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Regulation of E-cadherin and β-catenin by Ca^{2+} in colon carcinoma is dependent on calcium-sensing receptor expression and function

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An siRNA directed against the extracellular calcium-sensing receptor (CaSR) was used to down-regulate this protein in CBS colon carcinoma cells. In additional studies, we utilized a variant of the parental CBS line that demonstrates CaSR expression but does not upregulate this protein in response to extracellular Ca^{2+}. In neither the siRNA-transfected cells nor the Ca^{2+}-nonresponsive variant cells did inclusion of Ca^{2+} in the culture medium inhibit proliferation or induce morphological alterations. Extracellular Ca^{2+} also failed to induce E-cadherin production or a shift in β-catenin from the cytoplasm to the cell membrane. In mock-transfected cells and in a Ca^{2+}-responsive variant line derived from the same parental CBS cells, Ca^{2+} treatment resulted in growth-reduction. This was accompanied by increased E-cadherin production and a shift in β-catenin distribution from the cytoplasm to the cell membrane. Additionally, down-regulation of c-myc and cyclin D1 expression was observed in mock-transfected cells and in the Ca^{2+}-responsive variant line (along with reduced T cell factor transcriptional activation). Neither c-myc nor cyclin D1 was significantly down-regulated in the siRNA-transfected cells or in the Ca^{2+}-nonresponsive variant cells upon Ca^{2+} stimulation. In histological sections of human colon carcinoma CaSR was significantly reduced as compared to the level in normal colonic crypt epithelial cells. Where CaSR expression was high, strong surface staining for E-cadherin and β-catenin was observed. Where CaSR expression was reduced, β-catenin surface expression was likewise reduced.

Key words: β-catenin; calcium sensing receptor

Extracellular Ca^{2+} is a growth-regulator and cancer chemopreventative in the colon.[1–6] *In vitro* studies have demonstrated that maintenance of human colon carcinoma cells in Ca^{2+}-free medium allows the cells to remain loosely attached to the substratum and to one another and promotes proliferation. When extracellular Ca^{2+} (1.4 mM, final concentration) is included in the culture medium, growth slows, the cells take on a flattened appearance and behave as a cohesive epithelial unit.[7] How extacellular Ca^{2+} acts to down-regulate proliferation and alter morphological and adhesive features in colon carcinoma cells is not fully understood. Recent studies provide evidence that the extracellular Ca^{2+}-sensing receptor (CaSR) is required, based on the demonstration of reduced expression of this protein in colon carcinoma tissue as compared to normal colonic mucosa.[8–10] In a recent study with a colon carcinoma cell line (CBS), we isolated a subpopulation of cells that expressed CaSR immunochemically, but unlike the parental cells, did not upregulate CaSR upon exposure to extracellular Ca^{2+}. In these cells, Ca^{2+} failed to suppress growth.[7] The Ca^{2+}-treated cells also failed to express morphological changes (increased flattening and increased cell–cell contact) associated with Ca^{2+} treatment in the parental cells.

The present study continues our efforts to elucidate the role of CaSR expression and function in colon cancer cell biology. Here we present evidence using an siRNA directed against the CaSR as well as the variant cells with the Ca^{2+}-nonresponsive CaSR that in the absence of a functional CaSR, extracellular Ca^{2+} does not upregulate E-cadherin or induce cell surface accumulation of E-cadherin and β-catenin as it does in parental or mock-transfected CBS cells. Cells lacking a functional CaSR continue to proliferate and fail to flatten out or form cell–cell contacts as the parental cells normally do in the presence of extracellular Ca^{2+}.

Materials and methods

Cell culture

The parental CBS colon carcinoma cell line and 2 subpopulations referred to as Ca^{2+}-responsive (R1) and Ca^{2+}-nonresponsive (NR1) variants were available from previous studies.[7] The parental cells and the 2 variant lines were routinely maintained in Ca^{2+}-free Spinner modified minimal essential medium (SMEM, Sigma Aldrich, St. Louis, MO) supplemented with nonessential amino acids and 5% dialyzed fetal bovine serum (FBS). Growth was at 37°C in an atmosphere of 5% CO_2 and 95% air. Cells were subcultured by brief exposure to trypsin as required.

SiRNA treatment

A hairpin siRNA targeting the 3′ end of the CaSR gene transcript was used to down-regulate CaSR (Dharmacon RNA Technologies, Lafayette, CO). Transfection of the parental CBS cells with the siRNA was done using the GeneSilencer siRNA Transfection Reagent (Genlantis, San Diego, CA) according to the manufacturer's instructions. Briefly, cells were plated in 6-well tissue culture dishes at a density of 3×10^5 cells per well and transfected with 1 μg of siRNA. Mock transfection using the same reagent and a nonspecific control sequence was performed with each experiment. After transfection, cells were incubated at 37°C in an atmosphere of 95% air and 5% CO_2 in SMEM containing 5% FBS and allowed to recover for 2 days before further use in experiments. At this point, cells were plated on Lab Tek II chamber slides and incubated for a further 3 days in SMEM + 5% dialyzed FBS with or without the addition of 1.4 mM Ca^{2+} to the medium. After this incubation, cells were examined by immunofluorescence for expression of β-catenin as described later. Lysates were prepared from cells treated the same way and analyzed for CaSR and E-cadherin by Western blot.

T cell factor transcriptional activity

Constitutive T cell factor transcriptional activation activity (CTAA) was measured in R1 cells and in NR1 cells with a dual luciferase reporter assay (Promega, Madison, WI) as a ratio of luciferase activity from the pTOPFLASH vector to the pFOPFLASH vector.[9] All luciferase activities were normalized for transfection efficiency by cotransfection with pRL Renilla luciferase vector. Briefly, cells in 6-well culture plates were transfected with pTopFlash/pRL Renilla or pFopFlash/pRL Renilla and allowed to

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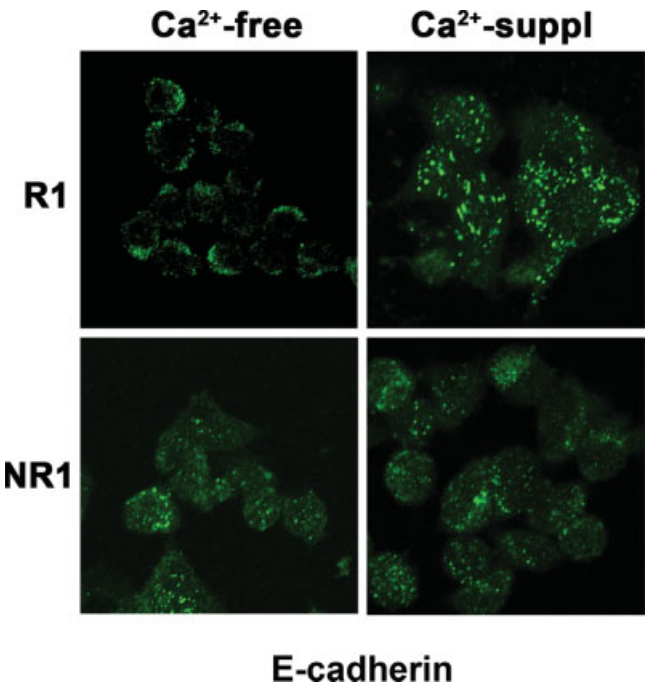

FIGURE 1 – E-cadherin expression in R1 and NR1 variants of CBS cells: Confocal fluorescence microscopy. R1 and NR1 cells were grown for 3 days under Ca^{2+}-free conditions or in the presence of 1.4 mM extracellular Ca^{2+}. At the end of the incubation period, immunofluorescence staining for E-cadherin was done. Confocal fluorescence microscopy was used for detection. In the absence of extracellular Ca^{2+}, diffuse staining was observed in both populations. In Ca^{2+}-containing medium, areas of intense focal staining was observed in R1 cells but the staining pattern in NR1 cells was similar to what was observed in the absence of Ca^{2+}. Results are representative of 3 independent experiments with consistent results.

recover for 24 hr. The medium was then replenished with either standard culture medium with no exogenous Ca^{2+} or with medium supplemented with 1.4 mM Ca^{2+}. Luciferase assays were then performed 24 hr later.

Immunofluorescence

Immunostaining was done as follows: Briefly, cells were grown on uncoated Lab Tek II chamber slides in Ca^{2+}-free SMEM (5% dialyzed FBS) or in the same medium supplemented with extracellular Ca^{2+} to a final concentration of 1.4 mM. After 3 days, cells were fixed with 4% formaldehyde for 20 min. After fixation, cells were washed twice 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 a monoclonal antibody to β-catenin (Chemicon, Temicula, CA) or with an antibody to E-cadherin (BD Biosciences; San Jose, CA) in blocking solution for 1 hr. After 3 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). After 3 additional washing steps, the cells were rinsed one time with water. Coverslips were mounted onto the 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.

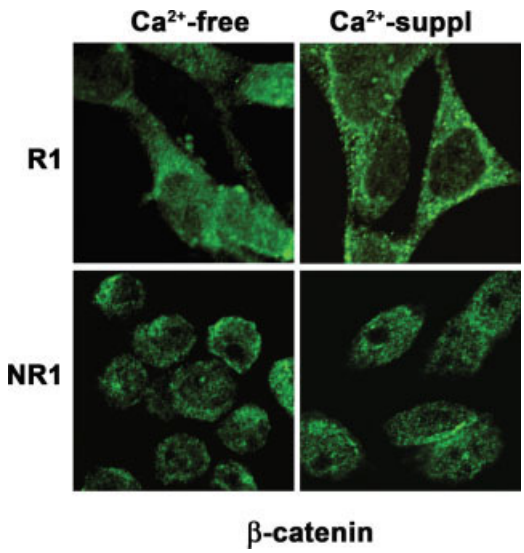

FIGURE 2 – β-Catenin expression in R1 and NR1 variants of CBS cells: Confocal fluorescence microscopy. R1 and NR1 cells were incubated in either Ca^{2+}-free medium or medium containing 1.4 mM Ca^{2+} for 3 days and stained with β-catenin antibody. Confocal fluorescence microscopy was used for detection. In the absence of extracellular Ca^{2+}, diffuse staining was observed in both populations. In Ca^{2+}-containing medium, peripheral staining was observed in R1 cells but the staining pattern in NR1 cells was similar to what was observed in the absence of Ca^{2+}. Results are representative of 3 independent experiments with consistent results.

Western blots

Extracts were prepared from parental CBS cells, from R1 and NR1 variants and from mock-transfected or siRNA-transfected cells as follows: Cells were washed once in DPBS before addition of lysis buffer (Cell Signaling Technologies, Danvers, MA). After 15 min of incubation in lysis buffer at 4°C, cells were scraped into the buffer and sonicated (5 pulses of 3 sec each) using a Fisher 550 Sonic Dismembrator (Pittsburgh, PA). The lysates were centrifuged for 15 min at 14,000*g* and the supernatant fluids were collected for Western blots. Western blotting was performed as described previously[7] using a rabbit polyclonal anti-β-catenin antibody (Chemicon, Temecula, CA), a rabbit polyclonal antibody raised against a synthetic peptide of the human CaSR molecule (Affinity Bioreagents, Golden, CO) or a mouse monoclonal anti-E-cadherin antibody (BD Biosciences, San Jose, CA). Anti-β-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA) served as control.

Proliferation assay

Proliferation studies were carried out as follows. Cells were plated in 24-well tissue culture dishes at a density of 4×10^4 cells per well, using Ca^{2+}-free SMEM with 5% dialyzed FBS as culture medium. Cells were allowed to attach overnight. The next day, cells were washed and incubated under the desired conditions for 3 days. Proliferation was measured by detaching the cells with trypsin and enumerating them with a particle counter (Coulter Electronics, Hialeah, FL).

Cell fractionation

Cells were plated in 100-mm culture dishes at a density of 1.5×10^6 cells per dish, using Ca^{2+}-free SMEM with 5% dialyzed FBS as culture medium. Cells were allowed to attach overnight. The next day, cells were incubated in fresh SMEM with 5% dialyzed FBS with or without 1.4 mM Ca^{2+}. After 3 days of incubation, cells were harvested and lysed. Membrane extraction was

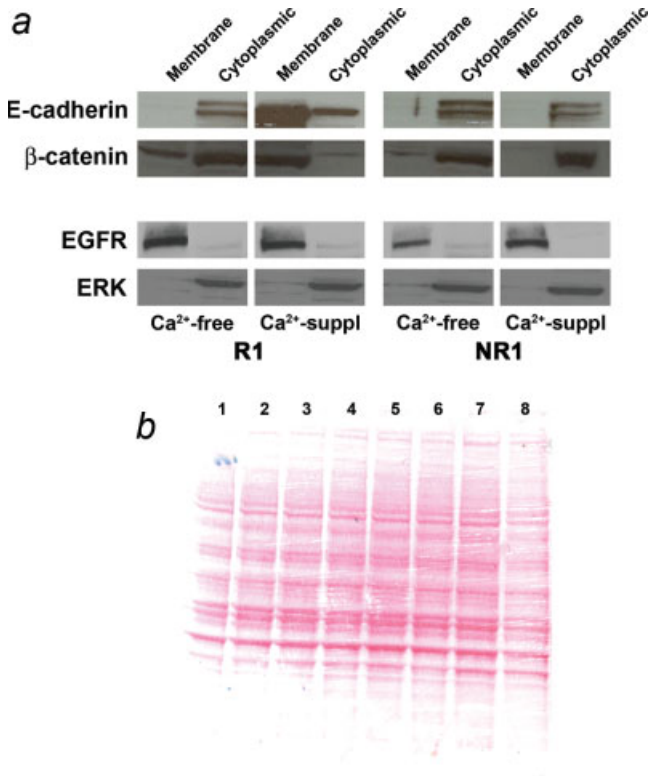

FIGURE 3 – E-Cadherin and β-catenin distribution in R1 and NR1 variants of CBS cells: Western blot analysis of cytoplasmic and membrane fractions. (*a*) R1 and NR1 cells were grown in Ca^{2+}-free medium or medium containing 1.4 mM Ca^{2+} for 3 days. Fractions were prepared using a phase-separation technique and E-cadherin and β-catenin were assessed by western blotting. The blots are representative of 3 independent experiments with consistent results. Controls for these studies include EGF receptor as a membrane marker and total ERK protein as a cytosolic marker. (*b*) The complete SDS-PAGE profiles of the extracts used are shown. Lanes 1–8 correspond to the 8 lanes (*n* order) presented in panel (*a*) above. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

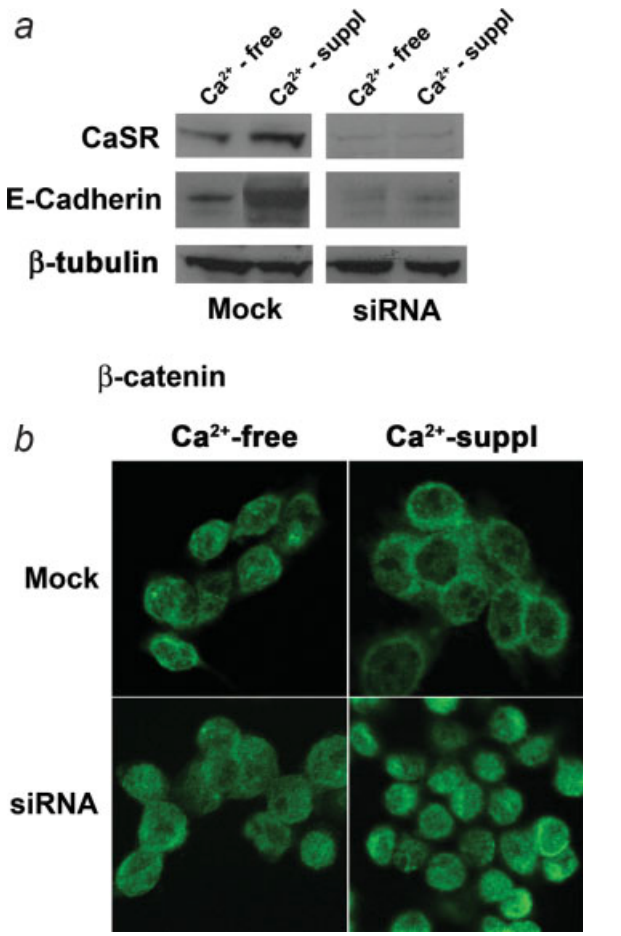

FIGURE 4 – Effect of extracellular Ca^{2+} on expression of CaSR, E-cadherin and β-catenin in siRNA treated CBS cells. (*a*) siRNA-transfected and mock-transfected cells were grown for 3 days under Ca^{2+}-free conditions or in the presence of 1.4 mM extracellular Ca^{2+}. At the end of the incubation period, lysates were prepared from the cells and probed for CaSR and E-cadherin expression by Western blot analysis. Blots are representative of 3 independent experiments with consistent results. (*b*) Immunofluorescence staining of siRNA-transfected and mock-transfected cells for β-catenin. Cells were incubated in either Ca^{2+}-free medium or medium containing 1.4 mM Ca^{2+} for 3 days and stained with β-catenin antibody. Confocal fluorescence microscopy was used for detection. Results are representative of 3 independent experiments with consistent results.

done using the Mem-PER Eukaryotic Membrane Protein Extraction Reagent Kit (Pierce Biotechnology, Rockford, IL) according to the manufacturer's 'phase-partitioning' protocol for mammalian cells. The membrane fraction and soluble fraction containing cytoplasm and nucleus were used for western blotting as described earlier. Prior to loading the gels, protein levels in each preparation were determined using the BCA protein determination kit (Pierce Biotechnology) and equal amounts of protein were loaded onto each lane. After electrophoresis and protein transfer to the nitrocellulose filters, we used Ponceau S reversible staining solution (Pierce Biotechnology) to visualize the transferred proteins and to confirm that comparable amounts of total protein were transferred.

To determine the degree to which membrane enrichment (or depletion) was achieved, we compared expression of a cell surface protein (epidermal growth factor receptor) and a cytosolic protein (total ERK) by Western blotting of cytoplasmic and membrane fractions. In every case, greater than 95% of the total ERK immunoreactivity was in the cytoplasmic fraction with barely detectable reactivity in the membrane fraction. At the same time, most of the EGFR (75–80%) was found in the membrane fraction with the remainder in the cytoplasmic fraction.

Immunohistochemistry

Five-μm thick sections of formalin-fixed, paraffin-embedded surgical specimens of 6 invasive colon tumors were obtained from the surgical pathology service at the University of Michigan Hospitals. The sections were stained by the immunoperoxidase method using the same antibodies to CaSR, E-cadherin, β-catenin indicated earlier. In each case, the reaction product was visualized using diaminobenzadine as the chromogenic substrate. Immunostained sections were examined by light microscopy. A hematoxylin and eosin-stained section for each tissue was examined in parallel.

Results

Effects of extracellular Ca^{2+} on E-cadherin and β-catenin distribution in Ca^{2+}-responsive and nonresponsive CBS cells: Confocal immunofluorescence

Figure 1 demonstrates the effects of extracellular Ca^{2+} on the distribution of E-cadherin in R1 and NR1 variants of the CBS line. The 2 isolates were incubated for 3 days in Ca^{2+}-free or Ca^{2+}-containing medium and then examined by confocal fluorescence

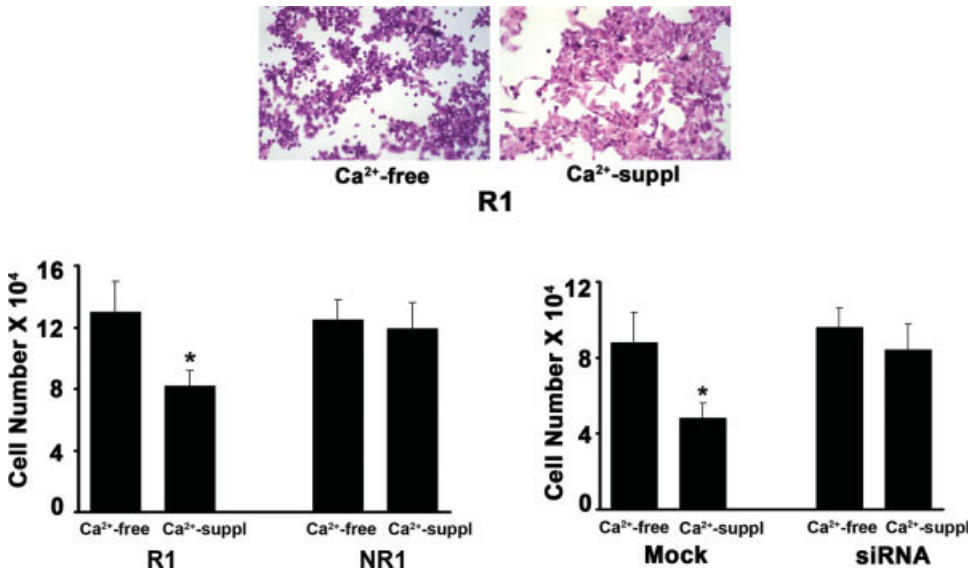

FIGURE 5 – Effect of extracellular Ca^{2+} on cell growth in the R1 and NR1 CBS cells and in mock-transfected and siRNA transfected CBS cells. Proliferation of R1 and NR1 cells and proliferation of mock-transfected and siRNA-transfected cells under Ca^{2+}-free or Ca^{2+}-containing conditions. Values shown are means and standard deviations based on triplicate samples in a single experiment. Statistical significance was determined by ANOVA, followed by paired group comparisons. *Indicates differences from control group (no Ca^{2+} supplementation) at the $p < 0.05$ level. Proliferation assays were conducted 5 times with consistent results. Insert: Morphological appearance of R1 cells grown for 3 days in the absence or presence of Ca^{2+}-supplemented medium. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

microscopy as described in the Materials and Methods Section. Under Ca^{2+}-free conditions, E-cadherin exhibited a diffuse staining pattern in both populations. In Ca^{2+}-supplemented medium, areas of concentrated focal fluorescence were observed in the R1 population. However, in the NR1 population, the fluorescence pattern remained diffuse (Fig. 1).

In parallel, β-catenin expression and distribution was assessed. In Ca^{2+}-free medium, β-catenin was detected throughout the cell in both R1 and NR1 cells. In the presence of extracellular Ca^{2+}, β-catenin was redistributed to the periphery of the cell in the R1 population. While it was impossible to prove that all of the β-catenin was localized to the cell surface, fluorescence in the perinuclear area of the cell was clearly reduced as compared to cells maintained in the absence of Ca^{2+} (Fig. 2). In contrast, NR1 cells showed no difference in the β-catenin immunofluorescence pattern between Ca^{2+}-free and Ca^{2+}-containing conditions (Fig. 2).

Effects of extracellular Ca^{2+} on E-cadherin and β-catenin distribution in Ca^{2+}-responsive and nonresponsive CBS cells: Cell fractionation

R1 and NR1 cells were grown in Ca^{2+}-free and Ca^{2+}-containing medium as earlier. At the end of the incubation period, membrane and cytoplasmic fractions were prepared from cell lysates and examined for E-cadherin expression (Fig. 3). Under Ca^{2+}-free conditions, E-cadherin expression in both variants was similar (*i.e.*, mostly in the cytoplasmic fraction). In the presence of extracellular Ca^{2+}, the distribution of E-cadherin was shifted from the cytoplasmic to the membrane fraction in R1 cells. In contrast, there was essentially no change in distribution pattern in NR1 cells under the same conditions (Fig. 3).

The same membrane and cytoplasmic fractions were examined by western blotting for β-catenin expression. Under Ca^{2+}-free conditions, the majority of the β-catenin in both variants was in the cytoplasmic fraction. In the presence of extracellular Ca^{2+}, the distribution of β-catenin was shifted from the cytoplasmic to the membrane fraction in R1 cells. In contrast, there was essentially no change in distribution pattern in NR1 cells under the same conditions (Fig. 3).

Effects of extracellular Ca^{2+} on E-cadherin and β-catenin expression and distribution in siRNA-transfected and mock-transfected CBS cells

The earlier experiments suggest that a functional CaSR is necessary for extracellular Ca^{2+} to modulate E-cadherin and β-catenin distribution in CBS cells. To provide direct evidence for the CaSR role, a vector-based siRNA technique was used to downregulate CaSR in parental CBS cells. In the initial experiments, we compared CaSR expression in cells maintained in Ca^{2+}-free medium with cells maintained in the presence of 1.4 mM Ca^{2+}. As seen in Figure 4*a*, mock-transfected CBS cells expressed detectable CaSR, and this protein was upregulated in medium containing Ca^{2+}. In contrast, the level of the protein was reduced in siRNA-transfected cells to virtually undetectable levels under Ca^{2+}-free conditions and not upregulated by Ca^{2+}.

E-cadherin expression was examined next. Previous studies have demonstrated Ca^{2+}-mediated upregulation of E-cadherin in parental CBS cells (as well as in several other colon carcinoma cell lines.[9] As seen in Figure 4*a*, E-cadherin was upregulated in mock-transfected cells in the presence of extracellular Ca^{2+}. In contrast, there was little E-cadherin expression under the low-Ca^{2+} conditions in siRNA-transfected cells and no upregulation with Ca^{2+}.

Next, we assessed intracellular distribution of β-catenin in mock-transfected and siRNA-transfected cells by confocal immunofluorescence. For these studies, CBS cells were transfected either with the control vector or with the siRNA-containing vector and incubated in Ca^{2+}-free medium or medium containing 1.4 mM Ca^{2+} for 3 days. Figure 4*b* demonstrates β-catenin localization in the absence or presence of extracellular Ca^{2+}. The results were similar to those described earlier with the R1 and NR1 populations. That is, in the mock-transfected cells, there was a redistribution of β-catenin away from the nucleus and to the periphery of the cell under the influence of extracellular Ca^{2+}. In

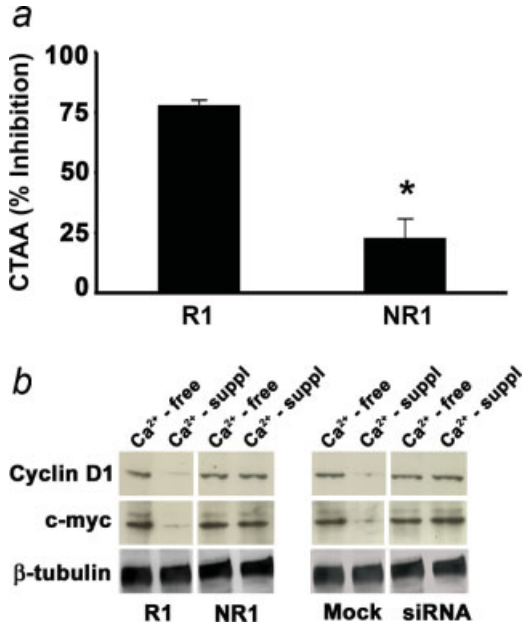

FIGURE 6 – Effect of extracellular Ca^{2+} on cell growth, CTAA and expression of c-myc and cyclin D1 in the R1 and NR1 CBS cells and in mock-transfected and siRNA transfected CBS cells. (*a*) CTAA was measured using the dual luciferase reporter assay. Results were expressed as percent inhibition of CTAA in the presence of extracellular Ca^{2+} relative to control cells cultured in the absence of Ca^{2+}. Values obtained represent the ratio of luciferase activity from the pTOPFLASH vector to the pFOPFLASH vector and normalized to transfection efficiency by cotransfection with the pRL Renilla luciferase vector. Error bars represent the standard error of the mean of triplicate determinations. Statistical significance was determined using the Student *t*-test. *Indicates difference from control group (no Ca^{2+} supplementation) at the $p < 0.05$ level. (*b*) Cells from each of the 4 populations were grown under Ca^{2+}-free or Ca^{2+}-containing conditions for 3 days. Lysates were prepared from these cells and probed for c-myc and cyclin D1 expression by Western blot analysis. Blots are representative of 2 independent experiments with consistent results. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

contrast, in siRNA treated cells, no peripheral localization of β-catenin was observed under either condition.

Effects of extracellular Ca^{2+} on CBS cell proliferation: Requirement for functional CaSR

In a final series of experiments with CBS cells, we assessed the effects of extracellular Ca^{2+} on proliferation. Figure 5 (left panel) demonstrates results with R1 and NR1 variants. Consistent with our previous results,[7] proliferation of the responsive cells (R1) was reduced by about 40% in Ca^{2+}-containing medium as compared to results obtained under Ca^{2+}-free conditions. In contrast, there was no change in proliferation in the nonresponsive (NR1) cells in Ca^{2+}-supplemented medium as compared to unsupplemented control medium. Proliferation of siRNA treated cells compared to the mock-transfected cells was also assessed. As shown in Figure 5 (right panel), statistically significant growth inhibition was observed in Ca^{2+}-treated mock-transfected cells as compared to the same cells cultured in Ca^{2+}-free medium. The degree of growth inhibition was similar to that reported earlier with the parental CBS cells[9] or with the R1 variant cells shown here. In contrast, there was no Ca^{2+}-mediated reduction in cell growth in siRNA-treated cells as compared to the same cells in Ca^{2+}-free medium.

In both the R1 variant cells and the mock-transfected cells, Ca^{2+}-mediated growth inhibition was accompanied by a change in morphology of the treated cells. That is, while the cells maintained a spherical appearance and were loosely attached to one another and to the substratum under Ca^{2+}-free conditions, both populations were more well-spread and more firmly attached to one another and to the substratum in the presence of 1.4 mM Ca^{2+}. In contrast, neither NR1 cells or mock-transfected cells demonstrated a change in morphology in response to extracellular Ca^{2+}. The insert in Figure 5 demonstrates the morphological differences in the Ca^{2+}-responsive CBS variant under Ca^{2+}-free (left) and Ca^{2+}-containing (right) conditions.

In parallel studies, we compared CTAA and expression of 2 growth-related proteins—*i.e.*, c-myc and cyclin D1-in R1 *versus* NR1 cells. For CTAA, cells in 6-well culture plates were transfected with pTopFlash/pRL Renilla or pFopFlash/pRL Renilla and allowed to recover for 24 hr. The medium was then replenished with either standard culture medium with no exogenous Ca^{2+} or with medium supplemented with 1.4 mM Ca^{2+}. Luciferase assays were then performed 24 hr later as described in the Materials and Methods. Western blotting was used to assess c-myc and cyclin D1 expression. As seen in Figure 6*a*, the ability of extracellular Ca^{2+} to suppress CTAA was reduced in the NR1 cells (23% reduction) as compared to R1 cells (78% reduction). Concomitantly, Ca^{2+}-mediated reduction in the levels of c-myc and cyclin-D1 was also much lower in the NR1 cells than in R1 cells. As shown in Figure 6*b*, expression of both proteins was significantly reduced in the mock-transfected cells in the presence of Ca^{2+} as compared to Ca^{2+}-free conditions. In siRNA-transfected cells, however, the reduction in response to extracellular Ca^{2+} was much less.

Immunohistochemical localization of CaSR, β-catenin and E-cadherin in colon carcinoma

Histological sections from 6 cases of invasive colon cancer were examined by light microscopy after staining with antibodies to CaSR, E-cadherin and β-catenin. In each section there were areas of normal colonic mucosa and areas of malignant tumor. To summarize, CaSR was strongly expressed in morphologically normal colonic crypt epithelium, particularly in the cells in the upper half of the crypt and at the surface. CaSR staining was significantly reduced in areas of malignant tumor. There was also variability within the tumors. In some areas of tumor, there was no detectable CaSR, whereas in other areas, staining was present, though weaker than seen in normal structures. Where staining was detected in malignant tissue, it was observed in areas of the tumor with evidence of lumen formation. Figure 7 shows an area of colonic mucosa, in which both normal and malignant epithelium are present. As seen in Figure 7*b*, CaSR reactivity can be seen in normal mucosa but is essentially missing from the tumor. These findings are consistent with past reports.[8–10]

Sections from the same 6 specimens were also stained for β-catenin. In all 6 cases, there was diffuse cytoplasmic staining and a more intense staining of the border between adjacent cells in the areas of normal mucosa. Nuclear β-catenin staining was routinely not observed in the normal crypts. In areas of malignant tumor, the β-catenin staining pattern was (in contrast to the consistent results obtained in normal colonic crypts) highly variable. In some areas, the malignant epithelial cells showed the same diffuse cytoplasmic and strong cell surface staining as did epithelial cells in normal crypts. However, even in these areas, strong nuclear staining was also observed in some of the cells. In other areas, strong cytoplasmic and nuclear staining (without evidence of junctional staining) throughout the entire section was observed. Figure 7*c* shows β-catenin staining patterns in normal and malignant epithelial cells from the same area shown in Figures 7*a* and 7*b*.

The E-cadherin staining pattern was significantly different from that seen with either CaSR or β-catenin. Specifically, E-cadherin was detected in virtually all of the cells in both normal and malignant areas in all of the histological sections. Where glandular structures were observed (*i.e.*, in either normal or malignant; whether or not CaSR was also detected), prominent staining along cell–cell junctures was observed (Fig. 7*d*). It was only in the most

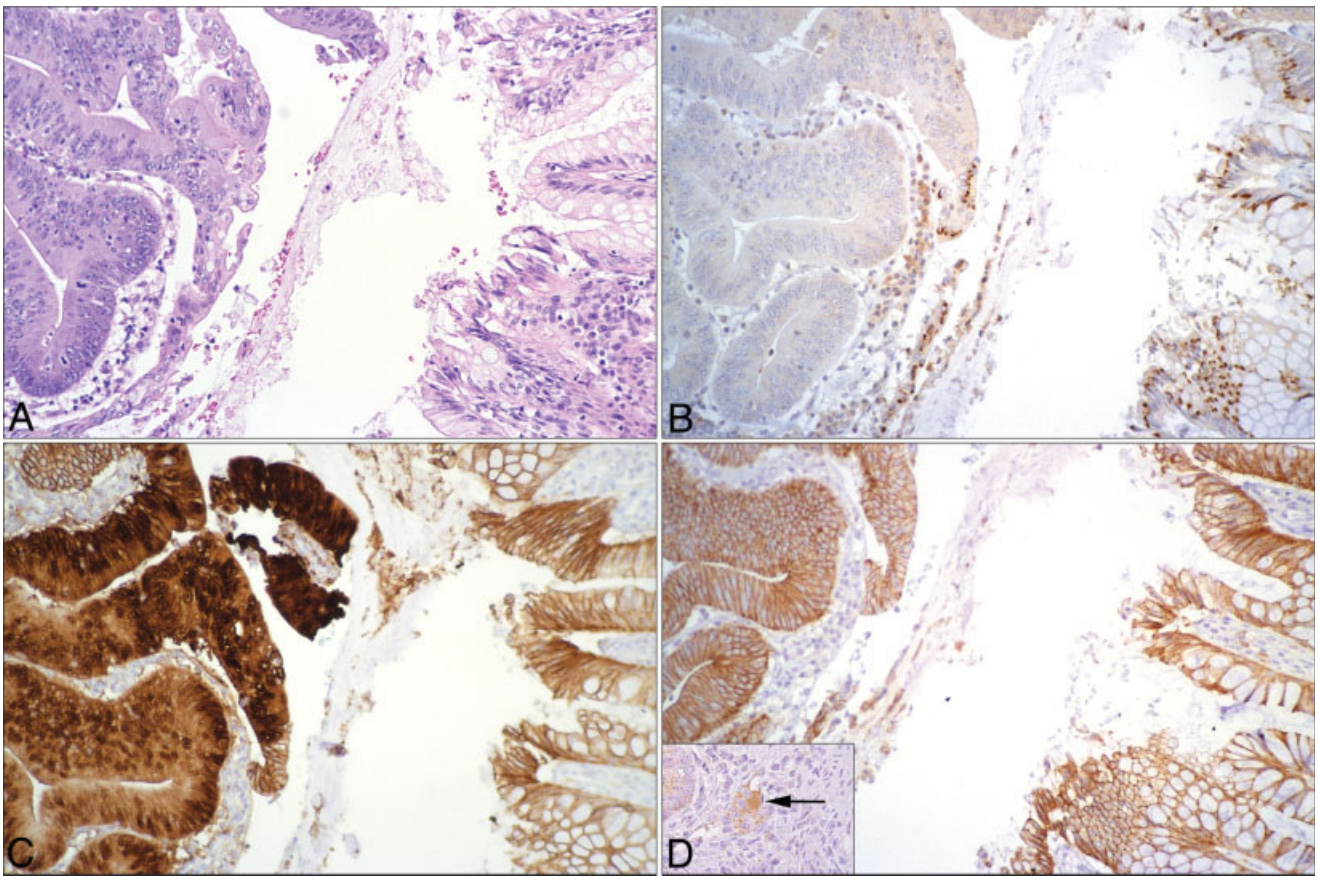

Figure 7 – CaSR, β-catenin and E-cadherin immunoreactivity in normal and malignant human colon tissue. Formalin-fixed specimens of recently diagnosed cases of colon carcinoma ($n = 6$) were stained with antibodies to CaSR, β-catenin and E-cadherin using the immunoperoxidase method. (*a*) Hematoxylin and eosin-stained sections through an area demonstrating normal (right side) and malignant (left side) epithelium. (*b*) CaSR: Strong immunostaining for CaSR was observed in normal colonic crypt epithelium. Tumor epithelial cells were virtually negative. (*c*) β-catenin: In normal crypt epithelium, staining was observed at the border between cells. In the tumor, strong cytoplasmic and nuclear staining was evident. The distinct border staining was lost. (*d*) E-cadherin: Junctional staining and intracellular staining were observed in both normal and malignant areas of the section. Insert: Where individual tumor cells were seen, border staining was lost and diffuse staining throughout the cell was evident. The findings presented here were typical of what was seen in all of the cases examined (All sections magnified X66; Insert X220).

advanced areas of the tumor, where glandular structures were not evident, that diffuse staining throughout the cell rather than staining of cell–cell junctures was seen (Fig. 7*d*, insert).

Discussion

CaSR is a G-protein-coupled. CaSR was initially identified in the parathyroid gland, where it senses minute changes in extracellular Ca^{2+} concentration.[11,12] Subsequently, this protein was demonstrated in a number of different types of epithelial cells (including those of the gastrointestinal tract).[13,14] A variety of indirect evidence suggests that CaSR plays a role in mediating the effects of extracellular Ca^{2+} on epithelial cell function.[7–9,14,15] Definitive evidence that CaSR is required for Ca^{2+}-mediated events is lacking, however. Furthermore, how CaSR regulates Ca^{2+} responses in epithelial cell is not fully understood. Here we have used an siRNA approach to down-regulate CaSR expression in a line of human colon carcinoma cells. In the CaSR-down-regulated cells, the addition of extracellular Ca^{2+} to the culture medium failed to suppress growth or induce cell flattening and cell–cell contact as it did in mock-transfected cells. In parallel studies, we used a variant of the CBS line that expresses detectable CaSR (immunochemically) but does not upregulate receptor expression in response to extracellular Ca^{2+}.[7] The Ca^{2+}-nonresponsive variant, like the siRNA-treated cells, did not undergo growth suppression or exhibit the other morphological changes seen in Ca^{2+}-responsive cells exposed to extracellular Ca^{2+}.

What are the critical down-stream events in the Ca^{2+} signaling pathway that depend on CaSR? Our data suggest that CaSR expression and function are necessary for Ca^{2+} to upregulate E-cadherin synthesis and to organize E-cadherin and β-catenin at the cell surface. When Ca^{2+}-responsive CBS (R1) cells were maintained in culture under Ca^{2+}-free conditions, both E-cadherin and β-catenin were diffusely expressed throughout the cell as detected by confocal fluorescence microscopy. When cell-fractionation was done, the majority of both proteins segregated into the cytoplasmic rather than membrane pool. However, in the presence of 1.4 mM extracellular Ca^{2+}, there was a redistribution of both proteins to the cell surface. In contrast, with both the nonresponsive variant cells and the siRNA-treated cells, exposure to extracellular Ca^{2+} failed to prevent a redistribution of E-cadherin and β-catenin from the cytoplasmic to membrane pool. The inability of the cells to properly organize these proteins at the cell surface could have important implications for epithelial cell function. It is well-established that E-cadherin is a critical component of cell surface adhesion complexes that form in differentiated epithelia and that β-catenin accumulates at the cell surface along with E-cadherin.[16–21] In the absence of β-catenin, E-cadherin is able to mediate a 'weak' cell–cell adhesive interaction.[22] Strong cell–cell adhesion and differentiation, however, do not occur. This requires stabilization of

the cell surface adhesion complex through interaction with the cytoskeleton; β-catenin is one of the critical components mediating this interaction.[23–28] Under conditions where both E-cadherin and β-catenin are localized at the cell surface and functional, epithelial cells behave as a cohesive unit. In contrast, when these proteins are not functional, discohesion is prominent. The epithelial cells behave as single cells. Under such conditions, cell motility and invasion of single cells and small groups of cells into the surrounding stroma can be seen.[18,20,24,25,27,28]

In addition to interfering with invasion-related properties, localization of E-cadherin and β-catenin at the cell surface could also act to suppress malignant outgrowth by influencing proliferation and the multiple signaling pathways that influence proliferation. Past studies have demonstrated a role for cytoplasmic/nuclear β-catenin in upregulation of NFκB, presumably through signaling events involving the p38 mitogen-activated protein (MAP) kinase pathway.[29] Other studies have shown a connection between E-cadherin and MAP kinase signaling.[30] Using an immortalized epithelial cell (HaCat), it was shown that when Ca^{2+}-dependent cell–cell contacts were formed, ligand-independent activation of the epidermal growth factor receptor occurred, leading to ERK phorphorylation. A role for E-cadherin-mediated cell–cell adhesion was implied, based on the finding that disruption of E-cadherin-mediated contacts between cells interfered with ERK activation.

Another important role for cytoplasmic/nuclear β-catenin involves activation of Wnt signaling. Past studies have shown that when β-catenin is translocated into the nucleus, it functions as a transcription factor activator for growth-promoting molecules in the Wnt signaling pathway.[31–33] Consistent with this, the data provided here demonstrate a relationship between CaSR expression and function, cell surface β-catenin localization, reduced CTAA and reduced expression of c-myc and cyclin D1 in CBS cells. Both c-myc and cyclin-D1 are down-stream targets in the Wnt signaling pathway.[34,35]

Although the findings presented here were obtained with a line of human colon carcinoma cells, the findings are likely to have implications for human colon cancer. We[8,9] and others[10] have shown in the past that expression of CaSR is reduced or lacking entirely in histological sections of colon carcinoma. Those cancers with the most anaplastic features exhibited the least CaSR.[8,9] In the small series of colon carcinomas studied here, we observed a relationship between CaSR expression and β-catenin staining at the cell surface. Where CaSR expression was detected (*i.e.*, in normal colonic mucosa and some areas of histologically differentiated tumor), β-catenin reactivity was seen primarily along the border between adjacent cells. Where CaSR expression was absent, β-catenin was primarily intracytoplasmic or intranuclear rather than cell surface. Thus, to the exent that cytoplasmic or nuclear β-catenin is associated with continued proliferation, the same mechanisms that limit proliferation of CBS cells *in vitro* may be operative *in vivo*, as well.

Of interest, there did not seem to be a similar relationship between CaSR and E-cadherin expression. E-cadherin immunoreactivity was observed between adjacent cells in areas of tumor that were negative for CaSR expression (as well as in areas where CaSR was expressed). Only at the invasive front, where all evidence of glandular structure was lost did we see a lack of surface E-cadherin expression. Perhaps in the CaSR-negative areas, the detectable E-cadherin is analogous to the E-cadherin that is loosely bound (and not fully functional) on epithelial cells *in vitro* in the absence of extracellular Ca^{2+}.[22] Immunochemical approaches are inadequate, of course, to assess functionality.

In summary, an intact Ca^{2+} signaling system is important for E-cadherin upregulation and for sequestration of E-cadherin and β-catenin at the cell surface in CBS colon carcinoma cells. When the signaling system is intact, the cells assume a flattened appearance and grow as a cohesive epithelial unit. Proliferation is reduced. When the signaling system is not intact, the cells do not behave as a cohesive unit. Cells remain loosely attached to one another, do not exhibit a flattened appearance and continue to proliferate. The present studies show that expression and function of the CaSR is a critical component of this signaling system in colon carcinoma cells. Observations in colon carcinoma tissue sections are consistent with the *in vitro* findings. The issue that needs to be addressed *in vivo* is whether the lack of CaSR expression in colon carcinoma reflects defects in CaSR or whether the lack of CaSR expression is, itself, the result of some other more fundamental defect. Further work is necessary to address this issue.

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