Expression of the Cdx1 and Cdx2 Homeotic Genes Leads to Reduced Malignancy in Colon Cancer-derived Cells

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We have previously described an inverse relationship between Cdx1 and Cdx2 mRNA levels and the extent of dysplasia and severity of clinical outcome in colorectal carcinoma, suggesting that altered expression of these genes was associated with colorectal carcinogenesis or tumor progression. To investigate further their involvement in the physiopathology of colorectal cancer, HT29 colon carcinoma cells that show very low Cdx expression were transfected with Cdx1 and/or Cdx2 cDNA to elicit their overexpression. Growth rate, tumorigenicity, resistance to apoptosis, and migration potential of the corresponding cells were analyzed. Growth rate of cells overexpressing Cdx2 decreased by half, whereas overexpression of Cdx1 had no effect. However, cells overexpressing both Cdxs had a growth rate reduced to 20% of control. In cells overexpressing Cdx1 or Cdx2, tumorigenicity and resistance to apoptosis induced by serum starvation, ceramide, or staurosporine were not changed compared with control cells; yet phorbol ester-stimulated cell migration was decreased by 50%. In cells overexpressing both Cdx1 and Cdx2, tumorigenicity was decreased by 50%, resistance to apoptosis was significantly lowered, and stimulated cell migration was further decreased to 15% of control compared with cells expressing Cdx1 or Cdx2. Finally, cells overexpressing both Cdxs showed strongly decreased Bcl-2 expression, which could account for their increased sensitivity to apoptosis. These findings show that, in HT29 cells, both Cdx1 and Cdx2 genes must be expressed to reduce tumorigenic potential, to increase sensitivity to apoptosis, and to reduce cell migration, suggesting that the two genes control the normal phenotype by independent pathways. This may explain why loss of Cdx1 or Cdx2 expression is associated with tumor development and invasiveness in colorectal tumors.

In an effort to characterize the mechanisms involved in colorectal cancer initiation and progression, we have developed a strategy based on the constitution of a large repertoire of transcripts from a colorectal tumor, all characterized by partial sequencing (1). Expression of these expressed sequence tags in normal and cancerous colon was compared, and those most differentially expressed were selected. Genes detected by these means may be causative or instrumental in tumor induction or/and progression. Looking for such genes, we found that the Cdx1 and Cdx2 homeotic genes were concomitantly down-regulated in about 85% of colorectal cancers (2). Such low expression of Cdx1 or Cdx2 in colon carcinoma was verified by immunohistochemistry (3, 4) and by reverse transcription polymerase chain reaction (5) studies. Cdx1 and Cdx2 are interesting candidates that could play a role in colon cancer pathologies because Chawengsaksophak et al. (6) recently reported the occurrence of multiple intestinal adenomatous polyps in the proximal colon of Cdx2−/− mice, suggesting that lowering Cdx2 levels in intestinal cells would suffice to induce intestinal tumors. Also, Suh and Traber showed that expression of the Cdx2 gene in an intestinal cell line could stimulate cell differentiation and growth arrest (7). A corollary of these findings might be that the down-regulation of Cdx2 would account for the loss of differentiation and exaggerated cellular growth observed in intestinal tumors.

In the present paper we have used HT29, a cell line derived from a colon tumor that shows very low Cdx1 and Cdx2 expression, to investigate the role of these homeotic genes in the physiopathology of colorectal cancer. To this end, HT29 cells were transfected with Cdx1 and Cdx2 expression plasmids, alone or in combination. The impact of Cdx overexpression on important events involved in tumor progression and invasion such as tumorigenicity, resistance to apoptosis, and cell migration was analyzed. It was found that Cdx1 and Cdx2 cooperate to reduce the malignancy of HT29 cells.

MATERIALS AND METHODS

Cell Culture—the human colon carcinoma cell line HT29 was obtained from Dr. A. Zweibaum (INSERM U.178). Cells were routinely cultivated at 37 °C in a 5% CO2, 95% air atmosphere in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum (Life Technologies, Inc.), 4 mM L-glutamine, 50 units/ml of penicillin, and 50 μg/ml streptomycin. Upon reaching 80–90% confluency, cells were dissociated with 0.05% trypsin and 0.02% EDTA in Puck’s saline A and replated into 100-mm Petri dishes.

Establishment of Stable Cell Lines—All cDNA inserts were generated by reverse transcription polymerase chain reaction using RNA from human small intestine as template, and primers were selected from the cDNA sequence of Cdx1 and Cdx2 (2). To synthesize the Cdx1 cDNA insert, the forward primer was 5′-GGGAATTCACGGTGAG-CAGTGCCTGTTGCTTC-3′ and the reverse primer was 5′-GGGAATTCACAGGGAGCCACTCAGAACCC-3′, which yielded a 942-base pair product (nucleotides 1–942 of human Cdx1). To synthesize the Cdx2 cDNA insert, the forward primer was 5′-GGGAATTCACGGCAGCTG- GTGAGGTCTG-3′ and the reverse primer was 5′-GGGAATTCACGGCAGCTGGTACAGGG-3′, which yielded a 1048-base pair product (nucleotides 32–1080 of human Cdx2). The coding sequences of Cdx1 and Cdx2 were amplified with oligonucleotides that incorporated EcoRI
restriction sites (underlined). The Cdx1 and Cdx2 polymerase chain reaction products were EcoRI-digested and subcloned into the EcoRI restriction site of the mammalian expression vector pCDNA3 (Invitrogen), downstream from the cytomegalovirus promoter. That plasmid also contained the aminoglucoze phosphotransferase 3' gene that confers resistance to G418. The antibiotic and G418 selection was performed at 65 °C during 16–20 h in the above solution supplemented with G418 (600 μg/ml) starting 48 h after transfection. Surviving colonies were picked, dried, and maintained in standard culture medium supplemented with G418 (400 μg/ml). To obtain cells expressing both Cdx1 and Cdx2, the HT29 pCDNA4/Cdx2S-transfected cells were transfected with the pCEP4/Cdx1 plasmid and selected with G418 (400 μg/ml) and hygromycin (300 μg/ml) concomitantly. Surviving clones were pooled and maintained in standard culture medium supplemented with G418 (400 μg/ml) and hygromycin (300 μg/ml).

The pcDNA3CAT plasmids were stably transfected into HT29 cells using the Lipofectin reagent (8) as described by the supplier (Life Technologies, Inc.). To select for stable transfectants, the cells were cultured over 3–4 weeks in media containing G418 (600 μg/ml) starting 48 h after transfection. Surviving colonies were picked, dried, and maintained in standard culture medium supplemented with G418 (400 μg/ml). The Cdx2 DNA insert was recovered from the pDNA4/Cdx2S plasmid by endonuclease restriction and directionally subcloned into the pCEP4 vector (Invitrogen), in which Cdx2 expression is under the control of the cytomegalovirus promoter but confers resistance to hygromycin. This construction was checked by restriction pattern. Plasmid DNA was purified with the Qiagen plasmid kit (Diagen, Hilden, Germany), and DNA concentration was measured by spectrophotometry.

The pCDNA3/Cdx1S, pCDNA3/Cdx2S, and pCDNA3/CAT plasmids were transfected into HT29 cells using the Lipofectin reagent (8) as described by the supplier (Life Technologies, Inc.). To select for stable transfectants, the cells were cultured over 3–4 weeks in media containing G418 (600 μg/ml) starting 48 h after transfection. Surviving colonies were picked, dried, and maintained in standard culture medium supplemented with G418 (400 μg/ml). The Cdx2 DNA insert was recovered from the pDNA4/Cdx2S plasmid by endonuclease restriction and directionally subcloned into the pCEP4 vector (Invitrogen), in which Cdx2 expression is under the control of the cytomegalovirus promoter but confers resistance to hygromycin. This construction was checked by restriction pattern. Plasmid DNA was purified with the Qiagen plasmid kit (Diagen, Hilden, Germany), and DNA concentration was measured by spectrophotometry.

To avoid clonal variations, after transfection and selection with antibiotics, more than 100 independent colonies of each transfection were pooled, replated, grown to confluence, dissociated, aliquoted, and stored at −140 °C. After replating, cells were used for less than six passages. Transfected cells were grown with appropriate selection antibiotic, except during experiments. We used Northern blot assay to test whether HT29 cells expressed the transfected genes. RNA was purified from HT29-transfected cells, and 20 μg of RNA was subjected to electrophoresis on 1% agarose containing 2.2 μM formaldehyde and transferred to nylon membranes (Nytran Plus) as described (9). For RNA probes, the membranes were prehydrated at 65 °C for 4 h in 5× SSC, 50 mM Heps (pH 6.8), 1% SDS, 5 μl 100 mM Heps (pH 7.4), 500 mM NaCl, and 5 mM dithiothreitol), 1 μl of glycogen, and hybridization was performed as described above. Specific hybridization of probes was analyzed by exposure to a Kodak X-Omat AR film.

Analysis of the Cdx1 and Cdx2 Proteins—Expression of Cdx1 was estimated by monitoring the activity of the Cdx1-inducible Hoxa-7 promoter in HT/Cdx1S and HT/Cdx1S2S cells. These cells express Cdx1 (see above). The Hoxa-7 LacZ (AX470 construct) and TK LacZ (control) plasmids (kindly provided by Dr. P. Gruss, Max Planck Institute for Biophysical Chemistry, Göttingen) have been previously described (10). The cells were transfected using the Lipofectin reagent as described above. At 48 h post-transfection cell extracts were prepared, and β-galactosidase assay was performed essentially as described previously (9).

Expression of Cdx2 was investigated by electrophoretic mobility shift assay in HT/Cdx2S and HT/Cdx1S2S cells. Nuclear extracts from HT/CAT, HT/Cdx2S, and HT/Cdx1S2S cells were prepared essentially as described by Dignam et al. (11) except that the buffers were supplemented with a mixture of 1 mM protease inhibitors (aprotinin, chymostatin, and peptatin, all from Boehringer Mannheim). Protein concentration was determined by the Bradford assay (12). The extracts were frozen in liquid nitrogen and stored at −80 °C. Protein aliquots were used for gel shift experiments. The synthetic oligonucleotides were used for the gel shift assays: 5′-GTC-CAATACAAAATCTTGTAGTA-3′ and 5′-TACTCCATAAGTTTTTATTG-CAC-3′ (13). 10 pmol of 5′ ends were phosphorylated with T4 polynucleotide kinase using the Ready-To-go kit (Amersham Pharmacia Biotech) with [γ-32P]ATP before annealing. The annealing was performed as follows: 10 μg of each strand was mixed in a 50-μl solution containing 10 μM Tris (pH 7.8), 1 mM EDTA, and 250 mM KCl. The mixture was boiled for 5 min and then incubated at 65 °C for 1 h. The water bath was turned off, and the hybridization mixture was allowed to cool slowly to room temperature. The binding reactions were performed in a mixture of 25 μl containing 6 μl of 10× binding buffer (100 mM Tris (pH 7.5), 500 mM NaCl, and 5 mM dithiothreitol), 1 μl of MgCl2, 500 μl of glycogen, 1 μl of labeled probe (about 1000 cpm), and 50 ng of single-stranded DNA. The reactions were incubated for 30 min at 4 °C. Then, 250 μl of 1% SDS-containing polyacrylamide gel electrophoresis (about 100 cpm) was added before a final 30-min incubation at 4 °C. Samples were loaded on a prerun nondenaturing 5% polyacrylamide gel. The following proteins were size markers: 70 kDa and 45 kDa. The gel was then dried and autoradiographed (overnight at −80 °C).

Cell Proliferation—HT/Cdx1S, HT/Cdx2S, HT/Cdx1S2S, and HT/CAT cells were plated in 12-well tissue culture plates at 5 × 103 cells/well. After 1, 2, 3, 4, 5, and 6 days, cell number was estimated by MTT assay (Sigma) and/or counted on a hemacytometer. Two sets of experiments were done, each one in triplicate.

Tumorigenicity Assays—Tumorigenicity was assayed by subcutaneous injection of 5 × 106 HT/Cdx1S, HT/Cdx2S, HT/Cdx1S2S, HT/CAT, or HT29 parental cells resuspended in 200 μl of phosphate-buffered saline into the flanks of 4-week-old nude mice. They were maintained under specific pathogen-free conditions and fed with sterile commercial chow (U.A.R., Villemoisson-sur-Orge, France) and water ad libitum. Tumors were measured in two dimensions with linear calipers at the indicated days.

Serum Starvation-, Ceramide- and Staurosporine-induced Apoptosis—To assess differences in resistance to apoptosis, the HT29-transfected cells were submitted to three apoptotic stimuli, i.e. serum starvation, ceramide, and staurosporine. For serum starvation-induced apoptosis, 5 × 105 cells were plated on 50-mm Petri dishes, and after 48 h, the cells were serum starved for 4 days. Surviving cells were quantified by MTT following the instructions of the supplier (Sigma), and DNA fragmentation was measured by gel electrophoresis and by flow cytometry. Gel electrophoresis was performed using standard protocols (9) and flow cytometry as follows. Cells were harvested from culture and permeabilized in 500 μl of 50 μg/ml propidium iodide, 0.05% Nonidet P-40, 4000 units/ml RNase in phosphate-buffered saline. Reagents were obtained as a kit from Coultronics France (Margency, France) and used according to the manufacturer’s recommendations. After washing, samples were allowed to equilibrate at room temperature in the dark for at least 1 h before analysis. Propidium iodide fluorescence analysis was performed in a flow cytometer (EPICS, Coulter Corporation).

Drug-induced apoptosis was elicited by treating cells with 20 μM ceramide (Euromedex) or 100 nM staurosporine (Sigma) for the indicated times. In preliminary studies, apoptosis induced by ceramide and staurosporine was confirmed by analysis of DNA fragmentation on gel electrophoresis and flow cytometry. The number of surviving cells was estimated by MTT assay. Data shown are the mean ± S.E. of three independent experiments performed in duplicate, and the percentage of surviving cells was defined as the number of ceramide- or staurosporine-treated cells relative to untreated cells.

Bcl-2 Expression in HT/Cdx1S, HT/Cdx2S, HT/Cdx1S2S, and HT/CAT Cells—Expression of Bcl-2 was analyzed by Western blotting. Cells were lysed in ice-cold lysis buffer containing 150 mM NaCl, 1% Triton X-100, 10 mM Tris (pH 7.4), 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 5 mM benzamidine for 20 min at 4 °C. Protein concentration was measured by the Bradford method (12), and 100-μg samples were mixed with 2× Laemmli buffer. Western blot analysis was performed according to standard procedures (9). Briefly, samples were resolved by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose filters (Schleicher & Schuell), and incubated with the primary antibody diluted 1:2500 in NaCl/F (pH 7.4) containing 9% nonfat dry milk for 2 h at room temperature under gentle shaking. The primary antibody was monoclonal antibody purchased from Boehringer Mannheim (Meylan, France; reference number 1 624 989). After incubation, the filters were washed and incubated for 1 h at room temperature with horseradish peroxidase-linked F(ab)2 fragment anti-mouse secondary antibody (1:4000, Amersham Pharmacia Biotech). The immune complexes were visualized with enhanced chemiluminescence imaging system (Amersham Pharmacia Biotech).

Cell Migration Assays—Migration of HT/Cdx1S, HT/Cdx2S, HT/Cdx1S2S, HT/CAT, or HT29 parental cells was studied by a modifications.
struct). The cells were transfected with the Hoxa-7 LacZ or TK LacZ expression by transactivation of the Hoxa-7 promoter (Hoxa-7 LacZ construct). The cells were transfected with the Hoxa-7 LacZ or TK LacZ construct using Lipofectin reagents, and β-galactosidase activity was measured 48 h later in cell extracts. The relative values of β-galactosidase activity (means ± S.E. of three independent experiments) are illustrated. c, analysis of Cdx2 protein expression by electrophoretic mobility shift assay. Nuclear proteins isolated from the indicated cells were analyzed by electrophoretic mobility shift assay using a double-stranded oligonucleotide corresponding to the Cdx2-responsive element of the sucrase isomaltase gene (13). As shown in Fig. 1, the DNA-protein complexes could form with nuclear extracts from HT/Cdx1S and HT/Cdx1S2S but not from HT/CAT control cells. In each case, competition with 100-fold molar excess of unlabeled specific oligonucleotide abolished the formation of the shifted complexes, whereas competition with 400-fold molar excess of unlabeled nonspecific oligonucleotide failed to compete with the formation of the retarded band (data not shown).

Establishment of Stable Cdx Transfectants—The transfection efficiency was evaluated by measuring chloramphenicol acetyltransferase activity in cell extracts for cells transfected with the chloramphenicol acetyltransferase gene (data not shown) and Cdx mRNA levels by Northern blot analysis for cells transfected with the Cdx1 and Cdx2 expression plasmids. Results shown in Fig. 1 indicate that Cdx1 and Cdx2 genes were expressed at very low levels in HT29 parental and HT/CAT-transfected cells because the corresponding signals were barely detectable after a 10 days exposure of the blots. By contrast, the transfected cell line expressed high levels of the corresponding transcript or of both transcripts in the case of Cdx1 and Cdx2 double transfectants.

To demonstrate that Cdx1 was functional in HT29 Cdx1-transfected cells, we performed transactivation experiments with constructs involving the Hoxa-7 promoter in HT/Cdx1S, HT/Cdx1S2S, and HT/CAT control cells. The Hoxa-7 promoter is regulated by Cdx1 (15). Compared with HT/CAT, transfection of the Hoxa-7 LacZ (AX470) construct led to an average induction of Lac Z expression reaching 5-fold in HT/Cdx1S and 7-fold in HT/Cdx1S2S, (Fig. 1), strongly suggesting that Cdx1 is indeed expressed in these cells. The control construct carrying the TK promoter instead of Hoxa-7 did not show induction.

The presence of Cdx2 was confirmed by electrophoretic mobility shift assay using a double-stranded oligonucleotide corresponding to the Cdx2-responsive element of the sucrase isomaltase gene (13). As shown in Fig. 1, the DNA-protein complexes could form with nuclear extracts from HT/Cdx1S and HT/Cdx1S2S but not from HT/CAT control cells. In each case, competition with 100-fold molar excess of unlabeled specific oligonucleotide abolished the formation of the shifted complexes, whereas competition with 400-fold molar excess of unlabeled nonspecific oligonucleotide failed to compete with the formation of the retarded band (data not shown).

Proliferation of HT/Cdx1S, HT/Cdx2S, HT/Cdx1S2S, and HT/CAT Cells—The rate of growth of each transfected cell line is shown on Fig. 2. Direct counting of the cells and indirect estimation of cell number by MTT assay led to similar results. Growth of HT/CAT cells was taken as reference because these cells have been transfected and selected under the same conditions as the Cdxs-transfected cells while expressing a nonrelevant protein. In fact, HT/CAT cells and the parental HT29 cells grew very similarly, suggesting little influence of transfection and selection, if any, on cell proliferation (data not shown).
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Increased Sensitivity to Serum Starvation-, Ceramide- and Staurosporine-induced Apoptosis of HT/CDx1S2S Cells—Because tumor growth and progression could be associated with a resistance to apoptosis of the transformed cells, we explored whether overexpression of Cdx1 or Cdx2 would change the sensitivity of HT29 cells to apoptosis. Apoptosis was quantified by measuring DNA fragmentation by flow cytometry and gel or by evaluating cell survival by MTT assay. When HT/Cdx1S or HT/Cdx2S cells were grown in the presence of serum, subdiploidy was observed in less than 1% of cells (data not shown), indicating that Cdx expression did not interfere with spontaneous apoptosis. When cells were starved from serum for 4 days, the percentage of apoptotic cells increased to 5–7% in HT/Cdx1S, HT/Cdx2S, HT/CAT, and HT29 cells. It was significantly higher (16%) in HT/Cdx1S2S cells (Fig. 4). Confirmation of these data was sought by measuring the apoptosis rate induced by ceramide and staurosporine, which are known to stimulate apoptosis. The optimal drug concentration that triggered apoptosis in HT29 parental cells was determined by DNA fragmentation and cell survival assays (data not shown). Then the survival of HT/Cdx1S, HT/Cdx2S, HT/Cdx1S2S, HT/CAT control, or HT29 parental cells treated by these amounts of staurosporine and ceramide was measured over a period of 18 h.

Under these conditions, cells cotransfected with Cdx1 and Cdx2 but not the cells overexpressing either Cdx1 or Cdx2 showed increased apoptosis rate, compared with parental or control HT29 cells (Fig. 4). These results confirm that sensitivity to stimulated apoptosis was increased only when Cdx1 and Cdx2 were concomitantly overexpressed.

Bcl-2 Is Down-regulated in HT/Cdx1S2S Cells—We investigated whether sensitivity to stimuliants of apoptosis was associated with modulation of Bcl-2 expression. Bcl-2 expression was monitored by Western blot analysis of HT29-transfected cells, using a specific monoclonal antibody. In cells expressing either Cdx1 or Cdx2, no change could be evidenced (Fig. 5). However, in cells in which Cdx1 and Cdx2 were concomitantly expressed, the Bcl-2 signal was significantly decreased.

Cdx1 or Cdx2 Overexpression Inhibits Phorbol Ester-stimulated Cell Migration—Cancer aggressiveness depends mostly on tumor invasiveness, and cell migration is a determining step in this process. This is why we studied the modulations of cell migration consecutive to Cdx1 or and Cdx2 overexpression. Cell migration was measured by counting the number of cells having crossed the margin of a wound made in a monolayer at a given time after wounding (14). HT29 cell migration was spontaneously very slow, but it was stimulated very significantly after cell treatment by PMA. As shown in Fig. 6, HT29 and HT/CAT migrated at a similar rate after stimulation by PMA. Under these conditions, the migration of HT/Cdx1S and HT/Cdx2S cells was profoundly impaired because it was only 40 and 37% of HT/CAT cells, respectively. Interestingly, the migration of HT/Cdx1S2S was only 16% that of HT/CAT cells, indicating that overexpression of both Cdx1 and Cdx2 resulted in a more profound inhibition of PMA-stimulated cell migration than overexpression of Cdx1 or Cdx2. Quite similar results were obtained upon treatment with mitomycin C (5 μg/ml), which inhibits cell proliferation, indicating that it was indeed cell migration that contributed dominantly to the wound closure process.

DISCUSSION

Several authors have proposed that altered expression of homeotic genes could be associated with cell transformation and cancer progression (16, 17) and that hypothesis was raised for various cancers (18, 19) including colon carcinoma (20). Previously, we (2) and others (3–5) have reported a decreased
expression of Cdx1 and/or Cdx2 in the tumors of several patients, compared with the adjacent normal colon. Furthermore, an inverse correlation was found between the severity of dysplasia and expression of Cdx1 and Cdx2. In order to get further insight into the role of these homeotic genes in colon carcinogenesis, particularly in tumor progression, we choose to analyze the consequences of re-establishing their expression in HT29 cells. These cells derive from a colon carcinoma, and they express extremely low levels of Cdx1 and Cdx2 mRNA (Fig. 1).

By appropriate transfections we obtained HT29-derived cell lines overexpressing Cdx1, Cdx2, or both and compared their capacity for tumor formation, their sensitivity to apoptosis, and their ability for migration.

The first parameter expected to be altered by Cdx expression was cell growth. Because Cdx mRNA expression is down-regulated in colon carcinoma (2–5), we looked into whether inducing Cdx overexpression would decrease the growth rate of HT29 cells. With respect to parental cells, the growth rate of HT/Cdx2S cells was indeed decreased by 50%, whereas that of HT/Cdx1S cells was not affected. This is consistent with previous reports showing a decreased growth rate in nontumoral intestine-derived cells overexpressing the Cdx2 (7) and a normal growth rate in epithelial intestinal cells of Cdx12/2 mice (15). In addition, we observed that overexpression of Cdx1 strongly potentiated the effect of Cdx2 overexpression, the growth rate of HT/Cdx1S2S being only 20% of control. Thus, Cdx2 seems to be directly involved in the control of cell growth, whereas Cdx1 would only enhance Cdx2 action.

Then we studied the relationship between Cdx expression and tumorigenicity. All cell lines, including controls, induced tumors when injected into nude mice. Cells overexpressing chloramphenicol acetyltransferase, Cdx1, or Cdx2 generated tumors that grew at the same rate as parental cells. However, the growth rate of tumors generated by cells overexpressing Cdx1 and Cdx2 was decreased by half. These results demonstrate that concomitant expression of Cdx1 and Cdx2 is required to reduce the tumorigenicity of HT29 cells but not sufficient to prevent tumor formation; conversely, they indicate that decreased expression of one of the two Cdxs may result in increased tumorigenicity. However, analyzing our findings in view of results obtained in vivo revealed some discrepancies. In Cdx12/2 mice, the growth rate of epithelial intestinal cells was unchanged, and the animals did not develop intestinal tumors (15). Cdx22/2 mice could not be analyzed because they died early after conception but 90% of Cdx2−/− mice developed intestinal tumors within the first three months of life (6). Hence, adequate expression of Cdx2 is also mandatory in vivo to pre-
When cells were exposed to serum starvation, ceramide, or staurosporine, apoptosis was induced to the same extent in control cells and cells overexpressing Cdx1 or Cdx2. By contrast, cells expressing both Cdx genes showed markedly increased sensitivity to induced apoptosis (Fig. 4). Thus, as reported above for tumorigenicity, underexpression of either Cdx1 or Cdx2 would confer a more severe phenotype to colon cells by increasing their resistance to apoptosis.

We found evidence that sensitivity to stimulated apoptosis was accompanied by down-regulation of the Bcl-2 protein in HT/Cdx1S2S cells (Fig. 5). Overexpression of Bcl-2 protein, obtained by transfection of the Bcl-2 gene into human colonic cells, renders these cells resistant to butyrate-induced apoptosis (22). These findings suggest that the anti-apoptotic gene Bcl-2 is involved in the pathway by which the Cdx control apoptosis in colon cells.

The ability of cells to migrate is the last parameter that we analyzed in conjunction with Cdx expression. This is an important aspect because cell migration is required for invasivity and metastasis (23). Migration is a complex function that depends on a dynamic regulation of the interaction of cell surface adhesion receptors with their ligands on the outside of the cell and with the cytoskeleton on the inside, the whole process being controlled by growth factors. In this study, it was found that overexpression of either Cdx1 or Cdx2 was sufficient to reduce the PMA-stimulated migration by 60%. Yet, here again, cooperation of Cdx1 and Cdx2 led to a much stronger effect, cell migration being inhibited by 85%. It is therefore very likely that loss of Cdx1 or Cdx2 expression from normal colon cells would significantly increase their migration potential.

It was concluded from these studies that concomitantly increasing the expression of Cdx1 and Cdx2 conferred to HT29 colon cancer cells a much less aggressive phenotype. Their rate of growth and tumorigenicity were decreased, their sensitivity to apoptosis was increased, and their migration upon stimulation by PMA was strongly reduced. Overexpressing only one of the Cdx genes produced a weaker effect or was ineffective. Hence, reduced expression of one of the two Cdx genes would suffice to maintain an aggressive phenotype, in agreement with previous findings that decreased expression of Cdx1 or Cdx2 was associated with colon cancer (2). These results suggest that restoration of Cdx expression in colon cancer cells would be a promising therapeutic strategy, provided that Cdx1 and Cdx2 are concomitantly induced.

The present findings could also be analyzed in terms of carcinogenesis because, if increasing the expression of the Cdx genes in colon cancer cells decreases the aggressivity of their phenotype, one can assume that in normal colon cells any event leading to decreased expression of Cdx1 or Cdx2 would participate in carcinogenesis. Hence, the two homeotic genes must independently control pathways that are involved in maintaining the normal phenotype. The mechanisms by which Cdx gene products influence the phenotype of the cells are presently unknown, but they could be part of the control of cell cycle and differentiation as reported for the retinoblastoma gene (RB1), because overexpression of that gene also reduced tumorigenicity and cell migration in many cancer-derived cell lines (Ref. 24 and references therein).

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REFERENCES

1. Frigerio, J. M., Berthelene, P., Garrido, P., Ortiz, E., Barthellemey, S., Vasseur, S., Sastre, B., Selenzieff, I., Dagorn, J. C., and Iovanna, J. L. (1995) Hum. Mol. Genet. 4, 37–43
2. Mallo, G. V., Recherehe, H., Frigerio, J. M., Rocha, D., Zweihaum, A., Lecas,

FIG. 6. Migration of Cdxs-transfected HT29 cells. a, HT29, HT/CAT, HT/Cdx1S, HT/Cdx2S, and HT/Cdx1S2S cells were plated in 35-mm culture wells and grown to 95% confluence. Then, the monolayers were wounded (see “Materials and Methods”), and cell migration was monitored after 24 h of culture in standard medium or in medium containing 100 nM PMA. In order to evaluate the contributions of migration and proliferation to the wound closure process, migration assays were also carried out in the presence of mitomycin (5 μg/ml). Cells migrating across the wound margin were counted in eight different areas randomly chosen. Results represent the mean values (± S.E.) of two independent experiments performed in triplicate. b, pictures from experiments reported in panel a, taken after a 24-h culture in the presence of 100 nM PMA. A, HT/CAT cells; B, HT/Cdx1S cells; C, HT/Cdx2S cells; D, HT/Cdx1S2S cells.

vent uncontrolled cell growth, but contrary to what was obtained in vitro with HT29, concomitant preservation of Cdx1 expression is apparently not essential. Partial complementation by another Cdx in Cdx1−/− mice might account for that discrepancy. Nevertheless, these findings support the hypothesis that situations leading to decreased Cdx expression in colon cells would likely be associated with increased tumorigenicity.

The third aspect that we investigated was whether Cdx gene overexpression altered cell response to apoptotic agents, because decreased susceptibility of most tumoral cells to apoptosis favors tumor growth and increases cell resistance to cytotoxic agents (21). In normal culture conditions, the apoptosis rate was less than 1% in HT/CAT control or HT29 parental cells and did not change upon overexpression of Cdx1 and/or Cdx2, suggesting that Cdx overexpression did not induce apoptosis.
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M., Jordan, B., Dusetti, N., Dagorn, J. C., and Iovanna, J. L. (1997) Int. J. Cancer 74, 1–10
3. Ee, H. C., Erler, T., Bhathal, P. S., Young, G. P., and James, R. J. (1995) Am. J. Pathol. 147, 586–592
4. Silberg, D. G., Furth, E. E., Taylor, J. K., Schuck, T., Chiu, T., and Traber, P. (1997) Gastroenterology 113, 478–486
5. Vider, B. Z., Zimber, A., Hirsch, D., Estlein, D., Chastre, E., Prevot, S., Gespach, C., Yaniv, A., and Gazit A. (1977) Biochem. Biophys Res. Commun. 232, 742–748
6. Chawengsaksophak, K., James, R., Hammond, V. E., Ko¨ntgen, F., and Beck, F. (1997) Nature 386, 84–87
7. Suh, E., and Traber, P. G. (1996) Mol. Cell. Biol. 16, 1475–1489
8. Schreiber, S. L., Gelfand, T. R., Hoh, T. Y., Roman, E., Pich, H. W., Wenz, M., Northrop, J. P., Ringold, G. M., and Danielson, M. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 7413–7417
9. Sambrook, J., Frisch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Edition, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
10. Knittel, T., Kessel, M., Kim, M. H., and Gruss, P. (1995) Development 121, 1077–1088
11. Dignam, J. D., Lebovitz, R. M., and Roeder, R. G. (1983) Nucleic Acids Res. 11, 1475–1489
12. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
13. Suh, E., Chen, L., Taylor, J., and Traber, P. G. (1994) Mol. Cell. Biol. 14, 7340–7351
14. Burke, R. R. (1973) Proc. Natl. Acad. Sci. U. S. A. 70, 369–372
15. Subramanian, V., Meyer, B. I., and Gruss, P. (1995) Cell 83, 641–653
16. Barba, P., Magli, M. C., Tiberio, C., and Cillo, C. (1993) Adv. Exp. Med. Biol. 348, 45–57
17. Lichty, B. D., Ackland-Snow, J., Noble, L., Kamel-Reid, S., and Dube, I. D. (1995) Leuk. Lymphoma 16, 209–215
18. Chariot, A., and Castronovo, V. (1996) Biochem. Biophys. Res. Commun. 222, 292–297
19. Tiberio, C., Barba, P., Magli, M. C., Arvelo, F., Le Chevalier, T., Poupon, M. F., and Cillo, C. (1994) Int. J. Cancer 58, 608–615
20. De Vita, G., Barba, P., Odar, Shety, N., Givel, J. C., Freschi, M., Bucciarelli, G., Magli, M. C., Boncinelli, E., and Cillo, C. (1993) Eur. J. Cancer 29, 877–893
21. Fisher, D. E. (1994) Cell 78, 539–542
22. Hague, A., Diaz, G. D., Hicks, D. J., Krajewski, S., Reed, J. C., and Paraskeva, C. (1997) Int. J. Cancer 27, 888–905
23. Straeke, M. L., Aznavoorian, S. A., Beckner, M. E., Liotta, L. A., and Schiffmann, E. (1991) Cell Motility Factors (Goldberg, I. D., ed) Long Island Jewish Medical Center, New York
24. Valente, P., Melchiori, A., Pagli, M. G., Masie, L., Ribatti, D., Santì, L., Takahashi, R., Albini, A., and Noonan, D. M. (1996) Oncogene 13, 1169–1178