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Growth Control in Colon Epithelial Cells: Gadolinium Enhances Calcium-Mediated Growth Regulation

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Abstract

Gadolinium, a member of the lanthanoid family of transition metals, interacts with calcium-binding sites on proteins and other biological molecules. The overall goal of the present investigation was to determine if gadolinium could enhance calcium-induced epithelial cell growth inhibition in the colon. Gadolinium at concentrations as low as 1–5 μM combined with calcium inhibits proliferation of human colonic epithelial cells more effectively than calcium alone. Gadolinium had no detectable effect on calcium-induced differentiation in the same cells based on change in cell morphology, induction of E-cadherin synthesis, and translocation of E-cadherin from the cytosol to the cell surface. When the colon epithelial cells were treated with gadolinium and then exposed to increased calcium concentrations, movement of extracellular calcium into the cell was suppressed. In contrast, gadolinium treatment had no effect on ionomycin-induced release of stored intracellular calcium into the cytoplasm. Whether these in vitro observations can be translated into an approach for reducing abnormal proliferation in the colonic mucosa (including polyp formation) is not known. These results do, however, provide an explanation for our recent findings that a multi-mineral supplement containing all of the naturally occurring lanthanoid metals including gadolinium are more effective than calcium alone in preventing colon polyp formation in mice on a high-fat diet.

Keywords

Calcium; Gadolinium; Colon; Differentiation; Cytotoxicity

Introduction

Past epidemiological studies [1–6] and interventional studies [7, 8] in human subjects have demonstrated the capacity of calcium to reduce colon polyp formation and reduce colon cancer risk. Animal studies have also demonstrated the protective activity of calcium supplementation with regard to colon polyp formation [9, 10]. In vitro studies have demonstrated the growth-regulating activity of calcium in a variety of epithelial cells and have provided insights into growth-suppressing mechanisms [11–17]. Although calcium has demonstrable chemopreventive activity against colon polyp formation, protection provided by calcium alone can be described as modest. It is estimated that under conditions of optimal use, a reduction in polyp incidence of 20–22 % might be achieved [18]. Additional interventions are needed.

In a recent study, we demonstrated that a multi-mineral-containing natural product derived from the skeletal remains of the red marine algae, *Lithothamnion calcareum*, suppressed colon polyp formation in mice on a high-fat diet more effectively than did calcium alone [19]. What accounts for the greater effectiveness of the multi-mineral product as compared to calcium alone was not addressed in the in vivo studies. Likewise, what minerals in the multi-mineral product are most important to growth suppression could not be determined using the complex in vivo model.

The lanthanoid metals constitute one potentially important group of elements in the multi-mineral product. The lanthanoid metals are a family of transition cationic metals. Certain lanthanoids, especially gadolinium, have an orbital size and configuration similar to that of calcium but a higher overall charge density [20, 21]. As a result, gadolinium (and other lanthanoids) interacts with calcium-binding sites on several important regulatory molecules [22–28]. In a recent study, we demonstrated that while the proliferation of human dermal fibroblasts was increased by gadolinium [29], gadolinium had no growth-stimulating activity for keratinocytes over the same concentration range but it decreased keratinocyte growth at high concentrations (50–100 μ M). The present study continues our effort to understand how the lanthanoid metals influence epithelial cell function. Here, we demonstrate that while micromolar amounts of gadolinium are ineffective in the absence of calcium, combinations of gadolinium and calcium suppress proliferation in human colonic epithelial cells more effectively than calcium alone. These results provide, perhaps, an explanation for the findings (noted above) that a mineral supplement containing calcium and all of the naturally occurring lanthanoid metals including gadolinium is more effective than calcium alone in preventing colon polyp formation in mice on a high-fat diet [19].

Materials and Methods

Reagents

Calcium-free minimal essential medium (SMEM) was obtained from Sigma Chemical Co., St. Louis, MO. It was supplemented with 10 % dialyzed (<10,000 molecular weight cutoff) fetal bovine serum from Thermo Scientific HyClone (Ogden, UT) (SMEM-dFBS). Gadolinium was obtained from Sigma Chemical Company as the chloride salt. Magnevist (Berlex Imaging), one of the gadolinium-based contrast agents used in magnetic resonance imaging, was obtained from In-Patient Pharmacy at the University of Michigan Hospitals. Magnevist is the *N*-methylglucamine salt of the gadolinium complex with the chelator, diethylenetriaminepentaacetic acid. Due to the effective chelation of the gadolinium ion, the chelated metal remains soluble in the face of the numerous anions present in biological fluids.

Human Colon Carcinoma Cells

Four different human colon epithelial cell lines (HCT-116, FET, SW480, and CBS) were used in the present investigation. Each of the lines was originally obtained from a moderately differentiated colon carcinoma, and all were available from previous studies in our laboratory [14, 15]. The cells were routinely maintained in monolayer culture using SMEM-dFBS as the culture medium. Incubation was at 37 °C in an atmosphere of 95 % air and 5 % CO₂. Cells were subcultured by brief exposure to trypsin and ethylenediaminetetraacetic acid (EDTA) as needed.

Proliferation

Proliferation studies were carried out as follows: Briefly, cells were plated in wells of a 24-well dish at 4×10^4 cells per well using SMEM-dFBS as the culture medium and allowed to attach. The cells were then gently washed two times with 1 ml of Dulbecco's phosphate-

buffered saline (DPBS). Duplicate wells were counted to provide accurate “zero-time” values. One milliliter of SMEM-dFBS was added to each remaining well. The desired amounts of calcium and/or gadolinium were then added. Incubation was up to 3 days at 37 °C in an atmosphere of 95% air and 5% CO₂. At the end of the incubation period, cells were harvested with trypsin–EDTA and counted using an electronic particle counter.

Cytotoxicity and Apoptosis

To assess viability, cells were incubated with calcium and/or gadolinium for a desired period of time and then harvested and counted. Immediately following this, cells were stained with Annexin V-FITC and propidium iodide and analyzed via flow cytometry as described previously [29]. For this, 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, and 10 µl of Annexin V-FITC (BD Pharmingen, San Diego, CA) and 5 µl of propidium iodide (Invitrogen Molecular Probes, Carlsbad, 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.

In parallel, cells were incubated under the same conditions and then harvested in the normal manner. The harvested cells were washed two times in DPBS, counted, and then added to wells of a 24-well dish in growth medium (SMEM-dFBS). Eighteen hours later, attached cells were harvested and counted as above. Trypan blue staining was done to eliminate nonviable cells. The number of viable cells recovered at each condition was compared to the number recovered from control wells (i.e., maintained throughout the original incubation period in SMEM-dFBS).

E-cadherin Analysis: Preparation of Cell Lysates and Immunoblotting

Cells were plated at 3×10^5 cells per well in six-well tissue culture dishes and allowed to attach. Following attachment, the cells were incubated for 1 day in SMEM-dFBS, with the desired amount of calcium and/or gadolinium as described in “Results.” The next day, cultures 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 the plate on ice for 5 min. After incubation, cells were scraped and samples were sonicated. Then, the extracts were cleared by microcentrifugation at $14,000 \times g$ for 15 min. Western blotting for E-cadherin was carried out. 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 anti-E-cadherin antibody (MAB 3197; Millipore-Chemicon, Temecula, CA), diluted 1:1,000 in 5 % nonfat milk/TTBS. Thereafter, the membranes were washed with TTBS, and bound antibody was detected using the Phototope-HRP Western blot detection kit (Cell Signaling Technologies, Inc., Beverly, MA). Images were scanned, digitized, and quantified. Prior to loading the gels, protein levels in each sample were determined using the BCA protein determination kit (Pierce Biotechnology, Rockford, IL) and equal amounts of protein were loaded onto each lane. In some experiments, the Ponceau S reversible staining solution was used to verify equal protein transfer from the gels to the Western blot membranes. An anti-β-catenin antibody (04–1002, Millipore-Chemicon), diluted 1:1,000, was used to detect β-catenin in the same lysates as a control.

E-cadherin Analysis by Confocal Immunofluorescence Microscopy

Cells were added to Lab-Tek II chamber slides and allowed to attach. Following attachment, the cells were incubated in SMEM-dFBS with calcium and/or gadolinium. The next day, cultures were washed and then fixed with 4 % formaldehyde for 20 min. After fixation, cells were washed 2× with wash buffer (0.05 % Tween-20 in DPBS), followed by permeabilization with 0.1 % Triton X-100 for 5 min. Cells were again washed and then exposed to a blocking solution consisting of 1 % bovine serum albumin in DPBS for 30 min. Next, cells were treated with monoclonal 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 rabbit antimouse IgG antibody (Invitrogen, Carlsbad, CA) in blocking solution and incubated for 30 min. Following three additional washing steps, the cells were treated with Alexa Fluor 488-conjugated goat anti-rabbit IgG antibody in the same solution to amplify the fluorescence signal. Finally, the cells were rinsed once with wash buffer and mounted with ProLong Gold Anti-fade Medium containing DAPI (Invitrogen). 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.

Intracellular Free Calcium Measurement

Intracellular calcium levels were assessed in HCT-116 cells by the change in the fluorescence intensity of fluo-3 [30, 31]. Fluo-3 acetoxymethylester (fluo-3 AM) was purchased from Molecular Probes (Invitrogen, OR, USA). Cells were grown in SMEM-dFBS, washed twice with DPBS (Ca²⁺- and Mg²⁺-free) and suspended at 1×10⁶ cells per ml. For loading with 4 μM fluo-3 AM, cells were incubated in the dark for 45 min at 37 °C. After incubation, cells were pelleted by centrifugation at 300 × *g* for 5 min, washed twice in DPBS, and resuspended at 5×10⁶ cells/ml in SMEM-dFBS. The resulting cell suspensions were kept in the dark at room temperature until use. Fluo-3 fluorescence was measured using BD Biosciences LSR II flow cytometer at 488-nm excitation and 515–545-nm emissions. The dye-loaded cells were heated to 37 °C and incubated for 1 min. Then, the dye-loaded cells were treated with different concentrations of calcium, as mentioned in “Results” and immediately examined in the flow cytometer. Twenty thousand events were collected (approximately 30-s time frame). To determine the effects of gadolinium on this response, cells were incubated with gadolinium for varying periods of time before adding calcium. In all the experiments, auto-fluorescence was assessed in control cells (not exposed to the dye). The maximum fluo-3 fluorescence intensity ($F_{\max}$) in HCT-116 cells was determined by adding 1 μM Ionomycin (Sigma, St. Louis, MO), and the minimum fluorescence ($F_{\min}$) was determined following the depletion of external calcium with the addition of 20 mM EGTA. Intracellular free calcium [Ca²⁺]_i was calculated according to the equation $[Ca^{2+}]_i = K_d (F - F_{\min}) / (F_{\max} - F)$, where K_d denotes the apparent dissociation constant (=392 nM) of the fluorescence dye–calcium complex.

Statistical Analysis

Data were analyzed using one-way analysis of variance (ANOVA) followed by the *t* test for selected pairs. Data were considered significant at $p < 0.05$. Asterisks have been added to the appropriate data in the figures and tables to denote values that are significantly different from the respective control value.

Results

Colon Epithelial Cell Growth in the Presence of Different Concentrations of Calcium and Gadolinium

In the first set of experiments, each of the four colon epithelial cell lines was incubated for 72 h in SMEM-dFBS with different levels of extracellular calcium (1.5, 3.0, or 4.5 mM) with or without gadolinium. As can be seen in Fig. 1, there was a fall off in cell number with increasing calcium concentration. In all cases, however, cell numbers at 72 h were still higher than zero-time counts. It can also be seen from the figure that when the cells were exposed to 25 or 50 μ M gadolinium in the absence of added calcium, there was minimal effect. However, when gadolinium was included along with millimolar amounts of calcium, there was a further reduction in cell number as compared to calcium alone. Cell numbers fell in some cases to levels below baseline, indicating a cytotoxic effect.

The HCT-116 and SW480 cell lines were used to establish a dose response for gadolinium-mediated growth suppression in the presence of calcium. With both cell lines, concentrations as low as 1 μ M were sufficient to see growth suppression, and statistically significant reductions were observed at 5 μ M (Table 1).

Since gadolinium rapidly forms insoluble precipitates with phosphate, carbonate, and other anions in biological fluids [32], experiments were conducted in which a chelated gadolinium compound (Magnevist; i.e., gadolinium diethylenetriaminepentaacetic acid *N*-methylglucamine) was used in place of gadolinium chloride. Similar to what we observed with gadolinium chloride, the chelated gadolinium compound (250 and 500 μ M) induced a decrease in the number of colon epithelial cells when combined with millimolar amounts of calcium (Fig. 2).

Colon Epithelial Cell Differentiation in the Presence of Calcium and Gadolinium

In the colonic epithelium, growth suppression is associated with differentiation [11, 14, 15, 17]. Colon epithelial cells (HCT-116) were exposed to different concentrations of calcium and gadolinium for 48 h and assessed for differentiation. Increasing the calcium concentration from basal to 1.5 mM led to an increase in E-cadherin production (Western blotting of whole cell lysates) (Fig. 3) and to a shift in E-cadherin distribution from the cytoplasm to the cell surface (confocal fluorescence microscopy) (Fig. 4). Gadolinium had no apparent effect on E-cadherin production or distribution under basal conditions or in the presence of 1.5 mM calcium. At 3.0 mM calcium, there was no further increase in E-cadherin production or distribution over that seen at 1.5 mM and also no increase when gadolinium was combined with calcium at 3.0 mM. Quite the contrary, the level of immunoreactive E-cadherin (Western blotting) decreased in response to the combination of 3.0 mM calcium and 50 μ M gadolinium. Accompanying the change in E-cadherin distribution was a change in cell morphology from round to flattened (Fig. 4). When each of the other three cell lines was examined in the same manner, results were similar to those presented with HCT-116 cells. This indicates that there was no change in E-cadherin synthesis or distribution between cytosol and surface and no change in cell shape over that occurring in response to 1.5 mM calcium alone. Taken together, these studies suggest that gadolinium, by itself, has little effect on the differentiation process and neither interferes with nor enhance calcium-induced differentiation in these cells.

Colon Epithelial Cell Cytotoxicity in the Presence of Calcium and Gadolinium

Combinations of gadolinium and calcium led to a reduction in cell growth such that by 72-h, the cell number was below the starting point. This indicates that cytotoxicity accounted for at least some of the combined effect. A number of experiments were carried out to directly

assess the cytotoxic effects of the calcium–gadolinium combination. HCT-116 cells were exposed to combinations of calcium and gadolinium for 5 h. After harvest, cells were stained with Annexin V and propidium iodide and examined using a flow cytometry-based assay for cell death and apoptosis. In parallel, washed cells were suspended in growth medium (SMEM-dFBS) and replated. Twenty-four hours later, viable cells from the replating assay were harvested and counted. As seen in the section A of Table 2, calcium alone at 1.5 or 3.0 mM induced only a slight increase (not statistically significant) in the percentage of nonviable cells. There was also no increase in the number of apoptotic cells. Whether or not gadolinium was present did not alter either parameter. The section B of the table shows results from the replating assay. The inclusion of gadolinium in the culture medium along with calcium had no significant effect on cell viability after 5 h. After 18 h, calcium alone (up to 3.0 mM) did not reduce cell number. In contrast, when cells were examined in the replating assay after 18 h of treatment, there was a significant decrease in cell number in the combination of calcium plus 50 μ M gadolinium as compared to calcium alone at either 1.5 or 3.0 mM.

Intracellular Calcium Changes in Response to External Calcium and Gadolinium

In a final set of experiments, intracellular calcium levels were assessed in HCT-116 cells under basal conditions (SMEM-dFBS) and in response to calcium and gadolinium. Figure 5 demonstrates that the intracellular calcium level increased from approximately 16 nM under basal conditions to 120 and 174 nM in the presence of 1.5 and 3.0 mM extracellular calcium. Exposure of cells in basal medium to gadolinium alone did not increase intracellular calcium. When gadolinium (50 μ M) was combined with extracellular calcium (1.5 or 3.0 mM) and the intracellular calcium level assessed immediately, there was no effect over that seen with calcium alone. However, when HCT-116 cells were exposed to gadolinium (50 μ M) in basal medium for 5 h prior to the addition of calcium, calcium entry into the cells was decreased compared to what was seen with calcium alone (Fig. 5). In contrast to the effects of gadolinium on calcium entry from the exterior, treatment of the cells with gadolinium for a 5-h period had little effect on the release of calcium from intracellular stores in response to ionomycin— $[Ca^{2+}]_i = 586$ nM in SMEM-dFBS without gadolinium and 534 nM in the presence of 50 μ M gadolinium.

Discussion

To summarize the results from the current study, gadolinium at concentrations up to 50 μ M had no effect on proliferation or differentiation under basal conditions (i.e., in SMEM-dFBS) in any of four human colon epithelial cell lines. However, when the calcium concentration was increased to 1.5, 3.0, and 4.5 mM, the cells underwent progressive growth suppression. With all four cell lines, growth suppression was increased by the combination of calcium and gadolinium over that seen in the presence of calcium alone. Gadolinium concentrations as low as 1–5 μ M were effective. Increasing the calcium concentration from basal level to 1.5 mM also induced a strong differentiation response as indicated by a change in cell shape from round to flattened, by increased E-cadherin production, and by translocation of E-cadherin from the cytoplasm to the cell surface. Neither higher calcium concentrations (3.0 and 4.5 mM) nor combinations of calcium (3.0–4.5 mM) and gadolinium (50 μ M) had additional effects on differentiation over that seen in the presence of 1.5 mM calcium alone. Taken together, these data indicate that the lanthanoid element can modulate growth without affecting differentiation.

Two questions are raised by this work. First is the potential to exploit these findings for improved growth control in the colon, and second is the mechanism(s) of enhanced growth inhibition in the presence of gadolinium. In regard to the first question, calcium (as noted above) has colon polyp chemopreventive activity, but alone, it is only modestly effective

[18]. Could a combination of calcium and gadolinium provide the basis for a more effective colon polyp chemoprevention strategy? This remains to be seen. A past study has shown that nifedipine, a calcium channel blocker used clinically in patients with cardiac disease, synergizes with calcium to induce apoptosis in the same colonic epithelial cells as used here [34]. The clinically used calcium channel blockers have significant side effects in their own right and would be unlikely to be used systemically as part of a long-term cancer preventive strategy. The gastrointestinal tract, however, provides a unique opportunity for intervention without systemic exposure. This would be especially applicable for a cationic substance such as gadolinium that would be unlikely to cross the gastrointestinal barrier to become systemic. In support of such an approach, we have recently shown that a natural product containing calcium and a number of trace elements (including all of the naturally occurring lanthanoids) was more effective than calcium alone in suppressing outgrowth of adenomatous polyps in C57BL/6 mice on a high-fat diet over an 18-month period [19]. We are currently assessing a combination of calcium and a mix of lanthanoids (formulated to include the same concentrations as found in the natural product) for colon polyp suppression in the same long-term animal model. Success in the animal model would support subsequent studies in human subjects at risk for polyp formation.

Gadolinium was examined here because this lanthanoid element has an orbital size and configuration similar to that of calcium but a higher overall charge density [20, 21]. While the current studies utilized gadolinium alone for “proof-of-concept,” other cationic metal ions, either alone or in combination, might prove to be more effective. This question would be difficult to address in an animal model, especially one that is inherently long term. However, the in vitro approach used in this manuscript should provide a way to assess multiple individual metals or combinations of metals for ability to synergize with calcium in suppression of colon epithelial cell growth. Currently, such studies are in process.

Regarding potential mechanisms of action, our studies showed that raising the calcium level to 1.5 mM induced differentiation (consistent with past reports [12–15, 17]). Raising the calcium further (with or without gadolinium) had no additional differentiation-inducing activity over that seen with calcium alone at 1.5 mM. In contrast, growth suppression increased with calcium alone up to 4.5 mM, and inclusion of gadolinium along with calcium enhanced growth inhibition at all three calcium concentrations (1.5, 3.0, and 4.5 mM). Gadolinium concentrations as low as 1–5 μ M were effective. Growth-suppressing combinations of calcium and gadolinium induced cytotoxicity in a fraction of the cells. Calcium, by itself, participates in numerous signaling events that modulate cell proliferation and differentiation [12–15] as well as cell death through both apoptotic and nonapoptotic mechanisms [33, 34 for reviews]. Since in the absence of calcium, gadolinium alone had little effect on cell function, our hypothesis is that gadolinium acts in some way to sensitize the target cells to calcium. This is consistent with the known action of gadolinium on numerous calcium-regulatory molecules [22–28].

CaSR is well established as a key calcium regulator in colonic epithelial cells. Our previous studies have demonstrated that calcium-induced growth suppression depends on the activation of CaSR [14, 15, 17]. This is associated with differentiation and depends on a transient rise in the level of intracellular calcium [35]. Based on these past data, we hypothesized that exposure of the colonic epithelial cells to gadolinium in conjunction with calcium would induce a rise in intracellular calcium over that seen with calcium alone. Our experiments failed to substantiate this, however. Quite the contrary, pretreatment with gadolinium reduced the subsequent influx of extracellular calcium that occurred in control cells exposed to 1.5 or 3.0 mM calcium. Ability of exogenous gadolinium to prevent extracellular calcium from entering the cell is consistent with calcium channel-blocking activity. Previous studies have shown that gadolinium interferes with receptor-gated calcium

channels, as well as voltage-gated and mechanical stress-activated channels in a variety of cells [22, 24, 36, 37]. While some studies have suggested that limiting the rise in intracellular calcium might prevent apoptosis and promote cancer development, most studies have demonstrated that blocking calcium movement into the cell induces cell death [reviewed in 32]. In the colon epithelial cells studied here, interference with calcium movement is clearly associated with reduced cell growth. That this relationship is cell specific is indicated by the almost contradictory findings in human dermal fibroblasts, where gadolinium is a potent growth inducer [29, 38–42].

In summary, a role for calcium in epithelial growth control is well established in the colon (as well as in other tissues). This is based on in vitro studies as well as on in vivo findings in both humans and in experimental animals. Calcium suppresses colonic epithelial cells, in part, by inducing a differentiation response. At higher calcium concentrations, processes that lead to cell death by apoptotic and nonapoptotic mechanisms are engaged. The present studies suggest that gadolinium enhances growth inhibitory/cytotoxic responses of colon epithelial cells to calcium but does not affect calcium-induced differentiation, per se. Whether the combination of calcium and gadolinium will, ultimately, prove to be useful as a colon cancer chemopreventive agent remains to be seen.

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Fig. 1. Effects of calcium and gadolinium on growth of human colon epithelial cells. Values shown are means and standard errors based on triplicate experiments with each line. * $p < 0.05$, compared to calcium alone at the same concentration

Fig. 2. Effects of calcium and Magnevist on growth of human colon epithelial cells. Values shown are means and standard errors based on triplicate experiments with each line. * $p < 0.05$, compared to calcium alone at the same concentration

Fig. 3.

E-cadherin expression in colon epithelial cells. **a** Lysates were prepared from HCT-116 cells and assessed by Western blotting for E-cadherin expression 48 h after exposure to the indicated conditions. **b** β -catenin served as a control. Lysates from three different HCT-116 preparations were assessed with virtually identical results

Fig. 4. E-cadherin expression in colon epithelial cells. HCT-116 cells were stained 48 h after exposure to the indicated conditions and examined by fluorescence confocal microscopy. **a** SMEM-dFBS, **b** SMEM-dFBS plus 50 μ M gadolinium, **c** SMEM-dFBS plus 1.5 mM calcium, **d** SMEM-dFBS plus 1.5 mM calcium and 50 μ M gadolinium, **e** SMEM-dFBS plus 3.0 mM calcium, and **f** SMEM-dFBS plus 3.0 mM calcium and 50 μ M gadolinium

Fig. 5. Intracellular calcium changes in response to calcium and gadolinium. Fluo-3 fluorescence was used to estimate intracellular calcium concentrations ($[Ca^{2+}]_i$) in HCT-116 cells. Values shown represent mean $[Ca^{2+}]_i$ along with standard errors based on three to five separate experiments for each data point. Values with 5-h gadolinium pretreatment were statistically significant at $p < 0.05$ relative to control. *Inset:* Histogram from a study showing the fluorescence values in HCT-116 cells under basal conditions versus 3.0 mM calcium and with a 5-h pretreatment with gadolinium

Table 1

Effects of calcium alone and calcium plus gadolinium at different concentrations on proliferation of HCT-116 cells and SW480 cells

Conditions	Number of cells $\times 10^{-4}$	
	HCT-116	SW480
1.5 mM calcium	13.2 $\pm$ 0.1	14.4 $\pm$ 0.2
3.0 mM calcium	9.9 $\pm$ 0.1	9.8 $\pm$ 0.2
+1 μ M Gd ³⁺	9.3 $\pm$ 0.2	9.6 $\pm$ 0.1
+5 μ M Gd ³⁺	7.5 $\pm$ 0.3 *	8.2 $\pm$ 0.1 *
+10 μ M Gd ³⁺	4.0 $\pm$ 0.1 *	7.9 $\pm$ 0.3 *
+25 μ M Gd ³⁺	1.3 $\pm$ 0.1 *	3.9 $\pm$ 0.1 *
+50 μ M Gd ³⁺	0.8 $\pm$ 0.1 *	1.9 $\pm$ 0.2 *
4.5 mM calcium	6.1 $\pm$ 0.2	6.4 $\pm$ 0.1
+1 μ M Gd ³⁺	6.0 $\pm$ 0.1	6.0 $\pm$ 0.3
+5 μ M Gd ³⁺	5.4 $\pm$ 0.4 *	5.9 $\pm$ 0.2
+10 μ M Gd ³⁺	4.8 $\pm$ 0.1 *	5.9 $\pm$ 0.1
+25 μ M Gd ³⁺	2.0 $\pm$ 0.2 *	4.0 $\pm$ 0.2 *
+50 μ M Gd ³⁺	1.7 $\pm$ 0.1 *	2.6 $\pm$ 0.4 *

Values shown are means and standard deviations based on a single experiment with three samples per data point for each condition. Statistical significance of the data was assessed by ANOVA, followed by paired-group comparisons. The experiment was repeated three times with similar results

* $p < 0.05$ (statistically significant decrease relative to controls)

Table 2

Assessment of cytotoxicity in HCT-116 cells treated with calcium and gadolinium

Group	A: Annexin V-propidium iodide			B: Replating		
	% live	% Dead	% Apoptotic	Number of viable cells ($\times 10^{-4}$)		
	5 hours			5 hours	18 hours	
SMEM + dFBS	96.2 $\pm$ 1.2	2.9 $\pm$ 1.0	0.7 $\pm$ 0.2	3.2 $\pm$ 0.9	3.9 $\pm$ 1.3	
+1.5 mM calcium	92.0 $\pm$ 2.9	4.6 $\pm$ 1.2	0.9 $\pm$ 0.1	4.6 $\pm$ 0.7	6.1 $\pm$ 0.6	
+50 μ M Gd ³⁺	92.1 $\pm$ 2.6	6.5 $\pm$ 1.9	1.0 $\pm$ 0.6	4.2 $\pm$ 1.1	3.2 $\pm$ 0.4 *	
+3.0 mM calcium	91.8 $\pm$ 1.6	6.6 $\pm$ 1.1	1.1 $\pm$ 0.9	4.4 $\pm$ 1.1	6.1 $\pm$ 0.7	
+50 μ M Gd ³⁺	91.6 $\pm$ 2.9	7.0 $\pm$ 2.8	0.8 $\pm$ 0.3	4.9 $\pm$ 0.6	2.5 $\pm$ 0.7 *	

Values shown are means and standard deviations based on duplicate experiments with three samples per data point for each condition in each experiment. Statistical significance of the data in the replating assay was assessed by ANOVA, followed by paired-group comparisons

* $p < 0.05$ (statistically significant decrease relative to controls)