Implication of Tyrosine Kinase Receptor and Steel Factor in Cell Density-dependent Growth in Cervical Cancers and Leukemias

Julio R. Caceres-Cortes,1 Jose A. Alvarado-Moreno, Kazuo Waga, Rosalva Rangel-Corona, Alberto Monroy-Garcia, Leticia Rocha-Zavaleta, Julia Urdiales-Ramos, Benny Weiss-Steider, Andre Haman, Patrice Hugo, Roland Brousseau, Trang Hoang

Laboratories of Oncology [J. R. C.-C., J. A. M.-A., R. R.-C., J. U.-R.] and Immunobiology, [A. M.-G.], Research Unit in Cell Differentiation and Cancer, Facultad de Estudios Superiores Zaragoza [B. W.-S.], and Molecular Biology Department, Biomedical Research Institute [L. R.-Z.] Universidad Autonoma de Mexico, Mexico 15000; Institute de Recherches en Biotechnologie, Montreal, H4P 2R2 Quebec, Canada [R. B.]; Procrea, Montreal, H4P 2R2 Quebec, Canada [P. H.]; and Laboratory of Hemopoiesis and Leukemia, Clinical Research Institute of Montreal, Montreal, H2W 1R7 Quebec, Canada [K. W. A. H., T. H.]

ABSTRACT

Cell-cell interaction is important in the expansion of leukemic cells and of solid tumors. Steel factor (SF) or Kit ligand is produced as a membrane-bound form (mSF) and a soluble form. Because both primary gynecological tumors and primary leukemic cells from patients with acute myeloblastic leukemia (AML) have been shown to coexpress c-Kit and SF, we addressed the question of whether mSF could contribute to cell interaction in these cancers. Investigations on primary cervical carcinomas have been hindered by the fact that the cells do not grow in culture. We report herein the establishment of two cervical carcinoma cell lines, CALO and INBL, that reproduce the pattern of SF/c-Kit expression observed in primary tumor samples. In addition, these cells exhibit marked density-dependent growth much in the same way as AML blasts. Using an antisense strategy with phosphorothioate-modified oligonucleotides that specifically target SF without affecting other surface markers, we provide direct evidence for a role of mSF and c-Kit in cell interaction and cell survival in these gynecological tumor cell lines as well as in primary AML blasts. Finally, our study defines the importance of juxtacrine stimulation, which may be as important, if not more, than autocrine stimulation in cancers.

INTRODUCTION

Cell-cell interaction is important in the expansion of leukemic cells and solid tumors. Ligand-receptor pairs are frequently coexpressed in human cancer cells, and the autocrine paracrine loops causing self-stimulation are thought to be involved in carcinogenesis (1–3). Human solid tumor cell lines have been shown to produce hematopoietic growth factors and display their receptors (4), suggesting that soluble or membrane-bound growth factors may contribute to cell interaction.

Mutations at the W1 locus in mice have deleterious effects on germ cells, melanocytes, and hematopoietic stem cells (reviewed in Ref. 5). The W locus encodes the c-Kit receptor tyrosine kinase of which the ligand is the product of the Steel (Sf) locus. SF is produced as a soluble form or a membrane-bound form (6, 7). There is considerable evidence to suggest that c-Kit and SF are involved in the pathogenesis of both hematological and nonhematological malignancies (8). For example, the murine hemopoietic cell line 32D is nonleukemogenic. Ectopic c-Kit expression confers to these cells a lethal leukemogenic potential in vivo without an obvious effect on cell proliferation in vitro (9). In addition, a very high percentage of primary AML blasts express c-Kit (10), and the majority of fresh leukemic blasts are growth stimulated by SF in vitro (11, 12). We and others have shown that the growth of AML blasts is density-dependent and that SF (Kit ligand) is the only cytokine that can replace cell interaction in AML culture (9, 12, 13). Together, these results suggest that cell interaction in AML may be mediated by SF/c-Kit interaction.

Molecular approaches have led to the concept that carcinomas arise from the accumulation of a series of genetic alterations involving the activation of proto-oncogenes and inactivation of tumor suppressor genes. Despite the evidence that HPVs are involved in uterine cervical carcinomas, the E6E7 genes of HPV16 alone cannot fully transform human and rodent primary cells in vitro or cause tumor development in immunodeficient animals. Full transformation requires cooperation of E6E7 with other activated oncogenes (14–16). It has been shown that the oncogenes myc and ras are activated and that the tumor suppressor genes p53 and Rb are altered, consistent with the concept of multistep carcinogenesis (17). Whereas these molecular events may confer a growth advantage to transformed cells, the mechanisms that underlie the process of cell interaction in cancers remains to be documented.

Transgenic mouse lines expressing HPV16 E6E7 oncogenes developed normally, but male mice exhibited a very high incidence of Leydig cell tumors at 8–10 months (18). The c-Kit receptor tyrosine kinase and its ligand SF were coexpressed in all of the tumors analyzed, and this coexpression of c-Kit/SF was also found in two other Leydig cell tumor lines. Moreover, the proliferation of transgenic tumor cells was attenuated by treatment with a c-Kit-neutralizing antibody in vitro, strongly suggesting that tumorigenesis is closely related to ligand-dependent receptor activation. A role for c-Kit in tumorigenesis came from the demonstration that tumor formation induced by the E6E7 transgene is attenuated in a Sf or W background (19). These results indicated that c-Kit activity is an important determinant of testicular tumor development, although the function of SF/c-Kit in the biology of HPV-induced gynecological tumors remains to be determined.

The incidence of cervical carcinoma in women in Peru is 54/100,000, which is the highest in the world (20). Similarly, ~30% of all of the malignant tumors in women in Mexico are uterine cervix carcinomas (21). Invasive cervical cancer occurs most often in women >40 years of age, and it is one of the main causes of cancer-related death in Latin America. Current treatment includes surgery and radiotherapy or chemotherapy. Radiations and chemotherapeutic agents induce apoptosis in cancer cells, but selectivity remains a key issue. Drugs that target nucleic acids through Watson-Crick base pairing should provide unique specificities. Among those, antisense oligonucleotides designed against a specific RNA have been modified for improved stability. The most promising are the phosphorothioates,
DNA analogues in which the oxygen atoms are replaced by sulfur atoms.

Both gynecological tumors (22) and colon tumor cell lines (23) have been shown to coexpress c-Kit and SF. It has been reported that autocrine/paracrine stimulation through this system may contribute to tumorigenesis in lung, breast, and testicular malignancies (24–28). Much less is known about cervical carcinomas because of limited availability of appropriate cellular models (29–31). We report herein the establishment of two carcinoma cell lines that reproduce the pattern of SF/c-Kit expression observed in primary tumor samples. Using an antisense strategy with phosphorothioate modified oligonucleotides directed against SF, we provide direct evidence for a role of mSF and c-Kit in cell interaction and cell survival in these gynecological tumor cell lines, as well as in primary AML blasts.

**MATERIALS AND METHODS**

**Source of Cells and Growth Factors.** Peripheral blood from AML patients (Hotel-Dieu Hospital, Montreal, Canada) were obtained by informed consent. AML blasts (Table 1) were isolated by centrifugation of peripheral blood cells on a Ficoll-Hypaque gradient (Pharmacia Fine Chemicals, Uppsala, Sweden). The cells were cryopreserved in FCS (Life Technologies, Inc., Grand Island, NY) containing 10% DMSO and stored in liquid nitrogen until use. On thawing, cells were >90% viable.

The human cell line TF-1, a kind gift of Dr. T. Kitamura (DNAX, Palo Alto, CA), was passaged 3 times weekly at 1.5 × 105 cell/ml in IMDM/10% FCS containing human GM-CSF at 5 ng/ml as described previously (32).

The mouse 3T3 cell line p220 expressing membrane-bound human SF (mSF) was a kind gift of Dr. D. Williams (Howard Hughes Medical Institute, Indianapolis, IN) (33). Purified recombinant human GM-CSF was generously provided by Dr. S. C. Clark (Genetics Institute, Cambridge, MA), and soluble recombinant human SF was produced through transient transfection in SV40 transformed African green monkey kidney cells.

**Cell Surface Analysis.** For fluorescence-activated cell sorter analysis, cells were labeled with a monoclonal mouse antihuman SF (1:10; Caltag, San Francisco, CA), or mouse antihuman HER-2 were labeled with a monoclonal mouse antihuman SF (7H6, IgG2a; kindly provided by Dr. D. Williams, Howard Hughes Medical Institute, London, England) (24). The cells were blocked with a mixture of human immunoglobulin (10% normal human serum, 1% methylcellulose (Fluka, Ronkonkoma, NY) as described previously (13).

**Sample FIGO**

| Sample | FIGO | Histopathology | mSF Mean fluorescence intensity
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<td>AML 72</td>
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**Histopathology**

The histopathology was assessed on H&E-stained tissue sections.
and TBS × 2, membranes were transferred to a visualization solution composed of 10 ml alkaline phosphatase buffer [100 mM Tris-HCl (pH 9.5), 100 mM NaCl, and 5 mM MgCl$_2$] containing 33 μl of nitroblue tetrazolium (50 mg/ml in 70% dimethyl formamide) and 66 μl of 5-bromo-4-chloro-3-indolyl phosphate (50 mg/ml in 100% dimethyl formamide). Membranes were soaked for 30 s to 1 min, and the reaction was stopped with water.

**Immunoprecipitation.** Exponentially growing CALO and TF-1 cells were washed and incubated in OPTI-MEM (Life Technologies, Inc.) containing 1% BSA (Life Technologies, Inc.) for 24 h at 37°C to deprive cells from growth factors; TF-1 cells were deprived from GM-CSF for 6 h. Before stimulation, the cells were harvested by trypsinization (0.1%), washed, and resuspended in appropriate medium. Tyrophostins 1 and B42 (10 μM) were added, incubated for 5 min, stimulated with SF for 3 min, and rapidly pelleted at 5,000 × g for 5 min in a microfuge (Eppendorf). Cells were resuspended in ice-cold lysis buffer [1% Triton X-100, 5 mM EDTA, 1 mM KCl, 140 mM NaCl, 2 mM MgCl$_2$, 50 mM Tris (pH 7.4), 1 mM phenylmethylsulfonyl fluoride, 1 mM NaF, 1% aprotinin, 1 μM leupeptin, and 100 μM Na$_3$VO$_4$] for 15 min. Lysates were clarified by centrifugation at 13,000 rpm at 4°C for 15 min. Total protein content of the lysate was determined by the Bio-Rad protein assay (Bio-Rad, Hercules, CA).

Total lysate containing the same amount of proteins was incubated with protein A-Sepharose (Life Technologies, Inc.) coupled previously to a specific mouse antihuman c-Kit antibody (Boehringer-Manheim, Mannheim, Germany; at 1 μg/ml) for 2 h at 4°C. Immunoprecipitated proteins were washed 5 times with lysis buffer, resolved by 7.5% SDS-PAGE, and transferred to nitrocellulose membrane. Western blots were analyzed using a mouse antiphosphotyrosine antibody (clone 3-365-10; Boehringer-Manheim) at 1 μg/ml, and proteins were visualized by alkaline phosphatase-conjugated antibodies system (Sigma Chemical Co.). Substrates were nitroblue tetrazolium (8 mg/ml) and 5-bromo-4-chloro-3-indolyl phosphate (1.6 mg/ml; Sigma Chemical Co.).

**Antisense Oligonucleotide Uptake and Stability.** Oligodeoxynucleotides were 5'-labeled with 5'-[γ-32P]thio) ATP (1276 Ci/mmol; Amersham) with bacteriophage T4 kinase and purified by polyacrylamide gel electrophoresis. AML blasts were incubated with 5 × 10$^6$ cpm of 32P-labeled oligonucleotide at 4 × 10$^5$ cells in 0.5 ml of IMDM supplemented with 10% FCS at 37°C. At the indicated times, cells were collected by centrifugation (3 min, 15,000 × g), washed twice in PBS, and the pellet was lysed in 0.1 ml of TBS [10 mM Tris (pH 7.4) and 150 mM NaCl] with 1% NaDodSO$_4$, and then extracted with phenol. The aqueous phase was saved and the phenol re-extracted with water. The combined aqueous phases were lyophilized, redissolved in 80% deionized water, and analyzed in a denaturing 20% polyacrylamide gel.

For the AS-SF, a 20-mer oligonucleotide was designed that starts at the translation initiation codon. A control oligonucleotide was synthesized in which the sequence was shuffled: AS-SF, CAA GTT GTG GTC TTC AT PAKL, TTG TTT ACT GCG TCT ACT AT.

**Whole Cell ELISA.** Translation of Sl into mSF was quantitated by whole cell immunosorbent assay. AML, CALO, and INBL cell lines were labeled for 30 min at 4°C with the mAb antihuman SF (7H6; 1 μg/10$^6$ cells). The cells were washed 3 times with PBS/BSA 1% and labeled with a second antibody (peroxidase-conjugated goat antimouse, 1:1000; Sigma Chemical Co.) for another 30 min. The cells were washed 3 times, and cell-bound antibodies were revealed with a 100-μl o-phenylenediamine (1 mg/ml) in phosphate buffered saline plus 14 μl of 30% H$_2$O$_2$. After 5 min (AML blasts) and 10 min (CALO and INBL cells) at 37°C, the reaction was stopped by addition of 50 μl of 4 N sulfuric acid. Then the cells were centrifuged, and the absorbance of the cleared supernatants was read with an ELISA plate reader at 490 nm.

The **TUNEL Reaction.** AML (1 × 10$^6$ cells), CALO, and INBL (5 × 10$^5$ cells) were analyzed for apoptosis using a quantitative DNA fragmentation assay, the TUNEL reaction. Briefly, cells were fixed with formaldehyde for 5 min at room temperature and permeabilized with 0.1% Triton X-100 and 0.1% sodium citrate at 4°C for 2 min. Cells were then incubated for 1 h at 37°C with 0.3 nmol of biotin-16-DUTP (Boehringer-Manheim), 3 nmol of dATP (Pharmac, Uppsala, Sweden), 20 units of TdT (Pharmac) in TdT buffer (0.1 mM potassium cacodylate pH 7.2, 2 mM CoCl$_2$, 0.2 mM dithiothreitol; Life Technologies, Inc.) in a total reaction volume of 50 μl. The reaction was stopped by the addition of 0.5 mM EDTA. DNA from apoptotic cells that contained free 3'-hydroxy ends was labeled by the TdT with biotinylated dUTP, which was then revealed with streptavidin-FITC (Amersham, Buckinghamshire, England). After washing, the cells were cytomsmeared and mounted in Vectashield for counting.

**RESULTS**

**Establishment of Human Cervical Carcinoma Cell Lines: Density-dependent Growth.** Both CALO and INBL cells form cobblestone areas in FCS-containing medium. Karyotype analysis (Fig. 1A) revealed that both cells were aneuploid, with CALO cells numbering on average 50 chromosomes with a range of 32–62 and INBL cells numbering on average 50 chromosomes with a range of 48–66. Cell cycle analysis by flow cytometry also confirmed the presence of an aneuploid cycle, which was 17.1% for CALO and 23.7% for INBL (data not shown). These observations are consistent with previous work indicating pronounced chromosomal imbalances in primary cervical carcinomas and cell lines (34).

We initially observed that cultures seeded at low density grew very slowly with a long lag time, whereas cultures seeded at high cell density propagated with a doubling time of 3–4 days. Limiting dilution analysis was therefore performed and revealed two distinct growth patterns for both CALO and INBL cells (Fig. 1B). At low cell concentrations, growth was extremely slow, whereas at concentrations >15,000 cells/ml, the growth curve showed a much steeper slope. This growth pattern was reproducibly observed, even after 50 passages. In contrast, HeLa cells, a cervical carcinoma cell line, exhibited
a single accelerated growth pattern similar to the second proliferative growth curve of CALO and INBL cells. Furthermore, conditioned medium harvested from cultures seeded at high densities did not accelerate the growth of cultures seeded at low densities (data not shown). These results indicate that for the two cervical carcinoma cell lines that we established, CALO and INBL, cell growth is density-dependent, consistent with a requirement in cell interaction for propagation in culture.

Because HeLa cells express HER-2 and primary cervical carcinomas express c-Kit, we investigated the surface phenotype of CALO and INBL cells. As shown in Fig. 1C, all three of the cervical carcinoma cell lines expressed HER-2 at comparable levels. In contrast, CALO and INBL expressed high levels of c-Kit (Fig. 1C, left and middle panels) comparable with those found on the CD34+ hematopoietic cell line TF-1 (data not shown), whereas HeLa cells were negative for c-Kit (Fig. 1C, right panel). We next investigated their growth factor requirement. Cell growth was clearly dependent on FCS, and serum withdrawal resulted in growth arrest, cell shrinkage, and cell death through a process resembling apoptosis (Fig. 2A). Interestingly, soluble SF added at 323 pM could substitute for FCS to sustain growth in both INBL and CALO cells (Fig. 2A). In these cells, soluble SF induced the tyrosine phosphorylation of c-Kit (M1, 145,000) as identified by immunoprecipitation and of two other proteins of M1, 95,000 and 120,000 (Fig. 2, B and C). Treatment with tyrosine kinase inhibitors (Tyrophostin B42) prevented c-Kit tyrosine phosphorylation (Fig. 2C) and induced apoptosis in both cell types, as assessed by the TUNEL reaction (Fig. 2D), whereas Tyrophostin 1, an inactive analogue, did not affect cell viability. Both Tyrophostin B48 and Tyrophostin B42, shown previously to inhibit c-Kit signaling (33), induced apoptosis to the same extent, suggesting that SF is a survival factor for cervical carcinoma cells as documented for AML blasts.

c-Kit and mSF are Coexpressed in Cervical Cancer Cell Lines and AML Blasts. Our results indicate that CALO and INBL cells express functional c-Kit receptors in the same way as primary AML blasts (11, 12). Therefore we addressed the question of whether these cells coexpress c-Kit and mSF that could contribute to the process of density-dependent growth (Fig. 1B; Ref. 12). A mAb specific for human SF brightly stained 3T3 cells that were engineered to express mSF (p220; Fig. 3B), whereas parental NIH-3T3 cells and TF-1 cells were negative for mSF. Interestingly, CALO, INBL, and HeLa cells expressed detectable levels of mSF (Fig. 3A). Six of seven primary AML samples expressed mSF to various extents (16–90%; Fig. 3B and data not shown). Samples 32, 45, and 55 stained most brightly and were classified as M1, M2, and M5, respectively. There was no obvious correlation between mSF levels and FAB classification.

The surface marker CD34 is expressed in AML stem cells (36). As the cells differentiate, they acquire myeloid surface markers such as CD13 and loose CD34 expression. Because mSF is not detected in all of the cells, we addressed the question of whether mSF expression is a property of leukemic progenitors and/or of differentiating leukemic cells. Double labeling indicated that both CD34+ and CD34- AML blasts expressed mSF (Fig. 4). Furthermore, most mSF+ cells are also CD34+. Thus, mSF is expressed by leukemic progenitors and differentiating leukemic cells, consistent with the fact that expression of mSF is not restricted to a FAB subtype.

Antisense Inhibition of mSF in AML Blasts and Cervical Cancer Cell Lines. To address the significance of mSF expression in these tumors, we sought to suppress mSF production through the use of phosphorothioate-modified oligonucleotides. We first determined the toxicity of control phosphorothioate oligonucleotide and found that concentrations <10 pM were well tolerated by INBL and CALO cells, and <4 pM by AML blasts (data not shown). Therefore, these concentrations were chosen for subsequent experiments. Kinetic analysis indicated that the oligonucleotides were efficiently incorporated into AML blasts and remained stable for ≥24 h (Fig. 5A), although at 8 h, the oligonucleotides were found in high molecular weight complexes. These complexes were resistant to detergent and heat denaturation, suggesting an irreversible association with cellular components. The level of inhibition of cell-associated SF was therefore quantitated by whole cell ELISA. Cells were harvested every day starting from 48 h after exposure. On day 2, half of the medium was changed, and the cells were re-exposed to the same concentrations of phosphorothioate-modified oligodeoxynucleotide. A 48-h treatment with AS-SF was not sufficient to reduce mSF expression (data not shown). After two cycles of 48 h of exposure to phosphorothioate-modified oligodeoxynucleotide, AS-SF induced a substantial decrease in mSF levels in CALO and INBL cells, as determined by whole cell immunoperoxidase (Fig. 5B) or by flow cytometry analysis (Fig. 5C). The same antisense was also efficient in abrogating the production of
mSF by the p220 3T3 transfectant (Fig. 5B) as well as in AML blasts (Fig. 5D). In contrast, a PAKL did not affect mSF levels in these cells (Fig. 5, B–D). To additionally determine the specificity of the AS-SF oligonucleotide, we monitored the levels of another surface marker, CD34, which is expressed on AML blasts. In contrast to SF, there was no significant difference in CD34 levels in cells treated with AS-SF, in the control oligonucleotide, or in parallel cultures that were left untreated (Fig. 5D). Similarly, the AS-SF oligonucleotide did not affect the level of HER-2 in CALO and INBL cells (data not shown), indicating its specificity for mSF. For sample AML 53, there was nonetheless some degree of apoptosis with the control oligonucleotide, consistent with [3H]thymidine incorporation studies (data not shown), indicating a narrow window of efficiency above which the oligonucleotide starts to have nonspecific toxicity (Fig. 5D). However, apoptosis was ≥2-fold higher with AS-SF. Taken together, our observations clearly indicate that AS-SF specifically targets mSF in AML samples as well as in gynecological tumors.

Antisense Inhibition of mSF in AML Blasts and Cervical Cancer Cells Prevents Density-dependent Growth and Induces Apoptosis. Our previous work indicates that autonomous cell proliferation in the culture of primary AML blasts occurs at high cell concentrations, whereas at low concentrations, cell proliferation is strictly dependent on an exogenous source of growth factor. Autonomous cell proliferation was first assessed by thymidine incorporation...
and was attenuated by treatment with AS-SF in a dose-dependent fashion (Fig. 6A). In contrast, the effect of the control oligonucleotide was not significant in the dose range tested. Similarly, blast progenitors, assayed through their capacity to form colonies in vitro in methylcellulose cultures either in the absence of growth factors or in GM-CSF-containing cultures, were significantly decreased after treatment with the AS-SF oligonucleotide but not the control oligonucleotide. To additionally support the specificity of antisense inhibition of mSF expression in AML blasts and CALO and INBL cells. We next addressed the role of mSF in the growth of cervical carcinoma cells in vitro (Fig. 7) in FCS-containing cultures. Treatment with the control oligonucleotide did not significantly affect the growth of CALO or INBL cells that remained adherent (Fig. 7A), viable (i.e., TUNEL−; Fig. 7B), and were able to sustain DNA synthesis as determined by thymidine incorporation (Fig. 7C). In contrast, AS-SF oligonucleotides caused the cells to apoptose and detach. Furthermore, antisense inhibition reduced thymidine incorporation to levels observed in cultures initiated at low cell density (Fig. 7C). Finally, HeLa cells that do not exhibit density-dependent growth, as shown in Fig. 1B, were not affected by the oligonucleotide (data not shown), consistent with the lack of c-Kit expression on these cells. Together, our results indicate that mSF contribute to the density-dependent growth of cervical carcinoma cell lines and of AML blasts.

**DISCUSSION**

Cell interaction is a process common to cancers of diverse origins. Cervical carcinomas and leukemic cells are shown here and elsewhere (11, 12) to coexpress mSF and c-Kit. In vitro culture assays for AML blasts revealed the importance of cell interaction in the process of autonomous growth (12). Interestingly, only irradiated cells or membranes isolated from AML blasts (37) could substitute for cell interaction in suspension culture. Indeed, our
attempts at replacing irradiated cells or membranes or at limiting cell concentrations by soluble growth factors such as IL-1 (38), IL-6 (39), tumor necrosis factor-α (40), and IFN-γ (41) were not successful. These results lead us to hypothesize that the presence of membrane-bound growth factors could mediate cell-cell interaction in AML. Our previous work indicated that colony formation as a function of cell concentration (12) was better described by a model with two limiting parameters, the colony-forming cell itself and an “interacting” cell. Interestingly, addition of high concentrations of soluble SF was sufficient to alleviate the requirement for cell interaction. These observations led us to investigate the possibility that these leukemic cells express mSF, which could participate in the process of cell-cell interaction. In the present study, we show that both undifferentiated (FAB M1, CD34+ and differentiating (FAB M4/5 and CD13+) AML blasts express mSF, shown here through an antisense strategy to be important in the process of cell-cell interaction in AML.

There is evidence to suggest that c-Kit and SF are also involved in the pathogenesis of nonhematological malignancies. Gynecological tumors (22), colon tumor cell lines (23), small cell lung cancers (26, 42), and testicular tumors (43) have all been shown recently to coexpress c-Kit and SF. To confirm the significance of these findings, a mutation of the SF gene in a laboratory mouse, the Steel-Dickie mutation, was introduced into a line of HPV-E6E7 transgenic mice that develop testicular tumors with full penetrance. In Steel-Dickie-E6E7 transgenic mice, tumorigenesis was initiated, but the tumor load was markedly reduced compared with transgenic mice carrying wild-type Sl alleles, showing that c-Kit activation by SF is essential for testicular tumorigenesis, especially in tumor promotion. These transgenic mice provide a useful in vivo model implicating growth factor autostimulation in carcinogenesis (18, 19). Various W mutations (W, W−, and W−2) were also introduced into the transgenic mouse strain carrying HPV oncocenes in which c-Kit/Steel-mediated tumorigenesis occurs with high incidence. In all of the transgenic strains carrying a W mutation, c-Kit deficiency affected the tumorigenic process to various degrees. Tumor development was markedly suppressed in transgenic strains carrying kinase defective mutations (W− and W−2) in a heterozygous condition. In null-type (W) heterozygous transgenic mice, tumorigenesis was suppressed to a lower level. Moreover, minimal focal lesions or, in some cases, no focal lesion were found in the testes of W/W− compound heterozygous transgenic mice, showing a close relationship between tumor cell growth and the degree of c-Kit inactivation. In this mouse model of testicular tumors, it is not clear, however, whether mSF-cKit interaction is important in paracrine or autocrine stimulation. Furthermore, there was no direct evidence for a role of c-Kit and mSF in cervical cancers, the most prevalent cancer in women in Latin America.

The role of mSF-Kit in AML blasts and in a mouse model of testicular tumors led us to address the role of this ligand-receptor pair in tumors of the cervix. Previous work indicates that transcripts for SF but not c-Kit are found in the commonly used cervical carcinoma cell lines (HeLa, Caski, and SiHa). We show here that HeLa cells do not require cell interaction for propagation in culture, possibly because of the fact that the cells have been extensively expanded in culture and may have acquired additional mutations bypassing this property. In contrast, the two cervical carcinoma cell lines that we have established, CALO and INBL, have retained the characteristics of most primary tumor cells, i.e., growth factor dependence for cell survival and density-dependent cell proliferation. Furthermore, we show that these two cell types coexpress mSF and c-Kit and that mSF is an important determinant of cell survival and cell-cell interaction in cervical carcinomas.

Tumorigenesis is a multi-hit process that involves several independent events, and evidence shown here and elsewhere suggests that autocrine growth factor production (1) is one of the more common perturbations, responsible for the final stages of uncontrolled growth and survival. Initially, autocrine stimulation was defined as the capacity of tumor cells to secrete and respond to growth factors (1). This concept later evolved to include a “private” autocrine loop in which growth factors interact with their receptors intracellularly (2). Our observations suggest that autocrine stimulation may frequently be a juxtacrine process mediated by membrane-linked growth factors. Such a process may in fact be amplified by signaling through additional “ligand-receptor”-type interaction belonging to the superfamilies of adhesion molecules. Consistent with this hypothesis, previous work indicates that mSF is more potent than soluble SF in maintaining the survival of multipotent hemopoietic stem cells in vitro (7). Therefore, we propose that juxtacrine stimulation by membrane-linked growth
factors may underlie the process of cell-cell interaction and density-dependent growth in cancers, which may be more common than anticipated previously.

As a consequence of autocrine stimulation in cancers, receptor antagonists became potential targets in cancer therapy. For example, IL-1R antagonist and soluble IL-1R were used in chronic myeloid leukemia aimed at inhibiting the action of autocrine IL-1β (44). However, antagonists are described for only a few growth factor receptors, and IL-1R does not appear to mediate cell-cell interaction. The antisense strategy described here aimed at interrupting mSF production by leukemic cells, and cervical carcinomas may prove useful as a novel adjuvant therapy with improved specificities. HPV16 E6/E7 antisense oligonucleotides were shown to decrease the proliferation of HPV-positive cells, albeit with low efficacy (45). It may therefore be possible to achieve high efficacy and specificity by targeting two genetic events in HPV-positive gynecological tumors, the HPV E6/E7 oncogenes and mSF. The in vivo efficacy of a targeted genetic therapy was shown recently in a clinical trial with patients suffering from advanced cancers. These patients were treated with a phosphorothioate antisense oligodeoxynucleotide directed to the 3′ untranslated region of the c-raf-1 mRNA, resulting in significant reductions of c-raf-1 expression by day 3 of treatment. Furthermore, the time course and depletion of c-raf-1 message in peripheral blood mononuclear cells paralleled the clinical benefit in two patients (46). Given that normal hemopoietic stem cells require SF in vivo, a strategy based on AS-SF will require accurate determination of its therapeutic window. The possibility of xenografting human AML blasts (36) and human cervical carcinoma cells (47) in immune-deficient mice and the availability of mouse models of testicular tumors (48) will allow for a preclinical evaluation of AS-SF on tumor burden in vivo and for its effect on normal hemopoiesis.

In summary, our results indicate that in both AML blasts and cervical carcinomas, exponential growth is dependent on cell concentrations suggesting a requirement in cell interaction. Antisense inhibition of cell-associated SF blocks this interaction by disrupting cell to cell contact mediated by the association of c-Kit with membrane-bound SF.

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Fig. 7. Antisense inhibition of mSF induces apoptosis in CALO and INBL cells and prevents density-dependent growth. A, CALO and INBL cells were plated at 15,000 cells/ml with AS-SF or PAKL as described in Fig. 5B. After 5 days of culture, a representative field was photographed from control and oligonucleotide-containing cultures. B, apoptosis was examined by TUNEL reaction. C, [3H]thymidine incorporation. Oligonucleotides (10 μM) were added after overnight adherence, and thymidine incorporation was determined as described in Fig. 1B. ■ untreated cultures; □ cultures treated with PAKL; ● cultures treated with the AS-SF. Data shown are typical of two independent experiments and in C, represent the mean of triplicate determinations; bars, ± SD. Differences between AS-SF and PAKL control-treated cells are highly significant (P < 0.05).
REFERENCES


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