**Drg-1 as a Differentiation-related, Putative Metastatic Suppressor Gene in Human Colon Cancer**

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**ABSTRACT**

A gene related to cell differentiation was identified by differential display as a candidate suppressor of metastases in colon cancer. This gene, with a full-length cDNA of 3 kb, is expressed in normal colon and primary colon cancer tissues and cell lines but not in their metastatic counterparts. A GenBank search found that it is identical to a recently cloned gene, differentiation-related gene-1 (Drg-1), isolated from differentiated HT-29 colon cancer cells. Stable transfection of the SW620 metastatic colon cancer cell line with Drg-1 cDNA induced morphological changes consistent with differentiation and up-regulated the expression of several colonic epithelial cell differentiation markers (alkaline phosphatase, carcinoembryonic antigen, and E-cadherin). Moreover, the expression of Drg-1 is controlled by several known cell differentiation reagents, such as ligands of peroxisome proliferator-activated receptor γ (troglitazone and BRL46593) and of retinoid X receptor (LG268), and histone deacetylase inhibitors (trichostatin A, suberoylanilide hydroxamic acid, and trichotyriam). A synergistic induction of Drg-1 expression was seen with the combination of trichotyriam and a low dose of 5′-aza-2′-deoxycytidine (100 nM), an inhibitor of DNA methylation. Functional studies revealed that overexpression of Drg-1 in metastatic colon cancer cells reduced in vitro invasion through Matrigel and suppressed in vivo liver metastases in nude mice. We propose that Drg-1 suppresses colon cancer metastasis by inducing colon cancer cell differentiation and partially reversing the metastatic phenotype.

**INTRODUCTION**

Metastasis consists of the spreading of tumor cells from the primary neoplasm to distant sites. Despite significant improvements in early diagnosis and surgical ablation, as well as local and systemic adjuvant therapies, the majority of cancer deaths are attributable to metastases that are resistant to conventional therapies. It is believed that the outcome of metastatic diseases is influenced by intrinsic changes of the tumor cell (seed) and by changes in host factors (soil; Ref. 1). The process of metastasis is not random but rather consists of a complex series of linked and interrelated steps involving multiple host-tumor interactions (1). Many proteins including proteases, adhesion molecules, angiogenesis, and growth factors are involved in metastasis. Therefore, understanding the gene expression changes in metastatic cancer cells may aid in early diagnosis and therapeutic intervention. In the last decade, considerable progress has been made in understanding these changes. Yet a sensitive and reliable method for detection of early metastasis in colon cancer is still not available, and clinicians still rely primarily on marginally sensitive pathological findings to predict metastasis (2).

To further define gene expression changes in metastatic colon cancer, we used differential display and identified 19 genes that are expressed differentially between primary and metastatic colon cancer. One of these genes is identical to a gene identified previously named Drg-2, which was found to be down-regulated in colonic adenomas and primary colon cancer (3). The expression of this gene was also found to be regulated by homocysteine, testosterone, and Ni²⁺ in different cell types (4–6). However, the function of this gene remains unknown. We now report that Drg-1 is further down-regulated in metastatic colon cancer cells to levels that are nearly undetectable when compared with the primary colon cancer counterparts. We have further demonstrated that stable transfection of a metastatic colon cancer cell line SW620 with Drg-1 cDNA induced morphological changes indicative of differentiation, up-regulated the expression of several colonic epithelial cell differentiation markers, and reduced in vitro invasion through Matrigel and in vivo liver metastasis in nude mice. In mechanistic studies, we found that the expression of Drg-1 was controlled by several differentiation reagents, such as ligands of PPARγ and the retinoid X receptor, as well as by reagents affecting DNA methylation and histone acetylation. These data suggest that Drg-1 may suppress colon cancer metastasis by inducing cell differentiation and reversing the metastatic phenotype.

**MATERIALS AND METHODS**

**Human Tissues and Cell Culture.** Sporadic human colon cancer tissues and their metastatic lesions were randomly obtained from the Pathology Department of Beth Israel Deaconess Medical Center. Tumor tissues were carefully dissected from adjacent normal colon tissues, snap frozen, and stored in liquid nitrogen before analysis. Colon cancer cell lines were purchased from American Type Culture Collection and cultured at 37°C in 5% CO₂ in a mixture of DMEM (½) and RPMI 1640 (½) with 10% fetal bovine serum and antibiotics. Drg-1 transfectants were maintained in the same culture medium containing 0.2 mg/ml of G418.

**Chemical and Biological Reagents.** AzA, all-trans retinoic acid, tributyrin, and TSA were purchased from Sigma Chemical Co. (St. Louis, MO). LG268, a retinoid X receptor selective ligand, was a gift from Richard A. Heyman (Ligand Pharmaceuticals, San Diego, CA). Troglitazone and BRL49653, ligands of PPARγ, were gifts from Dr. Bruce M. Spiegelman at Dana-Farber Cancer Institute. SAHA, a second-generation hybrid polar cytodifferentiation agent shown to inhibit histone deacetylation and induce terminal differentiation in transformed cells (7), was a gift from Dr. Paul Marks (Sloan-Kettering Cancer Center, New York, NY).

**DD.** SW480 and SW620 cell lines were both derived from the same colon cancer patient. SW480 was established from a primary colon cancer lesion, and SW620 was from a lymph node metastasis (8). To ensure that the observed differences were not an artifact of long-term cell culture, we also studied freshly isolated primary colon cancer tissue and lymph node metastasis from a single patient. DD was performed with a DD kit purchased from GenHunter Corp. (Nashville, TN), according to the manufacturer’s protocol (9). The anchor and arbitrary primers that led to detection of Drg-1 were 5′-AAGCTTTTTTTTTCG-3′ and 5′-AAGCTTTGGTT-3′. Band isolation and direct sequencing of the DD band were performed as described (9).

**RNA Isolations and Northern Blot Analysis.** RNA from colon cancer cells was isolated with TRIzol reagent (Life Technologies, Inc., Rockville, Maryland) and subjected to Northern blot analysis. The total RNA was electrophoresed in 1% agarose-formaldehyde gels and transferred to nylon membranes which were UV crosslinked. The membranes were baked at 80°C for 2 h, hybridized with radiolabeled cDNA probes, and autoradiographed. The membranes were stripped and rehybridized with a different cDNA probe. The cDNA used as probes were: carcinoembryonic antigen; CEA, carcinoembryonic antigen; RXR, retinoid X receptor. Arabic numerals and Roman numerals are used to designate paragraphs.
MD), according to the manufacturer’s protocol. RNA from colon cancer tissues was isolated by the guanidinium thiocyanate/CsCl method, as described (10). A multiple-tissue dot blot was obtained from Clontech (Palo Alto, California). Northern and dot blot analysis were performed as described (10), except ExpressHyb hybridization solution from Clontech was used. Nucleotides 4–337 of Drg-1 were 32P-labeled with a random labeling kit (Boehringer Mannheim, Indianapolis, IN) and used as a probe for Drg-1. Probes for E-cadherin and CEA were produced as described (11, 12). The probe for PPARγ was a gift from Bruce M. Spiegelman at Dana-Farber Cancer Institute (13). Membranes were hybridized in ExpressHyb hybridization solution (Clontech) with 32P-labeled probe, washed, and exposed to a PhosphorImager (Bio-Rad Laboratories, Richmond, CA) or X-ray films. The signal intensities were quantified with Imagequant software (Bio-Rad Laboratories) and normalized to 28S RNA expression.

Generation of Drg-1 Stable Transfectants. The coding region of Drg-1 (nucleotides 110-1346) was cloned from a human normal prostate cDNA library (Clontech) by PCR with Advantage cDNA Polymerase Mix from Clontech. The coding region of Drg-1 cDNA was inserted in-frame into the pcDNA3.1 vector, which contains the cytomegalovirus enhancer-promoter (Invitrogen Corp., Carlsbad, CA). The cDNA was then fully sequenced to ensure that no mutations were introduced during the PCR amplification. pcDNA3.1 vector, which contains the cytomegalovirus enhancer-promoter (Invitrogen Corp., Carlsbad, CA), according to the manufacturer’s protocol. After culturing in medium containing 0.8 mg/ml of G418 (Life Technologies, Inc., Valencia, CA), according to the manufacturer’s protocol. After culturing in medium containing 0.8 mg/ml of G418 (Life Technologies, Inc., Valencia, CA), according to the manufacturer’s protocol. 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cancer specimens (5 primary tumors paired with 5 metastatic colon cancer lesions; Fig. 2). Of the four cell lines with the lowest level of Drg-1 expression (Fig. 2A), three (SW620, LoVo, and Colo205) were derived from metastatic lesions of colon cancer, and the fourth, SW48, was derived from a poorly differentiated primary colon cancer. In contrast, the other four cell lines (SW480, DLD-1, HCT116, and CaCO2) were derived from primary colon cancer lesions (20–22).

Similarly, the expression of this gene was also found to be substantially down-regulated in two and completely undetectable in three metastatic lesions (Fig. 2B) when compared with the paired primary colon cancer lesions. In preliminary Northern blot studies of 36 clinical specimens of breast cancers (data not shown), the level of Drg-1 expression did not differ appreciably between primary breast cancers and metastatic lesions. These data suggest that Drg-1 may be specific for colon cancers.

To determine the pattern of expression of Drg-1 in normal human tissue, a master dot blot containing a total of 50 normal human tissues was probed with Drg-1. As shown in Fig. 3, the expression of Drg-1 was found in all tissues tested with a slightly higher expression level in the brain, prostate, and adult and fetal kidney, as well as placenta. The same blot was stripped and reprobed with ubiquitin to ensure equal loading (data not shown). The absence of signals in bacterial or yeast cDNA samples demonstrated the specificity of the probing. The presence of signals in human DNA (Fig. 3, right lower corner) suggests that Drg-1 is highly abundant in human tissue or belongs to a multigene family. The ubiquitous expression of Drg-1 suggests that this gene may function as a housekeeping gene.

**Overexpression of Drg-1 Induces Metastatic Colon Cancer Cell Differentiation.** To further investigate the function of Drg-1, we transfected the metastatic colon cancer cell line (SW620) with a pcDNA3.1 vector containing the 1.2-kb coding region of Drg-1 under the control of the cytomegalovirus promoter. Multiple SW620/T clones stably expressing transfected Drg-1 mRNA were selected for the subsequent studies. An in vitro translational study with pcDNA3.1/Drg-1 plasmid yielded a protein with a molecular weight of $M_r$, 43,000. This matches the predicted molecular weight of Drg-1, indicating that this plasmid construct functions in vitro (data not shown).

Although the growth rate of the transfected cells was very similar to that of the neo controls and parental nontransfected cells (data not shown), distinct morphological changes were seen in the Drg-1-transfected cells (Fig. 4). The transfected cells were larger, flatter, and spindle shaped (Fig. 4B), in contrast to the smaller, more round neo control cells (Fig. 4A). These morphological changes were consistently observed in multiple transfected clones.
expressing Drg-1 but not in those clones that did not express Drg-1. Similar morphological changes were seen when differentiation was induced in parental SW620 by known differentiation reagents such as tributyrin, a prodrug of butyrate (Fig. 4C), LG268, a ligand of RXR (Fig. 4D), and all-trans retinoic acid, a ligand of retinoic acid receptor (not shown). These findings suggest that the expression of Drg-1 induces changes characteristic of cell differentiation in colon cancer cells.

To substantiate this finding, the expression level of several colonic epithelial cell differentiation markers (14), including alkaline phosphatase, CEA, and E-cadherin, were determined. As shown in Fig. 5A, the activity of alkaline phosphatase was 2–3-fold higher in all five transfected cell clones (620/T) compared with the neo control cells (620/V). Similarly, the expression of E-cadherin and CEA was also up-regulated in all transfected cell clones, although the levels of expression varied among individual clones (Fig. 5B). Together, these findings suggest that the expression of Drg-1 induces colon cancer cell differentiation.

Regulation of Drg-1 Expression by Ligands of PPARγ, RXR, DNA Methylation, and Histone Acetylation. Because of the potential role of Drg-1 in the regulation of colonic epithelial cell differentiation, the effects of several known differentiation reagents on expression of the Drg-1 gene were sought. As shown in Figs. 2 and 6B, treatment with Aza, an inhibitor of DNA methylation and a known differentiation inducer (19), partially up-regulated the expression of Drg-1 in all colon cancer cell lines tested, suggesting that the expression of Drg-1 is controlled by DNA methylation. PPARγ is a ligand-inducible transcription factor.
known to control differentiation of a variety of cells including adipocytes and colonic epithelial cells (13). To investigate the potential functional association between the PPARy and Drg-1, we first studied the expression of PPARy in Drg-1-transfected and neo control SW620 cells. Northern blot studies with a probe specific for PPARy revealed that its expression level in both transfected and neo control SW620 cells is nearly identical (data not shown), suggesting that the expression of PPARy is independent of Drg-1. On the other hand, the expression of Drg-1 was up-regulated by troglitazone (Fig. 6A) and BRL46593 (not shown), two synthetic ligands of PPARy (13), suggesting that Drg-1 is actually down-stream of PPARy. In addition, LG268, a synthetic ligand specific for RXR (23) also up-regulated Drg-1 (Fig. 6A). A combination of troglitazone and LG268 induced Drg-1 expression by 10-fold, suggesting a possible synergistic effect from these two drugs. Moreover, the expression of Drg-1 was also markedly up-regulated by two histone deacetylase inhibitors, TSA and SAHA (7, 24, 25). The effect of another histone deacetylase inhibitor, tributyrin (a prodrug of butyrate), was only seen after 5 days of treatment (Fig. 6B). However, when cells were treated with tributyrin plus a low dose of Aza (0.1 μM), a marked increase in Drg-1 expression was seen (Fig. 6B), suggesting a synergistic effect from these two drugs. Together, these data suggest that Drg-1 may be a downstream element of the PPARy transcriptional pathway and is controlled by both DNA methylation and histone acetylation, two global mechanisms of gene regulation (26). We suggest that Drg-1 may suppress colon cancer metastasis by inducing cell differentiation and reversing the metastatic phenotype.

**Overexpression of Drg-1 Inhibits in Vitro Invasion through Matrigel and in Vivo Liver Metastasis in Nude Mice.** To test the hypothesis that Drg-1 may suppress colon cancer metastasis, we used an in vitro Matrigel assay (15) to examine the invasive capabilities of metastatic colon cancer cell lines transfected with the Drg-1 cDNA (SW620/T) or with an empty vector as a control (SW620/V). As shown in Fig. 7, the metastatic colon cancer cell line (SW620) migrated through the Matrigel at levels about five times greater than the primary colon cancer cell line (SW480), in agreement with data published previously (27). Expression of Drg-1 cDNA in SW620 cells (T1 and T5) reduced Matrigel invasion by up to 70% (P < 0.0001). Expression of the neo control vector had little effect on Matrigel invasion. These data suggest that the overexpression of Drg-1 inhibits the in vitro invasion ability of metastatic colon cancer cells.

To further investigate the role of Drg-1 in invasion and metastasis, three Drg-1-transfected cell clones (620/T1, 620/T5, and 620/T7) were each injected into the spleen of athymic nude mice. Two neo stably transfected cells (620/V and 620/V1) were injected to serve as controls. As shown in Table 1, 12 of 14 mice injected with neo control cells and 13 of 15 mice injected with transfected cells developed “primary” tumors in the spleen. The tumor burdens of the splenic primaries, as judged by their weights, were very similar between the transfected and the neo control group (data not shown). However, in the mice that developed “primary” tumors, 9 of 12 mice (75%) developed liver metastases in the neo control group, whereas only 3 of 13 mice (23%) had liver metastases in the Drg-1-transfected group. Statistical analysis using the Fisher exact test revealed a significant difference in the incidence of liver metastases between the groups (P < 0.0001).
Table 1 Overexpression of Drg-1 suppresses in vivo liver metastases in nude micea

<table>
<thead>
<tr>
<th>Cells</th>
<th>Total no. of miceb</th>
<th>Mice with splenic primariesc</th>
<th>Mice with splenic primaries and liver metastasesd</th>
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<tr>
<td>620/V</td>
<td>6</td>
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<tr>
<td>620/V1</td>
<td>10</td>
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<td>12</td>
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<td>620/T1</td>
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<tr>
<td>620/T7</td>
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<tr>
<td>Subtotal</td>
<td>15</td>
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a Viable colon cancer cells (5 × 104) were injected into the spleen of athymic nude mice. After 8 weeks, mice were euthanized, and the presence of tumors in the spleen and their liver metastases were determined as described in “Materials and Methods.”
b For the control group, a total of 14 mice were used. Twelve of 14 mice developed tumors; 1 had no tumor, and 1 mouse died right after injection. For the transfectected group, a total of 15 mice were used; Thirteen of 15 mice developed tumors; 1 had no tumor, and 1 died right after injection.
c The number of mice that developed splenic primaries or splenic primaries and liver metastases in the control and transfectected groups was used for statistical analysis using Fisher’s exact test. P was equal to 0.0169.
d The number of mice that developed splenic primaries or splenic primaries and liver metastases tested revealed a P of 0.0169, suggesting that the differences in liver metastases observed between the control and transfected groups are unlikely attributable to chance alone. Therefore, these findings suggest that Drg-1 may function as a suppressor of colon cancer metastasis. These results also indicate that Drg-1 did not alter the ability of cancer cells to form primary tumors.

**DISCUSSION**

Neoplastic transformation arises from multiple defects in cell growth and differentiation (28). Gene expression changes and/or genomic DNA mutations play a crucial role in the pathogenesis of cancer formation and in its progression (29, 30). Because the dispensable nature of the colon allows removal of the primary tumor, the prognosis of colon cancer directly correlates with the extent of tumor invasion and metastases (2). Molecules involved in cancer metastasis may serve as markers for early detection of metastasis and/or as targets for therapeutic intervention.

Using DD, we have identified 19 genes expressed differentially between primary and metastatic colon cancer. One of these genes, Drg-1, was found to be down-regulated in metastatic colon cancer tissues and cell lines. Overexpression of Drg-1 induced morphological and molecular changes consistent with colon cancer cell differentiation and suppressed in vitro invasion and in vivo liver metastases in nude mice. Drg-1 was initially identified by comparing gene expressions between undifferentiated and well-differentiated HT-29 colon cancer cell lines (3). Simultaneously, others found that Drg-1 was regulated by homocysteine in endothelial cells (4), testosterone in T-cell hybridoma 312.13 cells (5), and Ni2+ in human and rodent cell lines (6), implying that Drg-1 may be a housekeeping gene (4). In fact, a GenBank search revealed that the murine homologue of Drg-1 (named Ndr1, accession no. U60593) is a downstream target of N-myc, first suggesting that Drg-1 may be involved in cell growth and differentiation.

In the present study, we demonstrated that overexpression of Drg-1 induced distinct morphological changes similar to those observed during colonic epithelial cell differentiation. These morphological changes are associated with increased expression of several cell differentiation markers, suggesting that Drg-1 may function as a promoter of colonic epithelial cell differentiation. Moreover, the expression of Drg-1 is controlled by several known cell differentiation reagents. These results further support the notion that Drg-1 may be a key element in colonic epithelial cell differentiation. In addition, we have demonstrated that overexpression of Drg-1 in metastatic colon cancer cells suppress liver metastases in nude mice but do not alter the ability to form primary tumors. Together, these results suggest that induction of Drg-1 expression is capable of overriding the existing genetic defects and partially reversing the metastatic phenotype.

Our results indicate that the expression of Drg-1 is controlled by at least three mechanisms:

(a) PPARγ/RXR transcripational factor pathway. PPARγ is a member of the nuclear receptor superfamily that includes receptors for steroids, thyroid hormone, vitamin D, and retinoic acid (31). Ligands of PPARγ include polyunsaturated fatty acids such as linoleic, PGJ2, and the synthetic antidiabetic thiazolidinediones drugs, troglitazone and BRL 49653 (32, 33). Although dimerizing with the RXR receptor, PPARγ functions as a transcription factor, controlling differentiation of a variety of cells including adipocytes and colonic epithelial cells (34, 35). Therefore, the finding that the ligands of PPARγ and RXR activate Drg-1 suggests that Drg-1 may be a downstream target of the PPARγ/RXR differentiation pathway.

(b) DNA methylation pathway. It is well known that methylation of CpG islands in promoter sequences suppresses gene expression. Inhibition of DNA methylation by Aza induces differentiation of many cell types including colon cancer cells (36, 37). The 5’ end of the Drg-1 cDNA contains multiple CpG sites, which first suggested that Drg-1 may be controlled by DNA methylation. We have now cloned and sequenced 800 bp of the Drg-1 promoter region (data not shown). Analysis of this sequence reveals that there are multiple CpG sites, sufficient to comprise a CpG island (17). Additional studies to compare the promoter activity of Drg-1 with its methylation status will determine the role of DNA methylation in the regulation of Drg-1 expression. Our data also indicate that the expression of Drg-1 is only partially regulated by DNA methylation, implying that other mechanisms are involved in the down-regulation of Drg-1 in metastatic colon cancer cells.

(c) Histone deacetylation pathway. Inhibition of histone deacetylase by reagents such as butyrate and trichostatin has been shown to induce differentiation of many different cell types (7, 38). Our data demonstrate that inhibition of histone deacetylase induces the expression of Drg-1.

The synergistic effect of Aza and tributyrin on Drg-1 expression is of interest. The similar effect between an inhibitor of DNA methylation (Aza) and an inhibitor of histone deacetylation (TSA) also resulted in reexpression of genes such as p16 and MLH1, which are silenced in cancers (39). Together, these findings suggest that DNA methylation and histone acetylation, two key processes controlling gene regulation, cell growth, and cell differentiation, may be functionally linked. Because the degree of histone acetylation depends on the balance of acetylation and deacetylation, demethylated DNA may be a prerequisite condition for recruitment of acetyltransferase enzymes and histone acetylation. In this regard, recent studies by two independent groups have reported that MeCP2, a methyl-CpG-binding protein, interacts with histone deacetylase and induces transcriptional silencing by inducing histone deacetylation (40, 41). Additional studies of the regulatory mechanism of Drg-1 may provide insight about the interaction among transcription factors such as PPARγ as gene-specific regulatory mechanisms, as well as more global regulations such as DNA methylation and histone acetylation (42).
that plays a key role in controlling colonic epithelial cell differentiation. The fact that overexpression of Drg-1 induced expression of E-cadherin and two other cell differentiation markers, as well as induced morphological changes typical of differentiated cells, strongly suggests that SW620 metastatic colon cancer cells were “pushed” back into the differentiation pathway. Alterations of cell surface molecules, such as E-cadherin and possibly other cell surface molecules, may change the adhesion properties of cancer cells and result in the suppression of their in vitro and in vivo invasion capabilities (44, 45).

From a clinical point of view, decreased expression of Drg-1 in colon cancer cells may be used as a potential genetic marker to predict early metastasis. This can be achieved by analyzing the expression of Drg-1 in primary colon cancer using in situ hybridization or immunohistochemical studies, techniques that allow the identification of Drg-1 expression in individual colon cancer cells as compared with normal adjacent tissue. Moreover, ligands of PPARγ, RXR, or histone deacetylase inhibitors might be used as pharmacological agents to induce the expression of Drg-1 and thereby possibly reduce the invasion and metastatic abilities of colon cancer cells. Specifically targeting and manipulating the function of Drg-1 may offer a novel approach to the differentiation therapy of colon cancer.

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