Expression of the Carcinoembryonic Antigen Gene Is Inhibited by SOX9 in Human Colon Carcinoma Cells

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Abstract

The human carcinoembryonic antigen (CEA) is overexpressed in many types of human cancers and is commonly used as a clinical marker. In colon cancer, this overexpression protects cells against apoptosis and contributes to carcinogenesis. Therefore, CEA-expressing cells as well as CEA expression itself constitute potential therapeutic targets. In this report, we show that the transcription factor SOX9 down-regulates CEA gene expression and, as a probable consequence, induces apoptosis in the human colon carcinoma cell line HT29Cl.16E.

Introduction

The carcinoembryonic antigen (CEA), a member of the immunoglobulin superfamily, was originally identified in human fetal colon and colorectal cancers, and is widely used as a clinical tumor marker. This cell-surface glycoprotein is normally expressed in a variety of epithelial tissues such as the urogenital, respiratory, and gastrointestinal tracts. In the healthy human colon, the CEA protein is restricted to the apical side of the well-differentiated epithelial cells forming the luminal surface (1–3). Little is known about CEA function, but it acts at least as a homotypic adhesion molecule and is implicated in cell aggregation (3). CEA is overexpressed in numerous human cancers, where, in contrast with healthy tissues, it is present on the entire surface of cancer cells. Up-regulation of CEA occurs at the microadenoma stage in the colon of patients with APC mutations (4), and activation of oncogenic c-Ki-ras proteins during colon cancer progression results in up-regulation of CEA expression and disruption of basolateral polarity (5). Recently, Wirth et al. (6) have shown that CEA has antiapoptotic and prometastatic roles in colon cancer cells, and Ordonez et al. (7) reported that overexpression of CEA can protect tumor cells from undergoing anoikis (apoptosis induced by loss of cell contact with the extracellular matrix). Although decreasing CEA expression in cancer cells might lead to new approaches for the management of cancers of the colon and other organs (8, 9), little is known about the regulation of CEA expression. We show here that forced expression of SOX9, a novel intestinal crypt transcription factor (10), inhibits CEA gene expression and induces apoptosis in a human colon carcinoma cell line.

Materials and Methods

Cell Culture and Transfection. HT29Cl.16E cells (courtesy of C. Laboisse) were cultured in DMEM supplemented with 10% fetal bovine serum and glutamine. HT29Cl.16E cells were transfected using Tfx-50 reagents (Promega, Madison, WI). N-terminally flagged wild-type SOX9 and C-terminally truncated AC206SOX9 expression constructs (11, 12) were used to generate stable transfectant HT29Cl.16E cells inducibly expressing SOX9 or a dominant-negative form of SOX9 (AC206SOX9; T-Rex System, Invitrogen, San Diego, CA). The “SOX-luciferase” reporter construct consists of seven copies of the AACAAAG sox-binding sequence, inserted upstream of a minimal herpes simplex thymidine kinase promoter, and the control “SAC-luciferase” construct consists of seven copies of the CCGGGGT sequence (generous gift from Prof. H. Clevers). The pCMV-Luc and CEA424Luc vectors (13) were provided by Dr. W. Zimmermann.

Reverse Transcription-PCR. Total RNA was prepared with RNeasy kit (Qiagen, Courtaboeuf, France). One microgram of total RNA was used to prepare cDNA with M-MulV reverse transcriptase (New England Biolabs, Beverly, MA). Primers specific for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are forward 5’T-GAC CAG AGT CCA TGC CAT cac T-3 and reverse 5’T-ACC ACC ACC CTG TTG CTG TAG-3 and using CEA, forward 5’T-GGG CCA CTG TCG GCA TCA TGA TTG G-3 and reverse 5’T-GTG AGC TGT TGC AAA TGG TTT AAG GAA GAA GC-3. GAPDH primer set was amplified at 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1 minute for 15 cycles followed by a 10-minute extension at 72°C. CEA primer set was amplified at 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 1 minute for 30 cycles followed by a 10-minute extension at 72°C. Amplified PCR products were electrophoresed on a 2% agarose gel containing ethidium bromide (0.5 μg/ml).

Quantitative Reverse Transcription-PCR. RNA extraction and cDNA synthesis were done as above. Amplification was conducted in a LightCycler (Roche, Idaho Falls, ID) using the Quantitect SYBER Green PCR kit (Qiagen). Primers specific for GAPDH are forward 5’T-GAG AAG GCT GGG GCT CAT-3 and reverse 5’T-CTC TGA TGT TCA GGA TGC CTG-3; for CEA, forward 5’T-GGG CCA CTG TCG GCA TCA TGA TTG G-3 and reverse 5’T-GTG AGC TGT TGC AAA TGG TTT AAG GAA GAA GC-3; for human intestinal alkaline phosphatase, forward 5’T-TGG GTG TCC TCG TCT CTT ACT ACA-3 and reverse 5’T-GTG TGC CAA ACC GGT GGT A; for human intestinal alkaline phosphatase, forward 5’T-GGG GCA CTG TCG GCA TCA TGA TTG G-3 and reverse 5’T-GTG AGC TGT TGC AAA TGG TTT AAG GAA GAA GC-3; for human mucin 2, forward 5’T-TGG GTG TCC TCG TCT CTT ACT ACA-3 and reverse 5’T-GTG TGC CAA ACC GGT GGT A; for human intestinal alkaline phosphatase, forward 5’T-GGG GCA CTG TCG GCA TCA TGA TTG G-3 and reverse 5’T-GTG AGC TGT TGC AAA TGG TTT AAG GAA GAA GC-3; for human intestinal alkaline phosphatase, forward 5’T-GGG GCA CTG TCG GCA TCA TGA TTG G-3 and reverse 5’T-GTG AGC TGT TGC AAA TGG TTT AAG GAA GAA GC-3; and for human specificity protein 1, forward 5’T-ACC CAA CCA GTG TGG CTA TTA GAA GAA GC-3 and reverse 5’T-ACC CAA CCA GTG TGG CTA TTA GAA GAA GC-3. The cycling variables were 15 minutes at 95°C, followed by 40 cycles of 15 seconds at 94°C, 25 seconds at 62°C, and 15 seconds at 72°C. GAPDH, a common housekeeping gene, was used as an internal control for an equal amount of starting material.

Western Blot Analysis. After Bradford quantification, equal amounts of total cellular protein extracts were electrophoresed on an acrylamide denaturing gel and transferred by electroblotting onto a nitrocellulose membrane. Primary antibodies used were rabbit anti-SOX9 (11), rabbit anti-CEA (Neomarkers, Fremont, CA) and M2 monoclonal antibody anti-C. Blots were developed using the chemiluminescent substrate (Amersham Bioscience Europe). Immunohistochemistry. Sections of paraffin-embedded normal human colon were generously provided by Dr. C. Marty-Double (CHU, Nîmes, France). For immunohistochemistry, Envision+ (DAKO, Trappes, France) was used as a secondary reagent, staining was developed with 3,3’diaminobenzidine (brown precipitate), and a haematoxylin counterstain was used.

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Research Article
Results and Discussion

SOX9 Expression in the Human Colon. The epithelium of the adult human colon is a flat surface with many invaginations known as crypts. This epithelium is continuously renewed from undifferentiated multipotent stem cells located at the base of the crypts. Stem cells divide to produce three types of daughter cells (absorptive, goblet, and endocrine). Goblet cells are the most abundant cell type in the colon.

SOX transcription factors belong to the superfamily of high mobility group proteins, and are involved in tissue development and cell fate choice (14). Recently, we have shown that the SOX9 protein is expressed in the mouse intestine epithelium where it plays a crucial role in the preservation of its integrity, and that the Wnt/β-catenin/TCF4 pathway is needed for SOX9 expression in the intestine epithelium (10). As a consequence, SOX9 is also strongly expressed in all the colon carcinoma cell lines tested, which present a constitutive activity of the β-catenin/TCF4 complex, due to activating mutations in components of the Wnt pathway. We used immunohistochemistry to study SOX9 expression in sections of healthy human colon. As shown in Fig. 1A, a strong nuclear staining was observed only in epithelial cells, with an expression gradient from the bottom of the crypts to the top. No nuclear SOX9 staining was detected in the mature epithelial cells constituting the colon surface. Indeed, SOX9 is strongly and specifically expressed in crypt cells (Fig. 1A and B) whereas CEA is expressed only in the differentiated cells constituting the colon surface (2).

SOX9 Down-regulates CEA Expression. With the aim of determining whether the increase of CEA protein expression during differentiation was linked to the SOX9 down-regulation, we generated inducible stable transfectant cell lines expressing either a Flag-tagged wild-type SOX9 (HT29Cl.16E-SOX9) or a Flag-tagged dominant-negative form of SOX9 (HT29Cl.16E-ΔC206SOX9). The SOX9 dominant-negative protein still binds to DNA but does not activate transcription of target genes because the C-terminal transcription activation domain is lacking (11, 12). After doxycycline treatment of the HT29Cl.16E-SOX9 cells, the exogenous SOX9 protein was readily detected using an antibody against the FLAG tag (Fig. 2A, top). Induction of exogenous SOX9 expression resulted in a clear increase of the total SOX9 protein (Fig. 2A, bottom). The C-terminally truncated SOX9 protein (ΔC206SOX9) could not be detected with the anti-SOX9 antibody, directed against the deleted C-terminal region, but the expression of the doxycycline-induced ΔC206SOX9 protein was efficiently detected with the anti-FLAG antibody (Fig. 2A). To analyze the transcriptional activity of the doxycycline-induced SOX9 and ΔC206SOX9 proteins, inducible HT29Cl.16E-SOX9 and HT29Cl.16E-ΔC206SOX9 cells were transfected with a "sox-luciferase" reporter construct consisting of consensus Sox binding sites and a thymidine kinase minimal promoter, controlling the expression of a luciferase coding sequence. The basal luciferase activity was strongly increased after induction of SOX9 expression by doxycycline whereas it decreased by 50% on induction of ΔC206SOX9 expression (Fig. 2B). This indicates that the
exogenous SOX9 and ΔC206SOX9 proteins affect the transcription of the SOX reporter gene, as expected.

Next, to analyze the effect of inducing the expression of SOX9 on CEA expression level, HT29Cl.16E-SOX9 cells were grown for 2 days, in nonconfluent conditions, in the presence and absence of doxycycline. Reverse transcription-PCR (RT-PCR) analysis of CEA expression revealed that the basal level of the CEA mRNA decreased after induction of SOX9 expression (Fig. 2C). A typical experiment of real-time RT-PCR is represented in Fig. 2D. Quantification indicated a 3.45-fold reduction (three determinations; bars, SE; * P < 0.001) of the CEA mRNA levels on induction of SOX9 expression. Thus, SOX9 inhibits the expression of CEA.

HT29Cl.16E cells differentiate along the secretory lineage when maintained in confluent culture, and when treated with NaB, aspects of this lineage are repressed and markers of an absorptive cells may be induced (17). In both cases, as this differentiation results in a strong increase of CEA expression and a decrease of SOX9 expression, we asked whether forced SOX9 expression during the differentiation process, along either of the two lineages, would affect the expression level of the endogenous CEA protein. To test this, the CEA protein expression was analyzed before and after confluence-induction of NaB-induced differentiation and with or without doxycycline induction of SOX9 expression (Fig. 2E). After 5 days of culture, when the cells were not yet confluent, the level of CEA protein was significantly lower in cells with forced expression of SOX9. After 23 days of culture, when the cells are differentiated into goblet-like cells and endogenous SOX9 has been down-regulated, the CEA protein was strongly expressed in non-doxycycline-treated cells whereas it was almost undetectable in cells where SOX9 expression is forced. Expression of the CEA protein was also strongly increased when cells with a secretory phenotype were induced to switch to an absorptive phenotype by NaB treatment and this expression was also reduced on induction of SOX9 expression. Thus, independently of the cell lineage, the down-regulation of SOX9 expression is required to allow the up-regulation of CEA expression associated with differentiation of colon cancer cells.

**Endogenous SOX9 Limits the Basal CEA Expression Level in HT29Cl.16E Cells.** Inducible HT29Cl.16E-ΔC206SOX9 cells were grown for 2 days in nonconfluent conditions in the presence or absence of doxycycline. RT-PCR analysis revealed that the CEA mRNA level was increased in cells expressing the ΔC206SOX9 protein (Fig. 3A) and real-time RT-PCR quantified this increase at 1.46-fold (three determinations; P < 0.001; Fig. 3B). An increase of CEA protein level was also observed when ΔC206SOX9 expression was induced for 5 days (Fig. 3B). This indicates that the endogenous SOX9 protein present in exponentially growing colon cancer cells limits the expression of CEA. Interfering with the function of this endogenous SOX9 with a dominant-negative protein abrogates this inhibition and results in increased CEA expression. Thus, in exponentially growing HT29Cl.16E cells, strong SOX9 expression limits CEA transcription. During differentiation, SOX9 expression is down-regulated, probably as a result of the decrease of β-catenin/TCF4 signaling which was already described in Caco-2 cells (18). This down-regulation of SOX9, in turn, derepresses the expression of CEA. Consistent with this, in the human colon, SOX9 is expressed specifically in actively proliferating cells located at the bottom of the crypts, where CEA is absent. This
suggests that CEA expression might also be inhibited by SOX9 in vivo, but this remains to be shown.

**SOX9 Inhibits Differentiation of Both Secretory and Absorptive Cell Lineage.** We have previously shown that SOX9 inhibits expression of the mucin 2 (MUC2) gene, a goblet cell-specific differentiation marker (10), in human colon carcinoma goblet-like cells. We then asked whether SOX9 is also able to inhibit the differentiation of HT29CI.16E-SOX9 cells into the absorptive phenotype. For that purpose, we treated the HT29CI.16E-SOX9 cells with NaB, which is known to promote differentiation of the HT29CI.16E cells along the absorptive phenotype (17). The expression of the intestinal alkaline phosphatase gene (ALP), which is an enterocyte-specific marker, was analyzed by real-time quantitative RT-PCR upon induction of SOX9 expression with doxycycline. As previously reported (17), expression of alkaline phosphatase was increased by NaB treatment, and this increase was inhibited by forced SOX9 expression (Fig. 4). CEA expression, which is normally found in both the secretory and the absorptive cell lineages, was increased by NaB, and this increase was strongly inhibited by SOX9 (Fig. 4). As basal expression of mucin 2 was weak and not significantly modified by NaB (Fig. 4) or forced SOX9 expression, we concluded that the observed decrease in alkaline phosphatase expression upon SOX9 overexpression was not a consequence of a SOX9-mediated reorientation of the enterocyte fate into a goblet fate. Instead, SOX9 inhibits differentiation of HT29CI.16E-SOX9 cells into both the secretory and absorptive cell lineages.

**SOX9 Repression of the CEA Gene Promoter.** The minimal CEA promoter was first described as a 424 bp sequence upstream of the translation initiation site (−424 to +1) conferring cell-type-specific expression of a reporter gene (13). Later, it was shown that the sequence between −403 and −124 bp directed high levels of expression in colon carcinoma cells and that inclusion of an additional upstream sequence (−1,098 to −403 bp) repressed all the activity (19). Footprinting identified five putative cis-acting elements in the 1,098 bp sequence situated upstream of the initiation codon (ref. 19; Fig. 5A). Among the five cis-acting elements found in the CEA promoter, four are positive regulatory elements (FP1-FP4) and one is a negative regulatory element (FP5; ref. 19).

When transiently transfected into HT29CI.16E cells, the full-length SOX9 efficiently activated transcription of a SOX-luciferase reporter construct (three different experiments; P < 0.001). The SOX9 protein contains a high mobility group domain, which mediates sequence-specific DNA binding, and a C-terminally located transcription activation domain (12). The ΔC206SOX9 construct, lacking the C-terminal transcription activation domain (Fig. 5B), did not activate the SOX-luciferase reporter. On the contrary, the ΔC206SOX9 construct acted as a dominant-negative protein (three different experiments; P = 0.005) interfering with the basal luciferase activity driven by endogenous SOX9 (Fig. 5B). Next, these two constructs were transiently transfected into HT29CI.16E cells together with the CEA minimal promoter (CEA424Luc). Cotransfection of the CEA424Luc vector with the full-length SOX9-expressing vector produced a 3-fold significant decrease (three different experiments; P = 0.002) of the luciferase activity (Fig. 5C) whereas cotransfection of the ΔC206SOX9 truncated protein had no significant effect (three different experiments; P = 0.464). These experiments show that SOX9 inhibits the CEA minimal promoter activity in HT29CI.16E cells and that the transcription activation domain of SOX9 is required for this inhibition. Moreover, this inhibition is not due to the FP5 silencer element, which is absent from the 424 bp minimal CEA promoter.

When we analyzed the 424 bp sequence of the CEA minimal promoter, we found that it lacks the SOX9 optimal binding sequence AGAACAATGG (20). Furthermore, the SOX consensus binding sequence AACAAAG, present in the “SOX-luciferase” reporter construct, was not found in the 424 bp sequence. This analysis was then extended to the 10.8 kb of the CEA gene, situated upstream of the initiation codon (Genbank accession no. Z21818), and again no putative SOX9 or SOX consensus binding site could be detected.
SOX9 expression in exponentially growing or differentiated HT29Cl.16E cells, under conditions in which CEA expression was efficiently down-regulated by SOX9. No significant variation of upstream stimulatory factor-1 or specificity protein 1 could be detected, indicating that the SOX9-mediated repression of CEA does not involve regulation of the upstream stimulatory factor-1 and specificity protein 1 transcription factors. Several other still unknown transcription factors bind to the minimal CEA promoter (FP1-FP4; Fig. 5A; ref. 19), and the possible deregulation of these nuclear factors by SOX9 might explain the inhibition of expression of CEA by SOX9.

Effect of SOX9 Expression on Apoptosis. One of the well-described consequences of CEA deregulated overexpression in cancer cells is the inhibition of apoptosis (6). As SOX9 expression results in a reduction of CEA protein level, then it might be expected to induce apoptosis. To test this hypothesis, we stained the inducible HT29Cl.16E-SOX9 cells by the terminal deoxynucleotidyl transferase-mediated nick end labeling assay on induction of SOX9 expression. A typical microscope field of stained cells is shown on Fig. 6A. The analysis of a significant number of fields (n = 22) revealed that cells with forced expression of SOX9 were more inclined to undergo apoptosis (7% ± 1.2 for induced cells versus 0.8% ± 0.3 for the control without doxycycline; Fig. 6C). This result indicates that forced SOX9 expression increases the apoptotic rate of HT29Cl.16E cells, potentially due to the down-regulation of the CEA protein.

In summary, clinical studies have suggested that CEA expression is correlated with metastatic growth of colon cancer, and a preoperative elevated serum level of CEA is therefore associated with a poor prognostic (23). Moreover, CEA plays a direct role in metastatic behavior because, first, inhibition of its endogenous level inhibits the metastatic tumor growth of colon cancer cells (6), and second, CEA is antiapoptotic (24) and inhibits anoikis (7). All these functions of CEA in colon cancer cells are potentially

Direct binding of SOX9 to the minimal promoter of CEA, causing inhibition of its activity, is very improbable for at least three reasons. First, SOX9 function has been analyzed in a number of physiologic situations, including chondrogenesis (21), sex determination (11), intestinal epithelium physiology (10), and nervous system development (22), and it has always been found to be a transcriptional activator. Second, the transcription activation domain of SOX9 is required for the observed inhibition of the CEA gene promoter. Third, sequence analysis of the CEA gene promoter (10.8 kb) did not reveal any putative SOX9 binding sites. Taken together, these data indicate that most likely SOX9 does not regulate CEA expression by direct binding to the CEA promoter but rather regulates the expression of one or several nuclear factors implicated in CEA promoter activity.

Despite its medical importance, little is known about the transcriptional regulation of the CEA gene. Only two transcription factors are known to bind to the CEA promoter. Specificity protein 1 recognizes FP2 and FP3 regulatory elements, and upstream stimulatory factor-1 recognizes the FP1 regulatory element and activates the CEA gene promoter in vivo (19). To determine whether these factors are involved in the regulation of the CEA gene by SOX9, we used real-time PCR to monitor upstream stimulatory factor-1 and specificity protein 1 expressions upon doxycycline induction of HT29Cl.16E cells, under conditions in which CEA expression was efficiently down-regulated by SOX9.
reversible by SOX9, as it inhibits CEA expression. SOX9 is the first tissue-specific transcription factor shown to regulate the CEA gene. We also show that SOX9 induces apoptosis, and we propose that this is due to the abolition of the antiapoptotic effect of CEA. Recently, it has been described that overexpression of CEA provides a tumorigenic contribution to colon carcinogenesis (25), and the authors concluded their article by suggesting that “reversing the overexpression of CEA which occurs in so many different human cancers represents an appealing novel approach for cancer treatment.” Our study shows that the transcription factor SOX9 is able to reverse this CEA overexpression in colon cancer cells.

References

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