither cell line was accompanied by a significant increase in cell death with either the red algae extract or calcium chloride at Ca^{2+} concentrations up to 5.6 mM. Cell death, as determined in the stringent replating assay, was less than 7.5% in all cases and the percentage of apoptotic cells was less than 2.5% in all cases (Table 1). Furthermore, when cells were allowed to proliferate (under identical conditions) after exposure to the algae extract or to calcium chloride for one day, the rate of proliferation was similar (not shown).

Fig. 1 also demonstrates features of differentiation in the two CBS isolates in response to the red algae extract or calcium chloride. With the parental cells, both the mineral-rich extract and calcium chloride induced a change in cell shape from spherical to flattened (upper-right panels). Concomitantly, both treatments led to up-regulation and surface localization of E-cadherin (lower-left panels). Staining was more focal and more intense in the differentiating cells (compare staining pattern in C&E with pattern in A) The NR-1 variant cells responded to the high mineral extract (F) but not to calcium chloride (D). As shown in the lower-right panel, E-cadherin protein was increased (as indicated in western blot assays) in conjunction with altered cell surface expression. With the parent cells, up-regulation was seen with either agent. As expected, NR-1 variant cells responded to the red algae extract but not calcium chloride alone.

Based on these observations, we carried out more extensive concentration-dependent studies with NR-1 cells (Fig. 2). Cell growth was inhibited by the algae extract at a concentration of 0.6 mg/ml (equivalent to approximately 1.4 mM Ca^{2+}). In contrast, calcium chloride was ineffective at inhibiting the proliferation of NR-1 cells when used at concentrations below 5.6 mM (Fig. 2).

3.2. Effects of the mineral-rich red algae extract on proliferation of additional human colon carcinoma cell lines

Fig. 3 demonstrates growth-inhibitory effects of the algae extract with four additional human colon cancer cell lines. As controls for this experiment, both a physiological level of Ca^{2+} (1.4 mM calcium chloride) and the maximal amount deliverable in culture medium (5.6 mM) were used. As can be seen from the figure, statistically significant inhibition of proliferation was achieved in three of the lines with the algae extract. Of the three, the least sensitive line (SW480) demonstrated 28% growth inhibition. The most sensitive, HCT-116, demonstrated greater than 70% inhibition under the same conditions. Of the four lines, only Moser demonstrated a statistically-significant growth inhibition in response to calcium chloride (5.6 mM), while none of the lines were inhibited by calcium chloride at 1.4 mM Ca^{2+}. Table 2 provides a summary of growth-inhibition findings with each of the cell lines (including parental and NR-1 CBS cells) and compares the algae extract to calcium chloride.

With each of the cell lines, growth inhibition was accompanied by expression of differentiation features (shape change from spherical to flattened) and by increased E-cadherin production (based on Western blot of whole cell lysates). With SW480, a change in cell shape and up-regulation of E-cadherin were observed with calcium chloride at 5.6 mM Ca^{2+} (not shown) even though significant growth inhibition was not achieved. Thus, in all cases where growth inhibition was observed, features associated with differentiation were also present. In contrast, features of differentiation could be seen even when growth inhibition was not achieved.

4. Discussion

Marine algae (especially members of the coralline family of red algae) [30,31] constitute a rich source of minerals that are accumulated from seawater over the life of the organism. The present study demonstrates that a mineral-rich material derived from the red algae, *L. calcareum*, is capable of suppressing the growth of human colon cancer cell lines *in vitro*. The mineral-rich extract is effective in slowing proliferation and inducing differentiation under conditions in which cytotoxicity is not observed. The algae

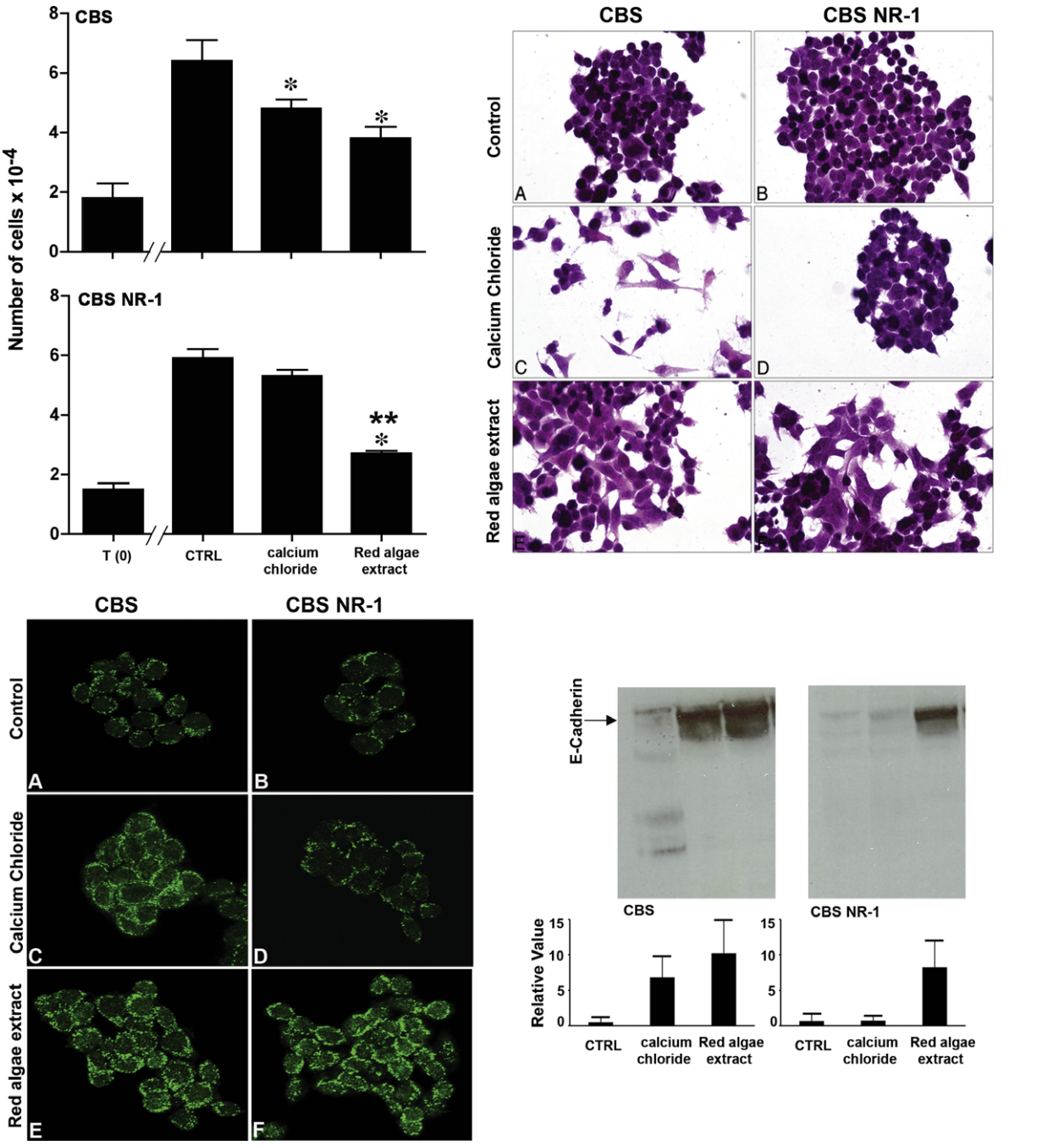

Fig. 1. Effects of a red algae extract on proliferation and differentiation of parental human colon carcinoma (CBS) cells and Ca^{2+}-resistant (NR-1) variant cells. Upper-left panel: cells were treated with the mineral-rich red algae extract (2.5 mg/ml) or with calcium chloride (1.4 mM) and cell numbers were determined after 72 h of incubation. Values represent means and standard errors based on nine independent experiments with both cell types. Statistical significance of the differences was determined by ANOVA followed by paired-group comparisons. *Indicates difference from control at $p < 0.05$. **Indicates difference from calcium chloride alone at $p < 0.05$. Upper right-hand panel: morphology: cells were stained with hematoxylin and eosin after 72 h of incubation. Lower left-hand panel: confocal immunofluorescence microscopy: E-cadherin was assessed after two days of treatment. Lower right-hand panel: western blot for E-cadherin: whole cell extracts made after 3 days of treatment with either the red algae extract or calcium chloride (1.4 mM) were assessed.

extract is as effective as calcium chloride alone in cells that are responsive to a physiological level of extracellular Ca^{2+}. More importantly, however, the algae extract is an effective growth inhibitor with colon cancer cells that do not respond well to physiological levels of Ca^{2+} alone. The findings presented here raise several issues.

One issue, in light of these findings, is the nature of the elements in the algae extract that are responsible for

Table 1
Comparison of the algae extract and calcium chloride for cytotoxicity and for induction of apoptosis in colon carcinoma cells.

Cell lines and conditions	Analysis	
	%Dead (replating)[a]	%Apoptotic (Annexin V)[b]
CBS		
Control	7.4 ± 0.7	1.1
Ca^{2+} (1.4 mM)	7.2 ± 1.1	1.1
Ca^{2+} (5.6 mM)	3.8 ± 0.4	1.9
Algae extract (2.5 mg/ml)	4.4 ± 0.5	2.0
CBS NR-1		
Control	5.5 ± 0.9	1.2
Ca^{2+} (1.4 mM)	5.1 ± 1.0	1.7
Ca^{2+} (5.6 mM)	4.5 ± 0.8	2.0
Algae extract (2.5 mg/ml)	6.9 ± 1.0	2.1

[a] The replating assay assesses the capacity of the cells to reattach to the culture dish after exposure to the algae extract or calcium chloride. Cells were exposed to the red algae extract or to calcium chloride for one day and then assayed. Cells that reattached were assumed to be viable. Values shown are means and standard deviations based on duplicate samples in three independent experiments.

[b] The Annexin V-FITC/propidium iodide is a fluorescence-based cytofluorimetric assay. Values shown are means of three separate experiments.

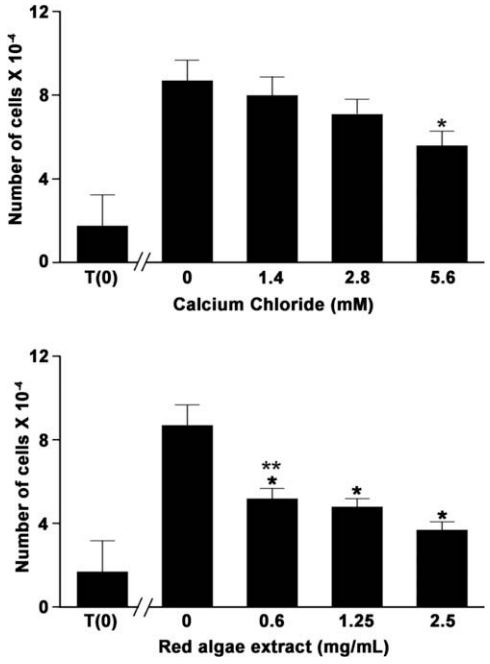

Fig. 2. Dose-responsive suppression of NR-1 proliferation with the red algae extract. Cell counts were made after 72 h of incubation under the indicated conditions. Values represent means and standard errors based on six independent experiments. Statistical significance of the differences was determined by ANOVA followed by paired-group comparisons. *Indicates difference from the control at $p < 0.05$. **Indicates difference from the equivalent concentration of calcium chloride at $p < 0.05$.

inhibition of colon carcinoma cell growth. The data are consistent with the suggestion that Ca^{2+} is an important contributor to the algae extract's effectiveness as a growth-suppressor. However, it is unlikely that the extract is simply a mixture of Ca^{2+} and other inert elements. Colon cancer cells that were resistant to calcium chloride supplementation (up to 5.6 mM Ca^{2+}) responded to the algae extract with growth inhibition at concentrations as low 0.6 mg/ml (equivalent to approximately 1.4 mM Ca^{2+}).

In addition to Ca^{2+}, the algae extract also contains a high level of Mg^{2+}. Recent studies have addressed the role of Mg^{2+} in colon cancer chemoprevention. While some studies have suggested no significant chemopreventive activity as a single variate, other studies have demonstrated a possible relationship between Mg^{2+} intake and reduced colon cancer incidence [32–35]. Recently it has been suggested that the Ca^{2+}:Mg^{2+} ratio is an important consideration [36]. By providing a source of Mg^{2+} as well as Ca^{2+}, the algae extract provides what appears to be a more optimal ratio of Ca^{2+} to Mg^{2+}, and this may have significant advantage over Ca^{2+} alone.

The algae extract is more than just a mix of Ca^{2+} and Mg^{2+}. It also contains measurable levels of 72 different trace elements accumulated from sea water over the lifespan of the algae. Some of these trace elements, such as manganese, selenium, zinc and copper, have been purported to promote health through one mechanism or another. In particular, these metals are critical components of cellular anti-oxidant enzymes [37]. In spite of this, only a few studies have attempted to obtain evidence relating levels of most trace elements with colon cancer rates [38]. How each of the moieties in the algae extract (either alone or in combination) contributes to tumor prevention will need to be investigated.

Are these findings relevant to human colon cancer? Similar to what is seen in individual tumor lines, reduced Ca^{2+}-responsiveness in evolving colon tumor lesions could underlie the loss of growth control in these lesions. Our own past studies have shown that the extracellular calcium-sensing receptor (CaSR), a critical regulator of cellular interactions with extracellular Ca^{2+} [39], is lost from the surface of colon carcinoma cells *in vivo* [26,28,29,40]. We have shown that in the presence of a functional CaSR, Ca^{2+} stimulation leads to E-cadherin production and membrane inclusion. This leads to incorporation of β-catenin in the cell surface-cytoskeletal complex and prevents translocation to the nucleus and Wnt stimulation [28]. Since Wnt pathway signaling is a major inducer of proliferation in colon epithelial cells, the sequestration of β-catenin at the surface during differentiation could be expected to reduce proliferation without being cytotoxic. The lack of a functional CaSR could explain the failure of even high levels of extracellular Ca^{2+} to exert effective chemopreventive activity as noted in some epidemiological studies [22,23]. By virtue of its additional components along with a high level of Ca^{2+}, the algae extract may function either by stimulating CaSR production and expression or by bypassing its requirement altogether. In any case, the expected result would be growth reduction without cytotoxicity. Whether the red algae extract can, in fact, be used under conditions needed for effective chemoprevention in the colon will require long-term studies, first in animals and then in humans. Currently we are in the midst of a 15-month study with rodents on a high fat, "Western style" diet that is known to promote adenoma formation in the animals [10–12]. All that can be said at this time is that a high-fat

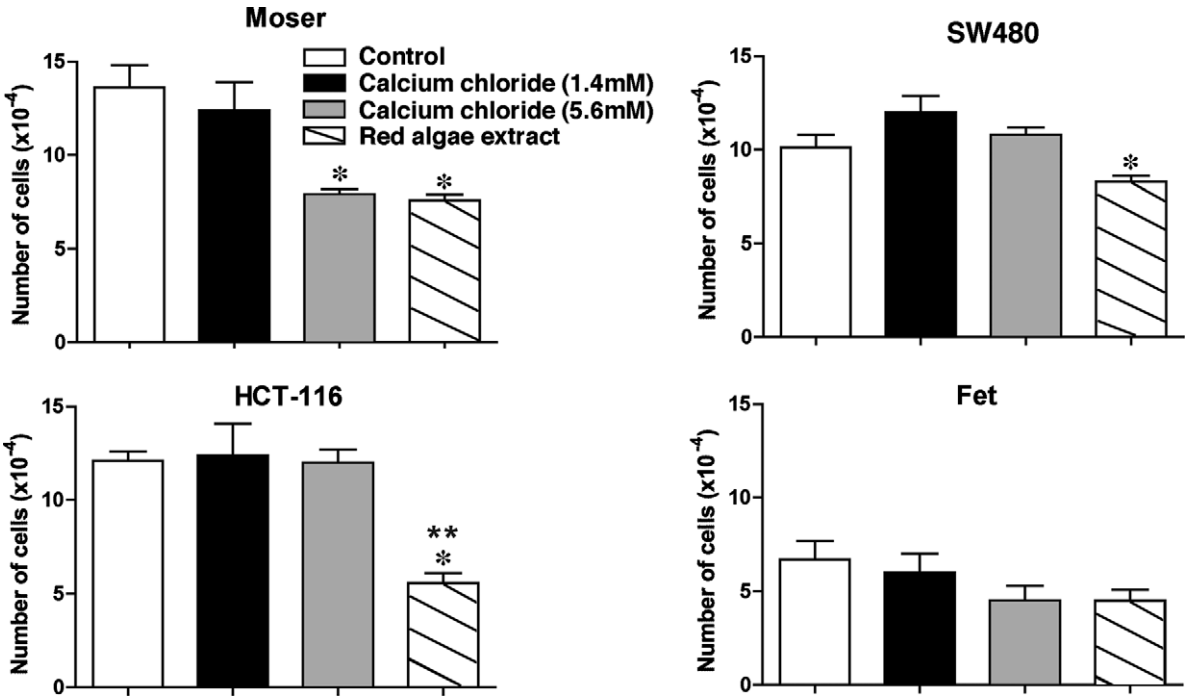

Fig. 3. Effects of a red algae extract on growth of four different human colon carcinoma cell lines. Cell counts were made after 72 h of incubation under the indicated conditions. Values represent means and standard errors based on 3–6 independent experiments with each cell line. Statistical significance of the differences was determined by ANOVA followed by paired-group comparisons. *Indicates difference from the respective control at $p < 0.05$. **Indicates difference from 5.6 mM calcium chloride alone at $p < 0.05$.

Table 2
Comparison of the algae extract and calcium chloride for ability to suppress growth of human colon carcinoma cells.

Cell line	Growth inhibition		
	Red algae extract		Calcium chloride (mM Ca^{2+})
	(mg/ml)	(mM Ca^{2+})	
CBS	1.9	(4.2)	2.5
CBS(NR-1)	2.0	(4.5)	>5.6
Moser	1.9	(4.2)	5.2
HCT-116	1.7	(3.8)	>5.6
Fet	2.5	(5.6)	5.6
SW480	>2.5	(>5.6)	>5.6

Inhibition values are presented as ED_{50}, based on 3–9 different experiments with each cell line. The level of Ca^{2+} in each concentration of the red algae extract is shown in parentheses.

diet supplemented with the *L. calcareum* mineral-rich extract at a level providing a 7-fold increase in Ca^{2+} (as well as the other elements in the extract) is well-tolerated by the animals. After 15 months on the diet, there is no detectable change in the appearance of the animals, no measurable effect on weight gain, and no other gross or metabolic abnormalities that can be attributed to the mineral-rich red algae extract (manuscript in preparation).

In summary, proliferation and differentiation were assessed in a series of human colon carcinoma cell lines in response to a mineral-rich extract derived from the red marine algae, *L. calcareum*. The algae extract was as effective as inorganic Ca^{2+} alone in suppressing growth and inducing differentiation in colon carcinoma cells that are responsive to a physiological level of extracellular Ca^{2+}. However, with cells that are resistant to calcium alone, the red algae extract was still able to reduce proliferation and stimulate differentiation. Based on the findings presented here, the use of the red algae extract in place of extracellular Ca^{2+} alone might be of value as a colon cancer chemopreventative.

5. Conflicts of interest statement

All named authors of the original research paper entitled "Growth-inhibitory effects of a mineralized extract from the red marine algae, *L. calcareum*, on Ca^{2+}-sensitive and Ca^{2+}-resistant human colon carcinoma cells" express that there exist no financial and/or personal relationships with other people and/or organizations that could inappropriately influence their work.

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