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Aberrant Activation of c-kit Protects Colon Carcinoma Cells against Apoptosis and Enhances Their Invasive Potential[1]

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ABSTRACT

Multiple genetic aberrations contribute to the development of biologically aggressive, clinically malignant colorectal carcinomas (CRCs). Some of these have been linked to inappropriate signaling through the tyrosine kinase moieties of growth factor receptors. We have described previously (G. Bellone *et al.*, J. Cell. Physiol., *172:* 1–11, 1997) that human CRCs overexpress both the receptor tyrosine kinase c-kit and its ligand, stem cell factor (SCF), relative to normal mucosa cells, thus establishing an autocrine c-kit-mediated loop. In addition, we noted that exogenous SCF contributes to anchorage-independent growth of HT-29 colon carcinoma cells in semisolid medium. Here, we investigated possible roles of the c-kit/SCF autocrine/paracrine system in survival and invasive capacity of DLD-1 colon carcinoma cells. We report that SCF was required for migration and invasion of DLD-1 cells through reconstituted basement membranes (Matrigel) and up-regulated gelatinase (matrix metalloproteinase-9) activity in DLD-1 cells. Furthermore, we describe that SCF supported survival of DLD-1 cells in growth factor-deprived conditions. These results suggest multiple roles of c-kit activation in support of the malignant phenotype of DLD-1 cells related to growth, survival, migration, and invasive potential.

INTRODUCTION

The receptor tyrosine kinase c-kit (1) and its ligand SCF[3] (2) are essential to the maturation of hemopoietic and primordial germ cells precursors and melanocytes during embryonic development. Inactivating mutations at either the *W* (*Dominant white spotting*) or the *Sl* (*Steel*) loci are associated with abnormalities affecting these three cell lineages (3). Multiple cellular functions are affected by c-kit-dependent signals including cell survival, proliferation, adhesion, differentiation, and functional maturation of hemopoietic cells (reviewed in Refs. 4–6).

Transduction of *c-kit* into NIH3T3 cells leads to transformation of these cells in the presence of SCF, indicating the oncogenic potential of c-kit (7). Aberrant expression of c-kit and/or SCF has been reported in human malignant cells derived from neural (8), breast (9), lung (10), prostatic (11), and CRCs (12). Most of these tumor forms derive from epithelial cell lineages that express c-kit during embryogenesis (13).

The failure of certain cell types to develop in animals with impaired SCF production or c-kit function suggests that c-kit, like other tyrosine kinase receptors, plays a role in differentiation or cell survival. In support of this view, SCF has been shown to significantly augment the derivation of melanocytes from embryonic stem cells (14). Furthermore, c-kit activation was found to suppress apoptosis of normal murine melanocyte precursors (15), soft tissue sarcomas of neuroectodermal origin (16), neuroblastomas (8), and normal and malignant human hemopoietic cells (17).

However, c-kit activation does not universally support cell survival, as demonstrated by the observation that HIV transactivation of c-kit in astrocytes is associated with increased cell death (18). Similarly, forced c-kit expression renders metastatic human melanoma cells susceptible to SCF-induced apoptosis and inhibits their tumorigenic and metastatic potential (19), and advanced melanoma cells express low levels of c-kit *in situ* (20).

In gastrointestinal tissues, c-kit expression has been observed in interstitial cells of Cajal in the small intestine (13) and in CRC cells (12, 21). Strong epidemiological evidence suggests a role of c-kit activation in gastrointestinal stromal tumors derived from Cajal cells either through overexpression or through activating mutations of *c-kit* (22). By contrast, little is known about functional aspects of c-kit/SCF expression in gastrointestinal carcinomas. In previous work, we noted coexpression of SCF and c-kit in human CRCs *in situ* (12). We also established that exogenous SCF stimulates anchorage-independent expansion of HT-29 colon carcinoma cells *in vitro*; this effect was abolished by treatment with transforming growth factor-β1, which down-regulated expression of c-kit in HT-29 cells. The present study further defines the contribution of SCF to the malignant phenotype of CRC cells. We demonstrate that SCF exerts multiple paracrine roles in the colon carcinoma cell line DLD-1 in supporting anchorage-independent growth, cell survival under anchorage-dependent conditions, and migration/invasion of these cells through a metalloproteinase (MMP)-dependent mechanism.

MATERIALS AND METHODS

Antibodies and Reagents. The following antibodies to c-kit were used: a MoAb (clone 104D2) from Serotech (Oxford, United Kingdom) for flow cytometric analysis and a MoAb (clone K44.2; Ref. 23) from Sigma Chemical Co. (St. Louis, MO) for neutralization experiments. Recombinant SCF was obtained from PeproTech (Rocky Hill, NJ).

Cells and Preparation of CM. Human colon carcinoma cells DLD-1 (American Type Culture Collection, Rockville, MD) were grown in DMEM supplemented with 10% FCS (Life Technologies, Inc., Grand Island, NY). The cells were *Mycoplasma* free as determined by staining with Hoechst dye H33258 (Sigma). To obtain serum-free CM, cells were detached with trypsin, extensively washed with PBS (pH 7.3), and seeded at 3×10^5/ml in 1 ml of serum-free RPMI 1640 supplemented with 0.25% of BSA. After 48 h incubation in the presence or absence of SCF (100 ng/ml) or TPA (10 mM) at 37°C in a humidified atmosphere containing 5% CO_2, cell-free supernatants were collected, centrifuged, and stored at −80°C until use.

RT-PCR Analysis. Total RNA was extracted from DLD-1 cells, megakaryocytic cell line M07e (kindly provided by Dr. L. Pegoraro, Department of Biomedical Sciences and Human Oncology, Torino, Italy), B-lymphoblastoid cell line RPMI 8866 (kindly provided by Dr. D. Santoli, Wistar Institute, Philadelphia, PA), and bone marrow stroma cells (12) using the single-step Trizol method (Life Technologies, Inc.). cDNA synthesis was performed at 37°C for 1 h using an oligo-dT primer in a final volume of 20 μl

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[3] The abbreviations used are: SCF, stem cell factor; CRC, colorectal carcinoma; MMP, matrix metalloproteinase; MoAb, monoclonal antibody; CM, conditioned medium; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; RT-PCR, reverse transcription-PCR; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling.

containing 20 units of reverse transcriptase (Superscript II), 1× reverse transcriptase buffer, 24 units of RNAsin, and 0.5 mM of deoxynucleotide triphosphate mix. PCR was performed on 10 μl of first-strand cDNA to which 20 μl of PCR mix were added containing 100 ng each of 5′ and 3′ primers and 1 unit of Taq DNA polymerase. All PCR reagents were from Life Technologies. Human c-kit, SCF, and β-actin primers and amplification conditions were as described by us previously (12). Multiplex PCRs were performed using the primer-dropping technique described by Wong *et al.* (24). Each reaction mixture contained template cDNA, the volume of which was determined by the amount necessary to equalize the intensities of β-actin bands visualized during agarose gel electrophoresis. Additional ingredients added to the reaction mixture were 5 μl of 10× PCR buffer, 1 μl of deoxynucleotide triphosphate mixture, 100 ng each of 5′and 3′ starter primer pair, and 1 unit of Taq polymerase. Human MMP-2- and MMP-9-specific primers were, respectively: 5′-TCCTTTCACAACCTTCTGTGG-3′ (5′) and 5′-GGGAACCATCACTATGTGGG-3′ (3′); 5′-ACCGCTATGGTTACACTCGG-3′ (5′) and 5′-GCAGGCAGAGTAGGAGCG-3′ (3′). The PCR protocol was as follows: 36 cycles at 94°C for 45 s for denaturation, 62°C for 45 s for annealing; and 72°C for 2 min for extension. The predicted sizes of the MMP-2 and MMP-9-PCR products were 316 and 584 bp, respectively. Aliquots of PCR products were analyzed by size fractionation using 2% agarose gels stained with ethidium bromide. Loading was equalized to the internal control mRNA (β-actin). Densitometric evaluation of the bands was performed on digital images using Sigmagel software (Jandel, Heidelberg, Germany). Values for each sample were expressed relative to the β-actin amplimers.

Immunofluorescence Staining and Flow Cytometric Analysis. DLD-1 cells, cultured in the absence or in the presence of SCF (100 ng/ml) for 24–48 h, were detached from plastic tissue culture flasks using 5 mM EDTA in PBS and incubated with appropriate amounts of anti-c-kit MoAb for 30 min at 4°C. Cells were washed twice with PBS and then incubated with FITC-conjugated $(Fab)_2$ goat antimouse IgG. After three washes, samples were analyzed on a FACScan (Becton Dickinson, San José, CA). A minimum of 10,000 events/sample was analyzed. As a negative control, an isotype-matched MoAb with irrelevant specificity was used.

Treatment of DLD-1 Cells with Blocking Anti-c-kit MoAb or Antisense Oligonucleotide. DLD-1 cells were treated for 30 min at room temperature with a neutralizing MoAb to c-kit (5 μg/ml) or with an isotype-matched control MoAb before the onset of the culture. In some experiments, commercially available c-kit-selective antisense and sense oligodeoxynucleotides (SCF receptor antisense oligonucleotide kit; Biognostik Antisense kit; Biognostik, Göttingen, Germany) were used according to the manufacturer's instructions. The cells were washed four times and resuspended at 2×10^4 cells/ml in cell culture medium. Aliquots of 100 μl/well were placed in 96-well plates and allowed to adhere for at least 1 h. Specific c-kit antisense and sense oligodeoxynucleotides were added directly to cells at a final concentration of 2 μM. The cells were maintained at 37°C in 5% CO_2, and antisense and sense oligodeoxynucleotides were added daily (2 μl/well) for different time periods, as indicated.

Anchorage-independent Growth Assay. DLD-1 cells (5×10^2), pretreated with blocking anti c-kit MoAb or c-kit antisense oligodeoxynucleotides and respective controls, were plated in triplicate wells in 35-mm Petri dishes in DMEM supplemented with 0.25% BSA and methylcellulose (0.9%) in the absence or presence of SCF (100 ng/ml). After 10 days of incubation at 37°C in 5% CO_2 humidified air, colonies were counted by using phase-contrast microscopy.

Matrigel Assay. *In vitro* cell migration was determined in the BioCoat Matrigel Invasion Chambers (Becton Dickinson, Bedford, MA) assay as described (25). DLD-1 cells were suspended in DMEM/0.1% BSA at a concentration of 5×10^4 cells/ml, placed in the upper compartments, and incubated for 24 h at 37°C in 5% CO_2 in the absence or presence of increasing concentrations of SCF. After incubation, the non-invading cells were removed from the upper surface of the membrane by scrubbing. Cells on the reverse side were stained with 0.1% crystal violet, and invading cells were counted under a microscope at ×100. The percentage cell migration was calculated from the ratio of the number of cells recovered from the lower compartment to the total number of cells loaded in the upper compartment. Each experiment was performed using at least four chambers for each DLD-1 cell sample and repeated at least twice. To examine the role of MMP in the migration through Matrigel, the DLD-1 cells were preincubated for 30 min with 0.5 mM *o*-phenanthroline (Sigma), a synthetic inhibitor of MMPs.

Gelatin Zymography. Gelatinase activity in CM was detected under nonreducing conditions by gelatin zymography, as described previously (26). Briefly, 30 μl of CM mixed with 10 μl of loading buffer (0.16 M Tris-HCl, 50% glycerol, 8% SDS, and 0.08% bromphenol blue) were applied onto a 10% polyacrylamide gel copolymerized with 1.2 mg/ml gelatin (Sigma) and subjected to electrophoresis under constant voltage (150 V) for 2 h at 4°C. The gels were washed three times in 2.5% Triton X-100 to permit renaturation of gelatinases before being incubated in zymography buffer (0.15 M NaCl, 5 mM $CaCl_2$, 0.05% NaN_3, and 50 mM Tris-HCl, pH 7.5) for 24 h at room temperature. The gels were then stained with 0.05% Coomassie Brilliant Blue in 2.5:1:7 ethanol:acetic acid:water and destained with 2:1:7 isopropanol in incubation overnight. Destaining visualized clear zones of lysis against a blue background, indicating gelatinase activity.

Apoptosis Detection Assay. Apoptosis detection was performed by JAM assay, in which loss of [^{3}H]thymidine-labeled DNA was measured. DNA labeling of DLD-1 target cells was performed by incubation of the cells in the presence of 10 μCi/ml [^{3}H]thymidine (DuPont New England Nuclear, Boston, MA) for 5 h at 37°C cultured in serum-free conditions. After washing with PBS, aliquots (100 μl) of the cell suspension were cultivated in serum-free medium in 96-well plates with or without of neutralizing anti-c-kit MoAb or with an isotype-matched control MoAb. Treatment of labeled DLD-1 cells with 200 ng/ml of TRAIL served as a positive control in apoptosis assays. All cultures were incubated for 48 h at 37°C, and cell-associated [^{3}H]thymidine was determined using a beta scintillation counter. The reduction in incorporated radioactivity in experimental samples as compared with untreated controls was used to calculate the percentage of specific target cell death: [(cpm untreated cells − cpm treated cells/cpm untreated cells) × 100]. In addition to the JAM assay, *in situ* detection of apoptosis by use of the TUNEL technique, which identifies cells with internucleosomal fragmentation of DNA, was performed using a commercially available *in situ* cell death detection kit (Roche Molecular Biochemicals, Indianapolis, IN) according to the manufacturer's instructions. Fixed and permeabilized cell cytospin preparations were subjected to the TUNEL reaction mixture containing terminal deoxynucleotidyltransferase and fluorescein-dUTP. After washing, the label incorporated at the damaged sites of the DNA was marked by an anti-fluorescein antibody conjugated with the reporter enzyme peroxidase and diaminobenzidine tetrahydrochloride as chromogen. Cells were considered positive when brown reactivity was detected in the nuclei. Acridine orange staining and fluorescence microscope analysis were used to examine whether DLD-1 cells exhibited nuclear changes characteristic of apoptosis after incubation with either neutralizing anti-c-kit MoAb or antisense and sense oligonucleotides.

Statistical Analysis. To assess statistically significant differences between data sets, Student's *t* test were performed using SigmaPlot (Jandel).

RESULTS

Expression of c-kit and SCF by DLD-1 Carcinoma Cell Line. Previously, we described coexpression of c-kit and SCF in HT-29 colon carcinoma cells (12). Here we extend this observation to DLD-1 colon carcinoma cells that expressed c-kit and SCF transcripts in a manner reminiscent of HT-29 cells. Specifically, by RT-PCR, amplimers of predicted size were generated corresponding to c-kit and the two known splice variants of SCF (Fig. 1*A*). In addition, flow cytometric analysis using an anti-c-kit MoAb showed cell surface expression of the c-kit protein in DLD-1 cells (Fig. 1*B*).

Down-Regulation of c-kit Expression or Function by Antisense Oligonucleotide and Neutralizing Anti-c-kit MoAb. To address potential paracrine and autocrine roles of SCF in DLD-1 cells, we tested whether a commercially available antisense oligonucleotide kit would reduce c-kit expression in DLD-1 cells. As shown in Fig. 1*C*, treatment with the c-kit antisense oligonucleotide was associated with marked down-regulation of c-kit cell membrane expression in DLD-1 cells.

Anchorage-independent Growth of DLD-1 Cells. Next, we tested whether the c-kit antagonists affected growth of DLD-1 cells in

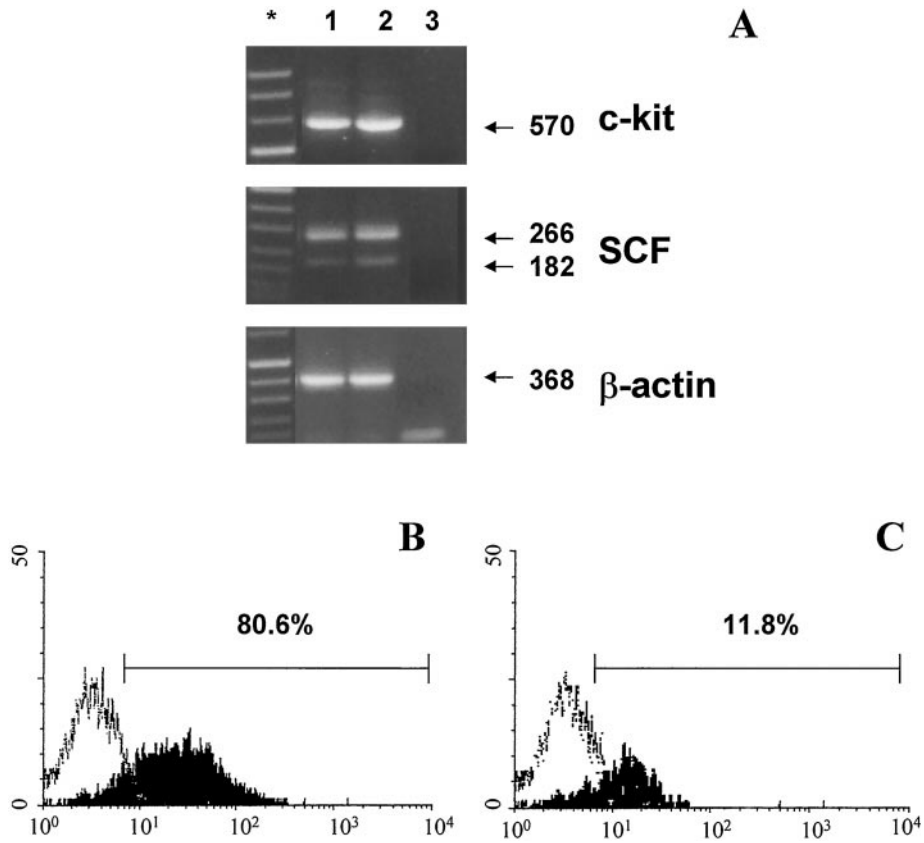

Fig. 1. Expression of c-kit and SCF by DLD-1 cells and down-regulation of c-kit expression by antisense oligodeoxynucleotides. RT-PCR analysis of c-kit and SCF mRNA expression in DLD-1 cells (*A, Lane 1*). M07e cells served as a positive control for c-kit expression (*A, Lane 2*), and stroma cells served as a positive control for SCF (*A, Lane 2*). *Lane 3* shows the absence of amplification products in the absence of a cDNA template. ∗, lane containing molecular weight markers (*A*). *B* and *C* show cell surface expression of c-kit by DLD-1 cells in the absence (*B*) and presence (*C*) of c-kit antisense oligonucleotide as determined by flow cytometric analysis.

soft agar. DLD-1 cells formed tightly packed colonies in the absence of exogenous SCF (Fig. 2). By contrast, exogenous SCF (100 ng/ml) induced large colonies of scattered cells. The addition of the neutralizing anti-c-kit MoAb (5 μg/ml) abrogated this effect of SCF, whereas an antibody with irrelevant specificity had no effect. Similarly, treatment of DLD-1 cells with c-kit antisense oligonucleotide markedly reduced the ability of these cells to form colonies in soft agar in response to exogenous SCF (Table 1). This effect was specific be-

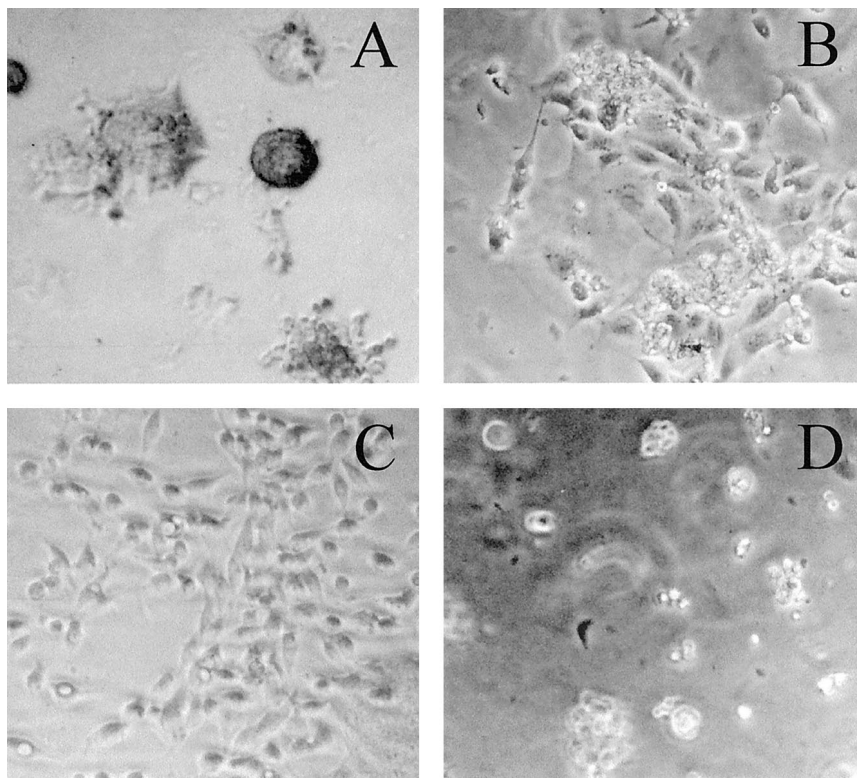

Fig. 2. Inhibition of SCF-induced anchorage-independent growth of DLD-1 cells by blocking anti-c-kit MoAb. The formation of tight colonies by DLD-1 cells in the absence of SCF is shown in *A*. SCF (100 ng/ml) induced large colonies of scattered cells (*B*). Addition of an anti c-kit MoAb (5 μg/ml) abrogated the effect of SCF (*D*), whereas an isotype-matched MoAb with irrelevant specificity had no effect (*C*).

Table 1 *Effect of c-kit antisense oligonucleotides on SCF-induced anchorage-independent growth of DLD-1 cells*[a]

Culture conditions	Colony numbers (1×10^3 cells/well)
Medium	59 ± 11[b]
SCF	128 ± 29
Medium + c-kit S ODN	63 ± 12
SCF + c-kit S ODN	118 ± 34
Medium + c-kit AS ODN	44 ± 19
SCF + c-kit AS ODN	73 ± 29[c]

[a] DLD-1 cells were exposed to the culture conditions indicated for 10 days. DLD-1 cells were pretreated with c-kit sense (S) and antisense (AS) oligonucleotides (ODN) as described in "Materials and Methods."

[b] Significantly different from control (SCF + c-kit S ODN, $P = 0.03$) as determined by Student's t test.

[c] Values represent mean ± SD of three separate experiments, each consisting of triplicate cultures.

cause the addition of control sense oligonucleotide had no effect on either basal or SCF-induced colony formation. Interestingly, the antisense c-kit oligonucleotide also reduced colony formation in control cultures maintained in the absence of exogenous SCF by ~30%, consistent with an autocrine role of DLD-1-derived SCF. This effect, however, was not statistically significant.

Effects of SCF on Migration and Invasion of DLD-1 Cells. In addition to the marked increase of the number of colonies in soft agar, SCF greatly increased their dispersal (Fig. 2). This observation raised the question of whether SCF affected not only growth but also the migratory and/or invasive capacity of DLD-1 cells under conditions of anchorage independence. To functionally test the effects of SCF on transmigration of DLD-1 cells through reconstituted basement membrane, we performed Matrigel invasion assays in the presence and absence of antisense c-kit oligonucleotide (Fig. 3 and Table 2). Control DLD-1 cells traversed Matrigel barriers at low cell frequencies (3%). By contrast, SCF-treated cells migrated through Matrigel at significantly higher rates (28%). This effect was markedly attenuated by treatment with antisense c-kit oligonucleotide, whereas sense c-kit oligonucleotide had no detectable effect.

Modulation of Metalloproteinase Expression and Function by SCF. Migration of epithelial tumor cells through Matrigel has been linked to the production of proteolytic enzymes capable of digesting the extracellular matrix barrier, including MMPs. CRC cells produce MMP-2 and MMP-9 in a tumor-associated fashion (27–29). To address whether MMPs functionally contribute to transmigration of DLD-1 cells through Matrigel, we used the broad-spectrum metalloproteinase inhibitor *o*-phenanthroline. This inhibitor significantly reduced the fraction of DLD-1 competent to invade through Matrigel (Fig. 4). This result led us to investigate whether SCF induced DLD-1 invasion by MMP-dependent mechanisms. We confirmed that DLD-1 cells expressed MMP-2 and MMP-9 mRNAs by RT-PCR analysis (Fig. 5*A*). To assess whether SCF affected MMP expression, we quantitated expression of MMP mRNA levels in DLD-1 cells relative to the expression of β-actin (Fig. 5*B*). MMP-2 expression was not affected by SCF treatment, whereas expression of MMP-9 was slightly but significantly increased in SCF-treated samples. As a control, we treated parallel cultures with the tumor promoter TPA, which, as expected, also increased MMP-9 levels. Next, we determined by zymographic analysis, MMP-2 and MMP-9 activities in media conditioned by DLD-1 cells in the presence and absence of SCF (Fig. 5*C*). This analysis demonstrated an increase in MMP-9 but not in MMP-2 activity in SCF-treated samples. Taken together, these results indicated that the effects of SCF on invasion by DLD-1 cells

Table 2 *Effect of c-kit antisense oligonucleotides on SCF-induced migration of DLD-1 cells through Matrigel*[a]

Culture conditions	% migration
Medium	3 ± 2[b]
SCF (100 ng/ml)	28 ± 9[c]
Medium + c-kit S ODN	3 ± 1
SCF + c-kit S ODN	32 ± 3
Medium + c-kit AS ODN	4 ± 1
SCF + c-kit AS ODN	9 ± 2[d]

[a] DLD-1 cells were exposed to the culture conditions indicated for 24 h. Cells were pretreated with c-kit sense (S) and antisense (AS) oligonucleotides (ODN) as described in "Materials and Methods."

[b] Values represent mean ± SD of three separate experiments, each consisting of triplicate cultures.

[c] Significantly different from control (Medium, $P = 0.04$) as determined by Student's t test.

[d] Significantly different from control (SCF + c-kit S ODN, $P = 0.01$) as determined by Student's t test.

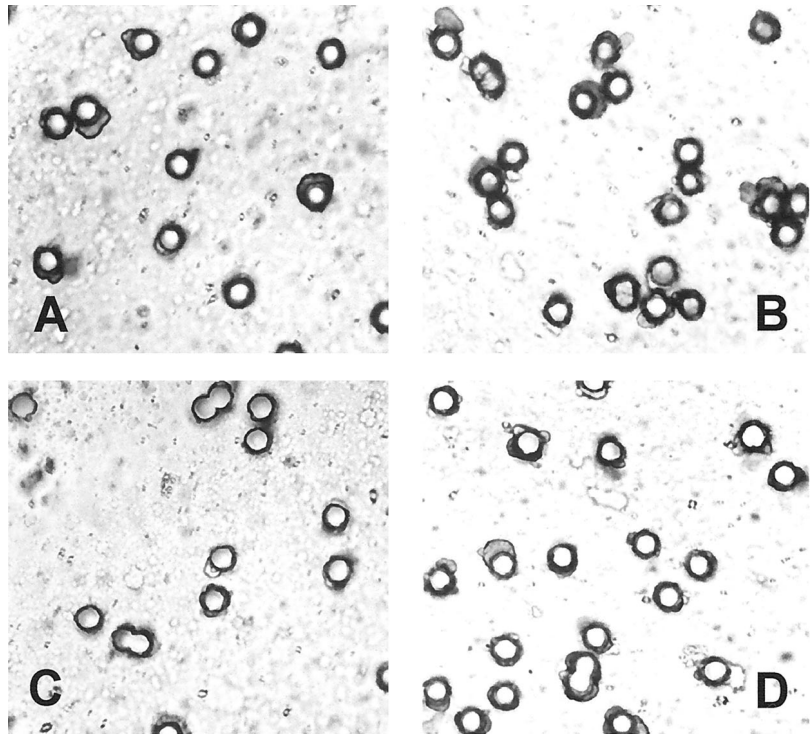

Fig. 3. Migration of untreated DLD-1 cells in the absence of SCF (*A*) and in the presence of SCF (*B*). Migration of anti-c-kit MoAb-treated DLD-1 cells in the absence of SCF (*C*) and in the presence of SCF (*D*). No effect was observed in the presence of an isotype-matched MoAb with irrelevant specificity used as control (data not shown).

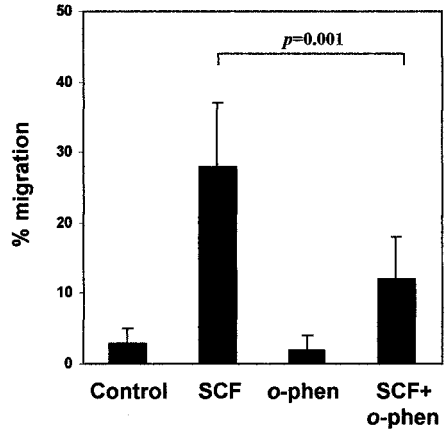

Fig. 4. Effect of MMP inhibitor *o*-phenanthroline on SCF-induced migration of DLD-1 cells through Matrigel. The final concentration of *o*-phenanthroline and the preincubation conditions are described in "Materials and Methods." A significant ($P = 0.001$) reduction in the percentage of migrated DLD-1 cells was observed in the presence of *o*-phenanthroline and SCF relative to controls treated with SCF only. Results are expressed as the means of three separate experiments; *bars*, SD.

was contingent on MMP activity and that SCF induced MMP-9 expression and functional activity.

Effect of Endogenous SCF on DLD-1 Cell Survival. Previously, we reported that exogenous SCF stimulated DNA synthesis of HT-29 colon carcinoma cells, whereas endogenous SCF produced by HT-29 cells had no detectable effect on DNA synthesis in these cells (12). To test whether endogenous SCF contributes to CRC cell survival, the DLD-1 cells were treated, in serum-free conditions, with neutralizing c-kit antibody or antisense c-kit oligonucleotide. The effect of these treatments was assessed by determining morphological and nuclear changes by acridine orange staining. DNA fragmentation was measured by the JAM tests and TUNEL assays. Both JAM tests and TUNEL assays revealed significant levels of DLD-1 cell death ranging between 38 and 52% over background induced by antisense oligonucleotide and by neutralizing c-kit antibody (Fig. 6, *A* and *B*). Consistent with apoptotic death, acridine orange staining of DLD-1 cells treated with antisense c-kit oligonucleotide revealed DNA condensation and nuclear fragmentation (Fig. 6*C*).

DISCUSSION

This study demonstrates that aberrant expression of the c-*kit* proto-oncogene in CRC cells serves to support the malignant phenotype of CRC cells through multiple mechanisms. Previously, we (12) and others (21) reported coexpression of c-kit and of SCF by CRC cells. In addition, SCF was shown to induce DNA synthesis and colony formation in soft agar of some CRC cells. Here, we not only extend these observations to DLD-1 colon carcinoma cells but also present evidence that c-kit-dependent signaling contributes to migration, invasion, and survival of CRC cells.

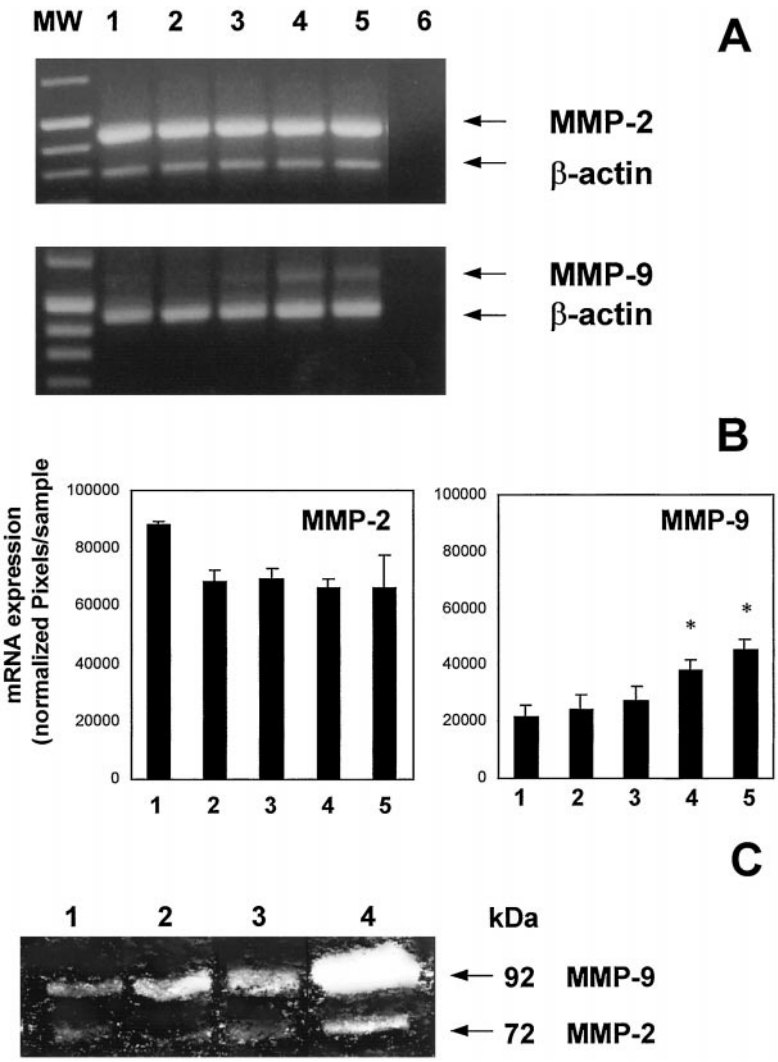

Fig. 5. Comparison of MMP-2 and MMP-9 mRNA expression and gelatinolytic activities produced by DLD-1 cells. *A*, RT-PCR analysis of MMP-2 and MMP-9 mRNA transcripts expressed by steady- DLD-1 cells (*Lane 1*), treated with SCF (*Lanes 2* and *4*) or TPA (*Lanes 3* and *5*) for 4 h and 6 h (*Lanes 4* and *5*). *Lane 6* shows the absence of amplification products in the absence of a cDNA template. *B*, graphic representation of results shown in *A*; results (in arbitrary units) represent signal intensity of MMP-2- and MMP-9-specific amplimers relative to the β-actin signal. *Bars*, SD. *C*, zymographic analysis of MMP-2 and MMP-9 activities in medium conditioned by DLD-1 cells in the absence (*Lane 1*) and presence of either SCF (*Lane 2*) or TPA (*Lane 3*). *Lane 4* shows human serum gelatinolytic activity, used as positive control. The cells were incubated for 24 h in serum-free medium with the supplements indicated, and the cell-conditioned medium was subjected to electrophoresis onto a 10% polyacrylamide gel copolymerized with 1.2 mg/ml gelatin.

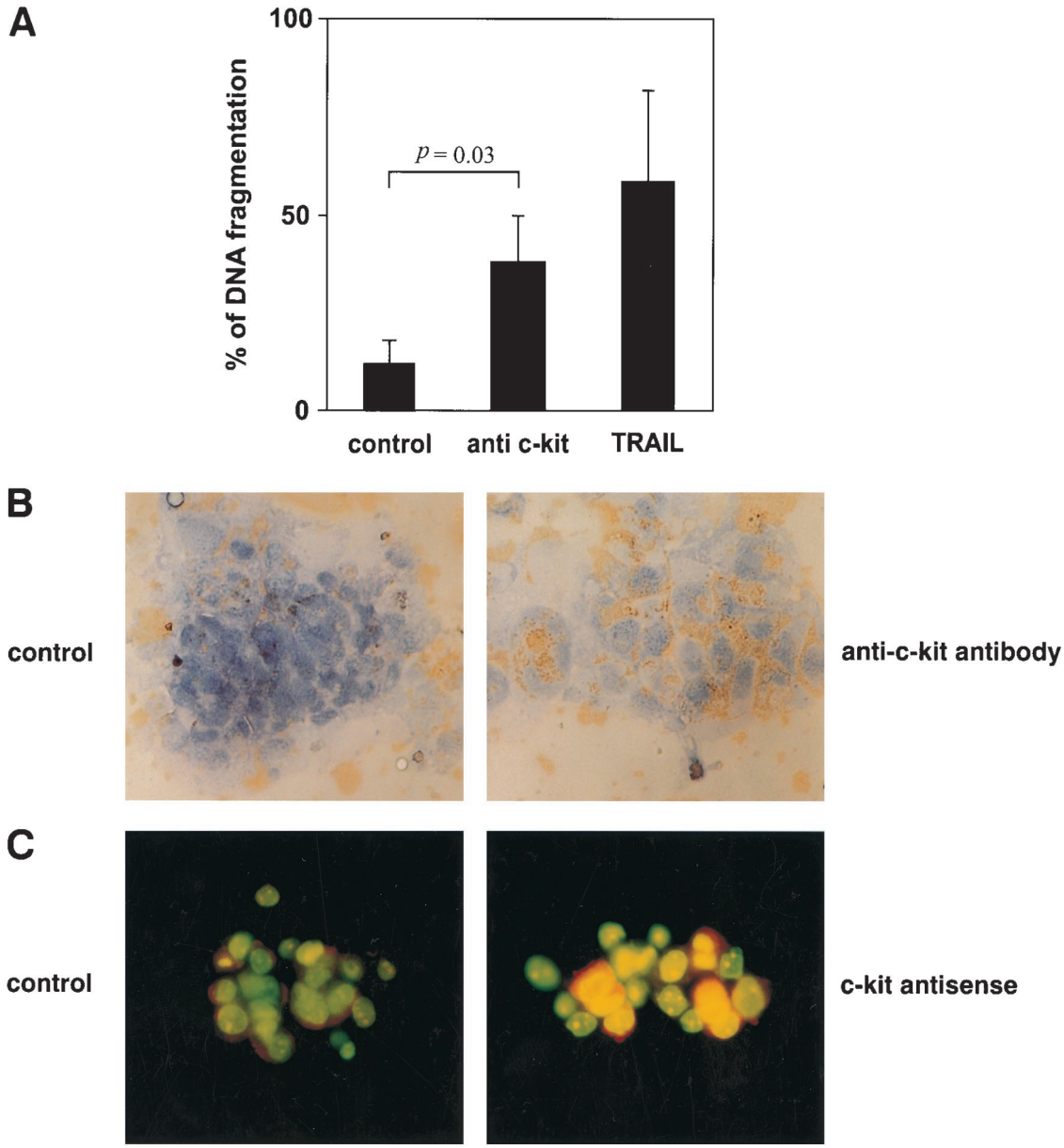

Fig. 6. *A*, DLD-1 cells were labeled with 10 mCi/ml [^{3}H]thymidine for 5 h, washed three times, and seeded in 96-well plates at 5×10^3 cells/well in the absence or in the presence of anti-c-kit MoAb or an isotype-matched irrelevant MoAb. As a positive control for apoptosis induction, TRAIL was used. After 48 h, cells were collected, and the percentage of specific DNA fragmentation was calculated. *Bars*, SD. *B*, representative light micrographs showing evident *in situ* detection of DNA nick ends by TUNEL assay in the DLD-1 cells treated with neutralizing anti c-kit MoAb but not in DLD-1 cells treated with an isotype-matched irrelevant MoAb (*control*). *C*, induction of morphological changes in DLD-1 cells consistent with apoptosis induced by c-kit antisense oligonucleotide treatment determined by staining with acridine orange. Chromatin condensation and nuclear fragmentation are evident in DLD-1 cells treated with the antisense oligonucleotide but not in the control.

The present studies were motivated by the observation that SCF-dependent colony formation in semisolid media is associated with dramatic dispersal of both HT-29 (12) and DLD-1 (this study) cells. These observations were consistent with an effect of SCF on migration and/or invasion of CRC cells. In support of this idea, we found that SCF markedly stimulated transmigration of DLD-1 cells through Matrigel-reconstituted basement membranes. This effect was dependent on c-kit activation because not only neutralizing antibodies to c-kit but also c-kit antisense oligonucleotide blocked the SCF-stimulated invasion. Although c-kit signaling has been implicated in migration of embryonal cells including melanoblasts and some germ cells (13), little is known about its contribution to migration and invasion of malignant cells except that SCF exerts chemotactic effects on lung carcinoma cells in Boyden chamber assays (30). However, SCF has been shown to contribute to the mobilization of hemopoietic CD34+ progenitor cells from the bone marrow by up-regulating the expression of the MMP-2 and MMP-9 (31). As in CD34+ progenitor cells, we observed that SCF up-regulated MMP-9 expression and enzymatic activity in DLD-1 cells, whereas inhibition of MMP activity by *o*-phenanthroline markedly inhibited transmigration of DLD-1 cells through Matrigel. However, in contrast to CD34+ cells, SCF treatment of DLD-1 cells did not detectably affect MMP-2 expression or activity. Taken together, our results point to c-kit-dependent MMP-9 production as a mechanism for invasive behavior shared between hemopoietic precursors and malignant gastrointestinal cells.

Several lines of evidence implicated SCF-dependent c-kit activation in survival of cells during embryonal development and in malignant tumors. For example, c-kit activation was found to suppress apoptosis of normal murine melanocyte precursors (15), soft tissue sarcomas of neuroectodermal origin (16), neuroblastomas (8), and

normal and malignant human hemopoietic cells (17). Our results support a role of c-kit activation in survival of DLD-1 CRC cells because both a neutralizing c-kit antibody and a c-kit antisense induced DNA nicks and nuclear changes characteristic of apoptotic death and markedly reduced viability of these cells. This result was significant because c-kit activation does not universally support survival of malignant cells. For example, activation of c-kit in astrocytes is associated with increased cell death (18), and forced c-kit expression renders metastatic human melanoma cells susceptible to SCF-induced apoptosis and inhibits their tumorigenic and metastatic potential (19). At present, the mechanisms underlying the apparent paradox of c-kit-dependent protection of melanocytes from apoptosis and induction of melanoma cell death by c-kit activation are unresolved. However, it is clear that effects of c-kit on cell survival are related to both cell lineage and the state of transformation; our results suggest that in CRC cells, c-kit serves an antiapoptotic role.

Most of the effects mediated by c-kit activation of DLD-1 cells were elicited by exogenous SCF added to these cells. This conclusion applies particularly to the induction of cell scattering in semisolid medium and the migration of DLD-1 cells through Matrigel. However, we observed coexpression of SCF and c-kit in DLD-1 cells, raising the issue of whether CRC cell-derived SCF serves autocrine functions. No reduction in migration of DLD-1 cells through Matrigel was observed in the presence of c-kit antisense oligonucleotide. This may in part be attributable to the fact that migration occurred at a very low rate in the absence of exogenous SCF. Also the cell-derived SCF is not likely to create a chemotactic concentration gradient required for directional migration. Thus, these results do not refute a role of CRC-derived SCF in invasion and MMP-9 production of DLD-1 cells. An autocrine role of SCF on DLD-1 growth and survival is suggested by the finding that the c-kit antisense oligonucleotide reduced colony formation in methylcellulose in the absence of added SCF from 63 ± 12 to 44 ± 19 (see Table 1), although this effect did not reach statistical significance. The modest reduction in colony formation associated with blocking c-kit is contrasted by a marked effect of exogenous SCF on DLD-1 colony formation. In summary, these results indicate that c-kit activation by autocrine or paracrine SCF is not a necessary requirement for DLD-1 survival and growth in anchorage-independent conditions but can serve to significantly amplify the capacity of DLD-1 cells to do so.

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Aberrant Activation of c-kit Protects Colon Carcinoma Cells against Apoptosis and Enhances Their Invasive Potential

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