

Extracellular Calcium and Calcium Sensing Receptor Function in Human Colon Carcinomas: Promotion of E-Cadherin Expression and Suppression of β -Catenin/TCF Activation¹

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ABSTRACT

Ca^{2+} has chemopreventive activity against colon cancer, albeit its mechanisms of action are not understood. In this study, we showed that four different human colon carcinoma cell lines (FET, SW480, MOSER, and CBS) expressed the human parathyroid calcium sensing receptor (CaSR) and that a function of extracellular Ca^{2+} and the CaSR in these cells was the promotion of E-cadherin expression and suppression of β -catenin/T cell factor activation. We also found that human colonic crypt epithelial cells expressed the CaSR, and histologically differentiated carcinomas (*i.e.*, where three-dimensional, crypt-like structures were present) expressed less receptor by comparison, whereas an almost complete loss of CaSR expression was observed in undifferentiated tumors. These results suggest that extracellular Ca^{2+} and the CaSR may function to regulate the differentiation of colonic epithelial cells and that disruption of this ligand receptor system may contribute to abnormal differentiation and malignant progression. In addition, the promotion of E-cadherin and suppression of β -catenin/T cell factor may be an important mechanism underlying the chemopreventive action of Ca^{2+} in colon cancer.

INTRODUCTION

The identification and cloning of the human parathyroid cell surface CaSR³ have shown that extracellular Ca^{2+} and the CaSR can serve as a first messenger system in controlling biological function (1). There is strong evidence to suggest that the function of the CaSR is diverse, extends beyond systemic control of Ca^{2+} homeostasis, and regulates diverse cellular processes in different cell types (2–5). The expression of CaSR in gastric mucous and colonic epithelial cells has been reported (2, 6), but the functional significance of this ligand receptor system in colonic cell physiology or in carcinogenesis is not understood. The chemopreventive properties of Ca^{2+} in colon carcinogenesis is well established (7, 8) and is the subject of recent interest. Exactly how Ca^{2+} acts at the molecular level to suppress or delay carcinogenesis is not known. We showed in this investigation that four different human colon carcinoma cell lines expressed the CaSR and that a function of this ligand receptor system in these cells was the promotion of E-cadherin expression, a tumor suppressor in colon cancer (9), and suppression of β -catenin/TCF activation, a prominent signaling pathway implicated in driving the malignant progression of colon cancer (10). Immunohistochemical staining showed that columnar epithelial cells in normal human colonic crypts stained strongly for CaSR expression, whereas there was significantly weaker staining in differentiated carcinomas and a greatly reduced or complete loss of staining in undifferentiated carcinomas. These results, taken together,

suggest that extracellular Ca^{2+} and the CaSR may play an important role in regulating colonic epithelial cell differentiation and that disruption of this ligand receptor system may contribute to malignant progression. In addition, promotion of E-cadherin expression and suppression of β -catenin/TCF signaling may be a mechanism underlying the chemopreventive action of Ca^{2+} in colon cancer.

MATERIALS AND METHODS

Cell Culture, RT-PCR, and Immunoblottings. Four different human colon carcinoma cell lines (*i.e.*, FET, SW480, MOSER, and CBS), available from previous studies (11), were used here. The carcinoma cells were maintained in Ca^{2+} -free SMEM medium (Sigma Chemical Co., St. Louis, MO) supplemented with sodium bicarbonate, peptone, vitamins, amino acids, and 5% fetal bovine serum. RT-PCR and agarose gel electrophoresis of the PCR products were performed as described previously (12). PCR amplification of the CaSR cDNA was accomplished by using a set of primers (forward [5'GGGCGCCGGGGATTGAGAAATTC-3'] and reverse [5'GGCGTGGGCAATGGAGTAG-3']) designed to amplify the NH₂-terminal extracellular domain of the human parathyroid CaSR (13). The expected size of the PCR product was 635 bp. Immunoblottings were performed as described previously (12) using rabbit polyclonal antibodies raised against a synthetic peptide of the human CaSR (Affinity Bioreagents, Inc., Golden, CO). Monoclonal anti-E-cadherin and anti- β -catenin antibodies (Transduction Laboratories, Lexington, KY) were also used in immunoblotting.

CTAA and β -Catenin/TCF4 Complex Formation. CTAA was measured by dual luciferase reporter assays (Promega, Madison, WI) using the constructs pTOPFLASH and pFOPFLASH and as a ratio of luciferase activity from the pTOPFLASH vector to the pFOPFLASH vector (14). All luciferase activities were normalized for transfection efficiency by cotransfection with pRL Renilla luciferase vector. β -catenin/TCF4 complex formation was determined by gel-shift assays using nuclear extracts and a ³²P-labeled, double-stranded 15-nt oligomer (CCCTTTGATCTTACC) as a probe and oligomer CCCTTTGGCCTTACC as the control probe (14). β -catenin in the complex was confirmed by electrophoretic transfer of the complex (obtained with nuclear extract and cold DNA probe) to nitrocellulose and immunoblotted with anti- β catenin antibodies.

Immunohistochemistry. Immunoperoxidase staining was performed as described previously using 5- μm sections of formalin-fixed, paraffin-embedded surgical specimens of human colon tumors (15). The immunoperoxidase reaction product was visualized using diaminobenzidine as the chromogenic substrate. Immunostained sections were lightly counterstained with hematoxylin and examined by light microscopy.

RESULTS AND DISCUSSION

The induction of differentiation of cancer cells *in vitro* may be defined as the use of specific chemical (or physical) agents to induce a more differentiated or "normal" cellular phenotype. Chemoprevention of cancer *in vivo* may be defined as the use of specific agents to prevent, inhibit, or reverse events in the process of carcinogenesis. Thus, differentiation-inducing agents often possess chemopreventative properties. Kallay *et al.* (16, 17) have shown that extracellular Ca^{2+} and the CaSR function to induce quiescence in the human colon carcinoma Caco-2 cell line and suggested this as a rationale for the use of Ca^{2+} supplements in the chemoprevention of colon cancer. Be-

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³ The abbreviations used are: CaSR, calcium sensing receptor; TCF, T cell factor; RT-PCR, reverse transcription-PCR; CTAA, constitutive T cell factor transcriptional activation activity.

cause the induction of colon carcinoma differentiation *in vitro* is linked to the suppression of malignancy (18), the goal of this study was to determine whether extracellular Ca²⁺ and the CaSR system could mediate its action through signal pathways that are known to modulate malignancy in colon carcinomas.

Agarose gel electrophoresis of RT-PCR products, using a set of primers directed against the NH₂-terminal extracellular domain of the human parathyroid CaSR (13), showed that the mRNA for CaSR was detected in all of the cell lines (Fig. 1A). The human parathyroid TT carcinoma cells (13) were used as a positive control in these experiments (Fig. 1A, Lane 3). The expected 635-bp PCR products amplified from the colon carcinoma cells were cloned, sequenced, and found to share 100% homology with the published sequence (data not shown). The expression of CaSR at the protein level was confirmed by immunoblotting using polyclonal antihuman CaSR antibodies (Fig. 1B). Thus, these human colon carcinoma cell lines express the CaSR at the protein level.

The homotypic cell-cell adhesion molecule E-cadherin functions as a tumor suppressor in colon carcinoma cells, and up-regulated E-cadherin expression is associated with the induction of differentiation (9, 19). We examined the effect of extracellular Ca²⁺ on the expression E-cadherin. Cells cultured in Ca²⁺-free SMEM medium (supplemented with 5% serum and thus containing a relatively low Ca²⁺ concentration of ~0.07 mM) did not express E-cadherin (Fig. 1C). However, when the medium was supplemented with extracellular Ca²⁺ to bring the final Ca²⁺ concentration to 1 mM, the expression of E-cadherin was induced (Fig. 1C). E-cadherin interacts with β -catenin, a component of the TCF/Wnt signaling pathway, and because the activation of Wnt is associated with malignancy (10) and extracellular Ca²⁺ induced the expression of the tumor suppressor E-cadherin, we hypothesized that extracellular Ca²⁺ would suppress β -catenin/TCF activation. Fig. 2, A–D shows that when the low Ca²⁺ culture medium was replenished with medium containing 1 mM Ca²⁺, there was a significant reduction in CTAA in all of the cell lines. The MOSER and CBS cells were found to be most sensitive to the down-modulation of CTAA by extracellular Ca²⁺ (Fig. 2, C and D) and were, therefore,

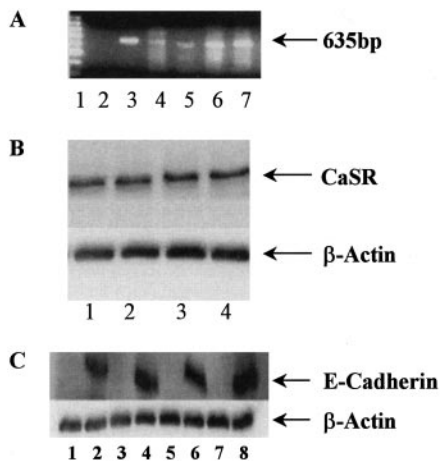


Fig. 1. CaSR expression and extracellular Ca²⁺ on E-cadherin expression. A, agarose gel electrophoretic analysis of RT-PCR products obtained with a set of primers directed against the NH₂-terminal extracellular domain of the human parathyroid CaSR (13). The expected size of the products was 635 bp. Lanes 1, molecular size markers; Lanes 2, control amplification in the absence of cDNA; Lanes 3, thyroid carcinoma TT cells; Lanes 4–7, human colon carcinoma cells FET, SW480, MOSER, and CBS, respectively. B, immunoblotting with anti-CaSR antibodies. Lanes 1–4, FET, SW480, MOSER, and CBS, respectively. C, immunoblotting with anti-E-cadherin antibodies. Lanes 1, 3, 5, and 7, FET, SW480, MOSER, and CBS, respectively, cultured in SMEM Ca²⁺-free medium supplemented with 5% fetal bovine serum. Lanes 2, 4, 6, and 8, FET, SW480, MOSER, and CBS, respectively, cultured in SMEM medium containing 1 mM Ca²⁺ and supplemented with 5% fetal bovine serum for 24 h.

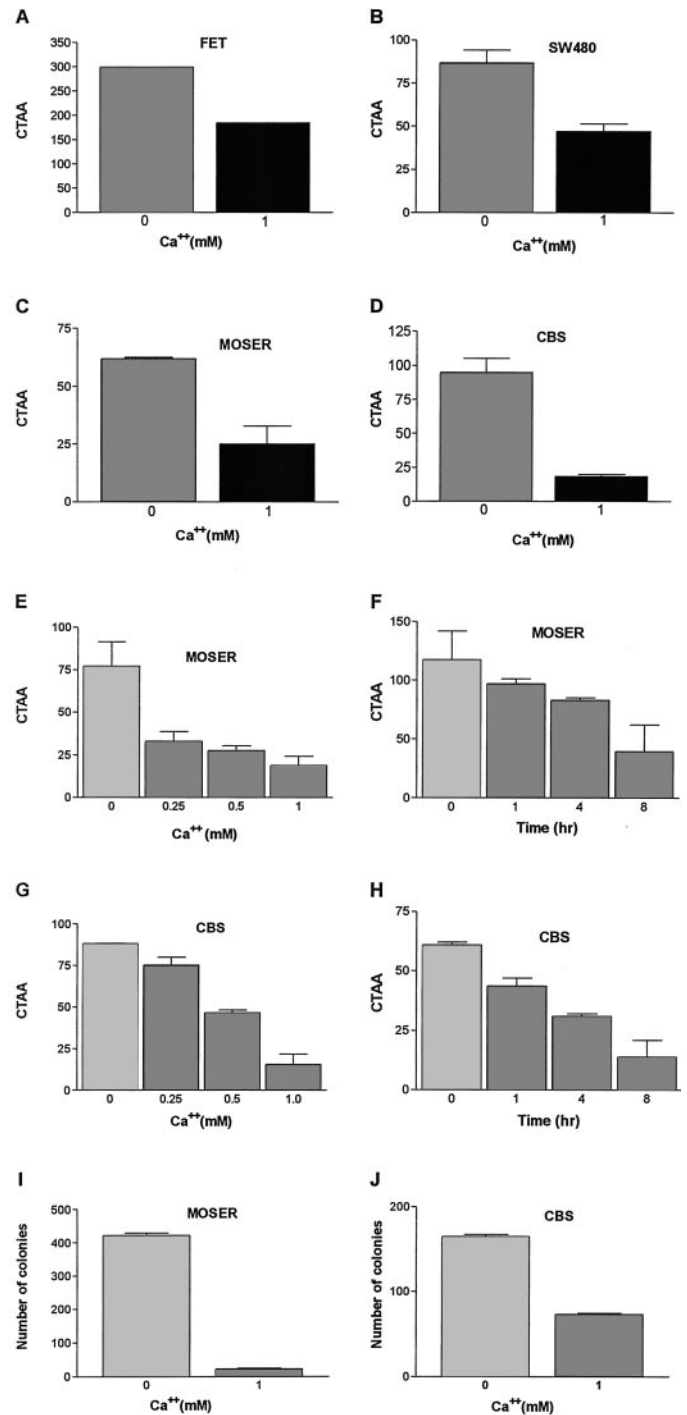


Fig. 2. Extracellular Ca²⁺ effect on CTAA and anchorage-independent growth. A–D, CTAA was measured by dual luciferase reporter assays as described (14). Cells in six-well culture plates were transfected with pTOPFLASH/pRL Renilla or pFOPFLASH/pRL Renilla and allowed to recover for 24 h. The medium was then replenished with either standard culture medium with no exogenous Ca²⁺ (0) or with medium supplemented with 1 mM Ca²⁺ (1). Luciferase assays were then performed 24 h later. Values shown 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 SE of triplicate determinations. E and G, effect of different Ca²⁺ concentrations on CTAA. These experiments were performed as described in A–D above with the exception that different concentrations of Ca²⁺ were used. F and H, kinetics of the changes in CTAA after the replenishment of medium supplemented with 1 mM Ca²⁺. I and J, effect of Ca²⁺ on anchorage-independent growth. The propensity of the cells cultured in medium without exogenous Ca²⁺ (0) or in medium supplemented with 1 mM Ca²⁺ for 3 days to grow in soft agarose was determined as described previously (18). A group of 5000 viable cells was seeded into each soft agarose culture plate and cultured for 10 days. The colonies were then stained with p-iodonitrotetrazolium violet and counted manually with the aid of a magnifying glass. The values shown represent the mean and SE of triplicate determinations.

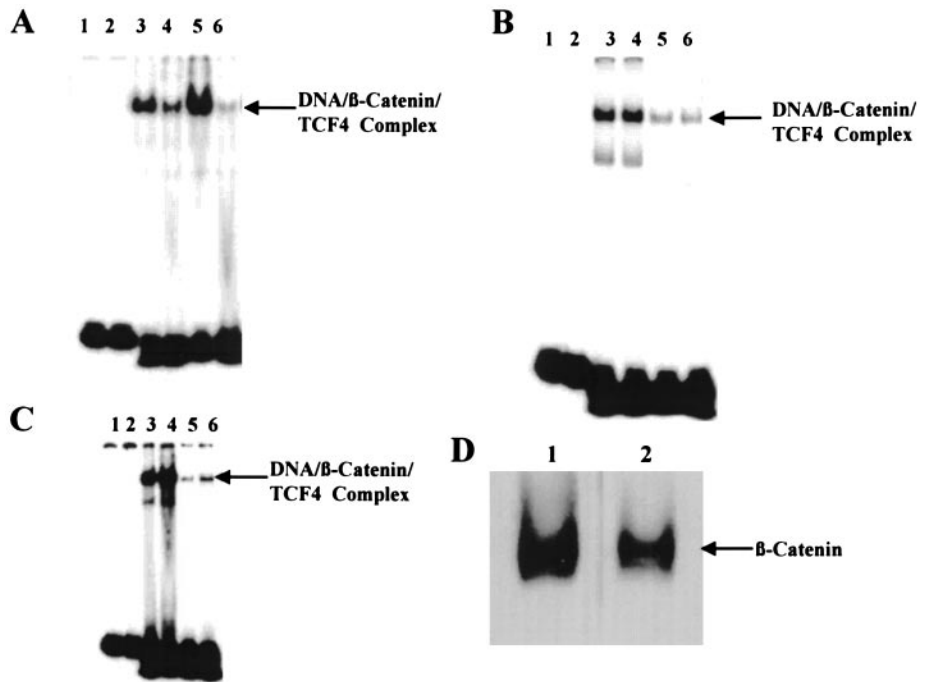


Fig. 3. Effects of Ca²⁺ and Gd³⁺ on β -catenin/TCF4 complex formation. A–C, gel mobility shift assays using ³²P-labeled DNA probes as described in “Materials and Methods.” Lanes 1 and 2, controls without nuclear extract and nuclear extract preincubated with molar excess of cold competitor, respectively. A, Lane 3, MOSER cultured in medium without exogenous Ca²⁺; Lane 4, MOSER cultured in medium supplemented with 1 mM Ca²⁺ for 24 h; Lane 5, CBS cultured in medium without exogenous Ca²⁺; Lane 6, CBS cultured in medium supplemented with 1 mM Ca²⁺ for 24 h. B, Lane 3, MOSER cultured in medium without exogenous Ca²⁺; Lanes 4–6, MOSER cultured in medium supplemented with 1 mM Gd³⁺ for 1, 3, and 6 h, respectively. C, Lane 3, CBS cultured in medium without exogenous Ca²⁺; Lanes 4–6, CBS cultured in medium supplemented with 1 mM Gd³⁺ for 1, 3, and 6 h, respectively. D, immunoblotting of cold DNA/ β -catenin/TCF4 complexes using anti- β -catenin antibodies. Lane 1, MOSER; Lane 2, CBS.

chosen for further investigation. Fig. 2, E–H shows that both the MOSER and CBS cells responded to extracellular Ca²⁺ with down-regulated CTAA in a dose- and time-dependent manner. In the presence of 1 mM Ca²⁺, the capacity of these cells for anchorage-independent growth (*i.e.*, propensity to grow in soft agarose; Ref. 18) was inhibited (Fig. 2, I and J). Thus, extracellular Ca²⁺ inhibited CTAA in these cells concurrent with inhibition of a trait associated with the malignant cell phenotype.

Next, gel shift assays were performed to determine the effect of extracellular Ca²⁺ on the formation of complexes between β -catenin and TCF4. Both the MOSER and CBS cells cultured in medium containing 1 mM Ca²⁺ for 24 h showed a significant reduction in the formation of β -catenin/TCF4 complexes by comparison with their counterparts cultured in Ca²⁺-free SMEM medium (Fig. 3A). Because Ca²⁺ can pass through plasma membranes via mechanisms not associated with the CaSR, the lanthanide element Gadolinium (Gd³⁺); a CaSR agonist that does not pass through plasma membranes; Refs. 1–4) was also used in these experiments to determine whether stimulation of the CaSR would mimic the effect of extracellular Ca²⁺. Treatment of the MOSER cells (Fig. 3B) or the CBS cells (Fig. 3C) with 1 mM Gd³⁺ for 3 or 6 h effectively suppressed the formation of β -catenin/TCF4 complexes. The presence of β -catenin in the complexes was confirmed by electrophoretically transferring cold protein/DNA complexes from the MOSER and CBS cells to nitrocellulose and blotting the nitrocellulose with anti- β -catenin antibodies (Fig. 3D). These results, taken together with the suppression of CTAA, described above, demonstrate that extracellular Ca²⁺ and the CaSR function to suppress the β -catenin-associated Wnt signaling in these colon carcinoma cells and that suppression of this signal pathway may be responsible for the abrogation of anchorage-independent growth. Because down-regulation of β -catenin/TCF signaling is linked to the promotion of differentiation in colon carcinoma cells (20), extracellular Ca²⁺ and the CaSR may work in concert to promote a more differentiated and less malignant phenotype in these cells.

The chemopreventive activity of Ca²⁺ in colon carcinogenesis is well known (7, 8), but how Ca²⁺ acts at the molecular level to prevent or suppress colon cancer development is not understood. These stud-

ies provide a molecular mechanism through which extracellular Ca²⁺ and the CaSR may function to prevent or suppress tumor outgrowth. It has been proposed that extracellular Ca²⁺ exerts its chemopreventive effect through suppression of cell proliferation acting through *c-myc* (17). We have also observed a suppression of cell proliferation in our cells when they were switched from Ca²⁺-free SMEM medium to medium containing 1 mM Ca²⁺ (data not shown). Interestingly, one of the downstream targets of activated β -catenin/TCF signaling is the stimulation of *c-myc* (10). Thus, the induction of quiescence by extracellular Ca²⁺ may be closely linked to the suppression of β -catenin/TCF activation.

In a final set of experiments, surgical specimens from 10 different human colon carcinomas were examined immunohistochemically for CaSR expression. In each of the specimens, there were areas of normal colonic epithelial cells. In each case, columnar epithelial cells of the normal colonic crypts stained strongly for CaSR expression. A representative CaSR staining profile from one case is presented in Fig. 4, A and B. In all of the specimens examined, there were areas of abnormal histology indicative of colon carcinoma. In many areas, the malignant epithelium consisted of abnormal glandular structures. These areas, which are referred to as differentiated or moderately differentiated tumor (Fig. 4, C and D), were positive for CaSR expression, but the degree of staining was reduced as compared with that seen in normal crypts (Fig. 4, A and B). Where individual invasive cells were seen in the same tissue sections, there was essentially no staining (Fig. 4D). In three of the specimens, there were areas in which malignant epithelial cells were present but in which there was no evidence of glandular structure. Rather, sheets of undifferentiated epithelial cells were present in these areas. In such areas, there was essentially no detectable CaSR staining, as shown in Fig. 4, E and F. These results suggest that loss of CaSR expression is associated with abnormal differentiation, malignant progression, or both *in vivo*.

An important issue raised by these findings is the mechanism by which the CaSR is lost as colonic epithelial cells progress from normal to malignant. Is the intact gene lost or mutated, or does the lack of expression reflect down-regulation? If CaSR expression is down-regulated in colon carcinomas, then it might be possible to

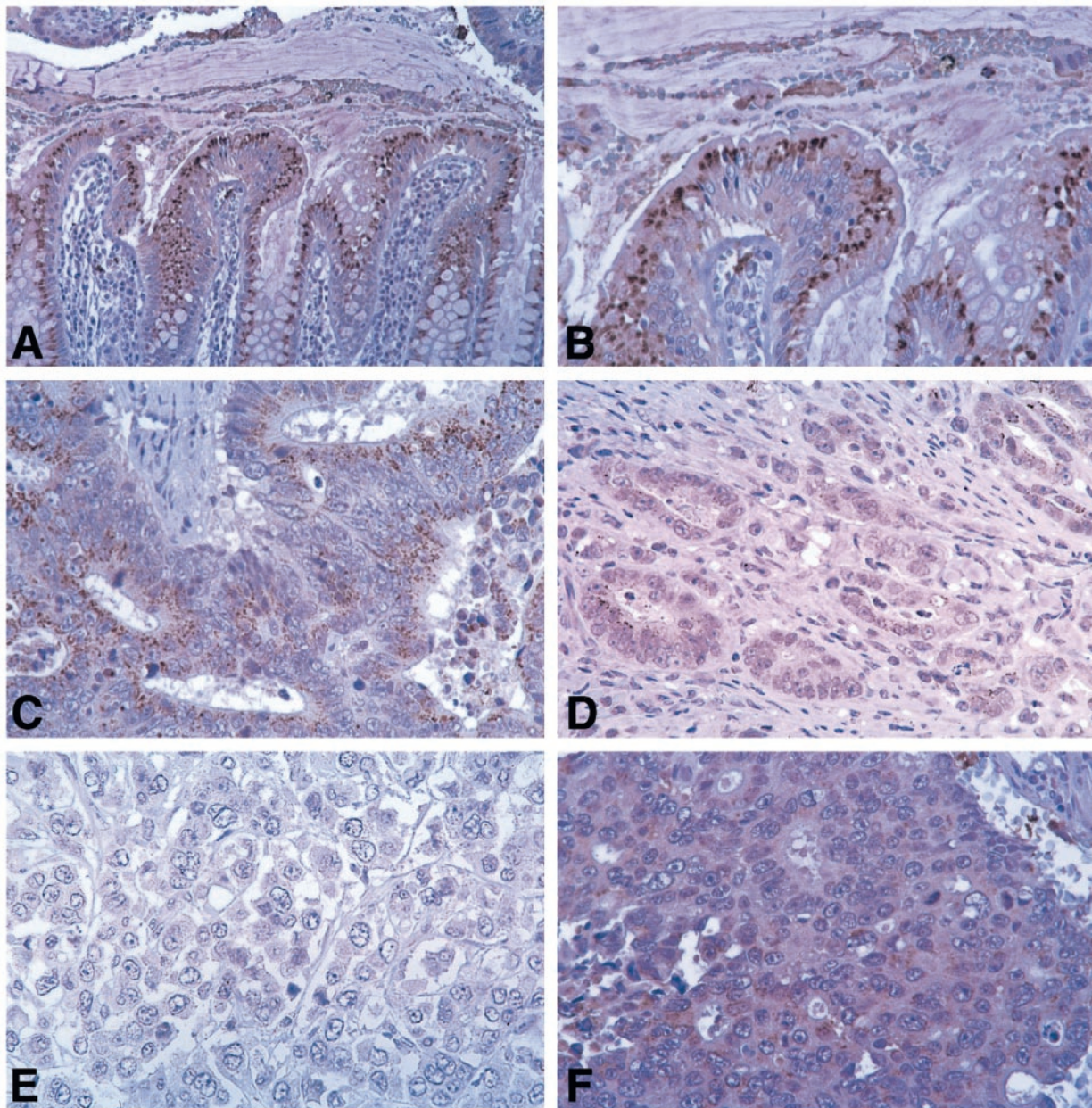


Fig. 4. Immunoperoxidase staining of 5- μ m sections of formalin-fixed, paraffin-embedded surgical specimens of human colon tumors with antihuman CaSR antibodies. A and B, normal colonic crypts showing intense staining of epithelial cells; C, histologically differentiated carcinomas with reduced staining; D, invasive front of a differentiated carcinoma showing little staining of differentiated cells in organized structures and essentially no staining of individual tumor cells at the invasive front. E and F, areas of undifferentiated carcinomas with little to no staining. All sections: $\times 850$.

induce expression in these tumors and thereby increase their sensitivity to the differentiation-promoting effects of extracellular Ca²⁺. On the other hand, if the functional gene is lost, then a gene therapy approach might ultimately prove useful. Another issue is the potential usefulness of CaSR as a prognostic factor. Knowing the relative expression of this protein in colonic epithelial cells in early carcinoma lesions or precancerous lesion might have predictive value in regard to which lesions would respond to the differentiation-inducing or chemopreventive activity of extracellular Ca²⁺.

In summary, we have shown that extracellular Ca²⁺ and the CaSR function in human colon carcinoma cells to promote E-cadherin expression, suppress β -catenin/TCF activation, and suppress the malignant behavior (*i.e.*, anchorage-independent growth) of these cells. Reduced or loss of CaSR expression was observed in undifferentiated primary carcinomas by comparison with normal colon epithelial crypt

cells. It is concluded that extracellular Ca²⁺ and the CaSR may function to regulate the differentiation of colonic epithelial cells, and disruption of this ligand receptor system may contribute to abnormal differentiation, malignant progression, or both. In addition, Ca²⁺ may mediate its chemopreventive action through the promotion of E-cadherin expression and suppression of β -catenin/TCF activation.

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