Overexpression of Normal c-Src in Poorly Metastatic Human Colon Cancer Cells Enhances Primary Tumor Growth but not Metastatic Potential

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Abstract
Whereas genetic paradigms are now defined for the development of human colon cancer, little is known regarding the mechanisms that regulate development of the metastatic phenotype. Recent reports have indirectly linked the expression and activation of c-Src to the process of human colon cancer metastasis. Whereas v-Src, a highly activated mutational derivative of c-Src, has been shown to induce metastasis, normal c-Src has not been tested for this property. We hypothesized that c-Src overexpression in the milieu of a poorly metastatic cancer cell might permit the development of a highly metastatic cell. Two poorly metastatic human colon cancer cell lines were stably transfected with expression vectors encoding normal human c-Src. Clones producing 4–10-fold more c-Src than controls were injected s.c. and intraspinally into the nude mouse to assess primary tumor growth and liver metastatic potential. Whereas metastatic potential was unaffected, primary tumor growth in vivo was significantly enhanced by c-Src overexpression. No effects on rates of tumor cell proliferation were seen in vitro. Our findings suggest that normal c-Src may be necessary but is insufficient for the induction of the metastatic phenotype.

Introduction
Current models of tumor initiation and progression suggest that colon cancer is the result of numerous genetic alterations that include both oncogenes and tumor suppressor genes. Principally implicated are the APC and MCC genes localized to chromosome 5, RAS on chromosome 12, p53 on chromosome 17, and DCC on chromosome 18 (1). This model delineates a multistep process that permits the transgression of normal colon mucosa to invasive cancer; however, little is known regarding the molecular events that promote development of the metastatic phenotype. Recently, using comparative genomic hybridization, we have identified numerous chromosomal alterations present in tumors with metastatic potential (2). Whereas many of these alterations are inclusive of genes known to be involved in colon cancer tumorigenesis, new loci have been identified that may harbor oncogenes and tumor suppressor genes not yet identified. One common finding was a gain of chromosome 20q, which contains the locus for SRC. This genetic gain may provide the molecular mechanism for the modest increases in c-Src overexpression associated with colon cancer (3). c-Src has been strongly, albeit indirectly, linked to the process of colon cancer tumorigenesis and progression (4–6). It has been shown to be overexpressed and incrementally activated in precancerous adenomatous polyps, primary cancers, and metastatic lesions (3, 9). Whereas the initial activation of c-Src seems to be important for tumorigenesis, additional increases in activation have been observed in metastases (10).

There are numerous additional reasons to believe that c-Src may have a pivotal role in governing the process of metastasis. Analysis of its many reported substrates results in a list of factors that have been directly linked to metastatic behavior (11). For example, Src substrates include focal adhesion kinase, β1 integrin, p130Cas, β-catenin, and annexin II, all of which have been linked to the process of metastasis. These and other substrates have been implicated in cytoskeletal organization, cell-substrate adhesion, and cell-cell homotypic adhesion and communication. c-Src has also been linked to enhanced expression of the metallocproteinase family (12), enzymes that are critical to the invasive process that is required for entry into the vascular and lymphatic systems and precedes metastatic spread. Moreover, it has been suggested that oncogenes such as mutant K-ras and v-src might be capable of inducing tumor angiogenesis (13, 14). Interestingly, recent studies have demonstrated that inhibition of c-Src expression reduces the expression of VEGF, an angiogenesis factor thought to be important for both tumor progression and metastasis (15–17).

Despite reports of a potential role for c-Src in colon cancer tumor progression, numerous investigators have reported that overexpression of normal c-Src has little, if any, trans-

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4The abbreviations listed are: VEGF, vascular endothelial growth factor; Ab, antibody; mAb, monoclonal Ab.
forming potential on fibroblasts (18–22), and the effect of c-Src overexpression on colon cancer metastatic potential has not yet been directly tested. These observations are in contradistinction to reports that v-Src (23, 24) and other forms of mutationally activated c-Src (23, 25) are not only capable of transforming fibroblasts but also may increase the metastatic potential (26, 27) of cancer cells. Based on our recent observation (28) that c-Src is overexpressed and highly activated in human colon cancer cell lines with high metastatic potential and that this activation may be regulated by numerous growth factor tyrosine kinase receptors such as the epidermal growth factor receptor, c-Neu, and the hepatocyte growth factor receptor, we hypothesized that c-Src overexpression in the milieu of a cancer cell might increase metastatic potential. To test this hypothesis, we selected two different human colon cancer cell lines, KM12 C (2, 29, 30) and SW 480 (31–33), known to be poorly metastatic to the liver in the nude mouse intrasplenic injection assay. Both cell lines express c-Src, but in low levels when compared to those of highly metastatic tumor cell line variants (KM12 SM, KM12 L4A, and SW 620). Normal human c-Src was transfected into both poorly metastatic cell lines, and stably expressing clones were selected for further analysis. Clones expressing approximately 4–10-fold more c-Src than controls were used in subsequent in vitro and in vivo studies designed to determine the effect of normal c-Src overexpression on tumor growth and metastatic potential.

Results

c-Src Expression and Activation in Stably Transfected Human Colon Cancer Cells with Low Metastatic Potential. To study the potential effect of c-Src on human colon cancer metastasis, we selected a well-studied metastasis model (34) that uses the intrasplenic route of injection to test the potential of tumor cells to metastasize to the liver in the nude mouse. Two different cell lines (KM12 C and SW 480) that are known to be poorly metastatic in the nude mouse model were selected for stable transfection studies. Both were stably transfected with an expression vector containing the normal human c-Src cDNA coding sequence. Two clones, C* (Fig. 1A) and 480.7* (Fig. 1B), which demonstrated significantly greater levels of c-Src expression than controls, were selected for further study. Northern analyses confirmed significant expression of the 1.6-kb c-Src messenger RNA in stable transfectants versus controls that demonstrated no detectable 1.6-kb message, although a larger (4 kb) endog-

Fig. 1. Northern analysis of c-Src mRNA expression in c-Src transfec-
tants (C* and 480*) and controls (C and 480). c-Src message was easily detected in cell lines (C* and 480*) selected for stable c-Src overexpression but was not detected in controls. A, c-Src expression in a selected overexpressing clone was compared with four different control cell lines producing low levels of endogenous c-Src. B, three different transfec-
tant clones overexpressing c-Src were compared with a vector only-transfected control; 480.7* was subsequently used for all experiments. Blots were probed with glyceraldehyde-3-phosphate dehydrogenase to control for loading.

Fig. 2. Representative analysis of c-Src levels and kinase activity in tumor cell lines stably overexpressing c-Src grown in vitro and in vivo. a, panel A, c-Src kinase activity seems linked to protein levels and is significantly increased in overexpressing cell lines (C* and 480*) versus controls (C and 480). Panel B, c-Src protein levels are significantly greater in c-Src transfec-
tants (C* and 480*) than in controls (C and 480). Panels C and D, c-Src kinase activity seems to mirror c-Src protein levels, but neither is affected by c-Src protein overexpression. Both c-Src kinase activity and protein levels are increased in the SW 480 cell lines when compared to the KM12 C cell lines. Panel E, cellular lysates from tumors grown in nude mice (2 months) show preservation of large differences (10-fold) in c-Src expression between 480* controls (C*) and controls (C). N.D., no data. b, both autokinase and enolase phosphoryl-
ing activity are increased in cells overexpressing normal human c-Src. Enolase phosphorylating activity for cell lines overexpressing c-Src is 11-fold greater than controls for C* and 4-fold greater than controls for 480*.
enous Src message is faintly visible in all cell lines. Controls included additional KM12 cell lines that were not transfected for reference and SW 480 cell lines with lower levels of Src expression. To analyze the levels of c-Src protein expression, Western blots (Fig. 2a, panel B) were performed using both c-Src overexpressing cell lines and their appropriate controls. Laser densitometry demonstrated a 10-fold and 4-fold increase in c-Src protein expression by C' and 480', respectively, when compared with controls. Src kinase assays confirmed increased protein kinase activity in c-Src overexpressing transfectants versus controls (Fig. 2a, panel A). Again, just as protein levels were increased in c-Src transfectants over controls, so were levels of c-Src kinase activity as measured by autophosphorylation and by phosphorylation of the exogenous substrate, enolase (Fig. 2b). Fold increases in enolase phosphorylating activity (11- and 4-fold) in fact closely approximated fold increases in Src protein expression (10- and 4-fold).

As an additional control, c-Yes kinase activity (Fig. 2a, panel C) and protein levels (Fig. 2a, panel D) were analyzed. c-Yes expression was easily detected in the SW 480 cell lines but was nearly undetectable in the KM12 C cell lines. c-Src overexpression, however, had no effect on c-Yes kinase activity or protein expression.

Fig. 3. H&E stains of tumors overexpressing c-Src identify increases in tumor necrosis. A, large portions of tumors derived from cells overexpressing c-Src (C') were necrotic (×200). B, significantly smaller portions of tumor were necrotic in tumors derived from control (C) cell lines (×200).
Src expression levels were maintained for at least 8 months in tissue culture while under the selection pressure of G418. To test the stability of expression in vivo, tumors derived from s.c. injection were analyzed for Src expression by Western blot and routine histochemistry as well as by immunohistochemistry after 2 months of in vivo growth. Western analysis of cell lysates derived from tumors found that high levels of c-Src overexpression were preserved in tumors and were significantly higher than control tumors that produced little c-Src (Fig. 2a, panel E).

Routine histochemistry identified two significant differences between controls and tumors overexpressing c-Src: (a) H&E stains showed significantly more necrosis in the c-Src-overexpressing transfectants (Fig. 3A) than in controls (Fig. 3B); and (b) special stains (mucicarmine) for mucin demonstrated an approximately 40–50% reduction of mucin production by tumors overexpressing c-Src (data not shown).

Immunohistochemical analysis with anti-Src mAbs identified significant membranous staining of cells overexpressing c-Src in tumors produced by s.c. inoculation of cells overexpressing c-Src (Fig. 4A) versus controls (Fig. 4B). This staining pattern was reproduced with Abs directed against phosphotyrosine (Fig. 5, A and B), suggesting that the en-
enhancement of staining was secondary to increases in Src protein expression.

**c-Src Overexpression Significantly Enhances in Vivo s.c. and Intrasplenic Tumor Growth but not Liver Metastatic Potential.** Using a nude mouse model to test for liver metastatic potential, tumor cells were injected intrasplenically, and, after a period of incubation, primary splenic tumors and liver metastases derived from the splenic tumors were enumerated and evaluated. Tumor cells overexpressing c-Src were equally tumorigenic when compared with controls injected intrasplenically in the nude mouse; nearly all mice developed tumors in their spleens that were left in situ after tumor cell inoculation. We found that tumors derived from cells overexpressing c-Src seemed to grow larger than those of controls, as reflected by a significant difference in tumor weights (Table 1) measured at necropsy. However, despite the larger sizes noted for the c-Src-overexpressing splenic tumors when compared with those of controls, no increase in liver metastatic potential was observed.

Similar to results of tumors cells injected intrasplenically, tumor cells injected s.c. showed significant differences in in vivo growth rates, but only after approximately 30 days of

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**Fig. 5.** Immunohistochemical analysis of phosphotyrosine tissue levels. A, elevated levels of phosphotyrosine are seen in tumors derived from c-Src-overexpressing cells (C) (×200). B, tumors derived from control cells (C) show no detectable phosphotyrosine (×200).
Table 1  Measurement of tumor weights (g) as an estimate of tumor burden in KM12 C c-Src transfectants and controls

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<td>Mean + SE</td>
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* P < 0.05, one-sided unpaired t test.

growth (Fig. 6, A and B). Measurements before this time showed no real differences between controls and c-Src transfectants.

Effect of c-Src Overexpression on in Vitro Tumor Growth. To determine if the effect of c-Src expression seen on tumor growth in vivo was also reflected in cell proliferation in vitro, tumor cells were seeded and pulsed with [3H]thymidine at different time points during log growth. Whereas no real differences in cell proliferation rates were noted between cells overexpressing c-Src and their respective controls, the KM12 C cell lines grew faster than the SW 480 cell lines (Fig. 7), an observation that was also seen in vivo when tumor growth rates were studied (Fig. 6, A and B). The lack of any in vitro growth advantage conferred by c-Src transfection was also confirmed by cell counts when tumor cells were enumerated (data not shown).

Discussion

We hypothesized that c-Src overexpression in the milieu of a poorly metastatic cancer cell would enhance metastatic potential. To test this hypothesis, we chose to overexpress normal human c-Src in a cell line with low metastatic potential rather than suppress it in a cell line with high metastatic potential, because the inhibition of c-Src would lead to decreased tumor growth and tumorigenicity, (16) and, for this reason, metastatic potential would be difficult to evaluate with this experimental design. Our hypothesis was based on the observations that c-Src is overexpressed and highly activated in metastatic tumors (28) and that cancer cells transfected with v-Src (26, 27) are more metastatic than controls. Because v-Src and other mutant forms of c-Src have not yet been detected in human colon cancer (35), we decided to overexpress normal human c-Src in human colon cancer cells.

Our data showed that there was no difference in metastatic potential when cells overexpressing normal human c-Src were compared directly with controls expressing 4–10-fold less c-Src. Both c-Src-overexpressing cell lines and their controls remained poorly metastatic in liver metastasis assays. Furthermore, both controls and c-Src transfectants were equally tumorigenic, as judged by the formation of splenic tumors; however, the c-Src transfectants produced significantly larger splenic tumors than did controls. The observation that tumors grew faster in the spleens of mice was also confirmed by the s.c. inoculation of tumor cells. These positive effects on tumor growth are entirely consistent with reports that suppressing c-Src expression by antisense methodology results in the opposite effect, a decrease in tumor growth (16).

The lack of an observed effect of c-Src overexpression on metastatic potential could not be explained by the absence of tumorigenicity or the absence of prolonged c-Src overexpression in vivo as documented by anti-Src immunohistochemistry performed on tumors 2 months after inoculation. Estimates of c-Src kinase activation suggest that in vitro kinase activity (as measured by enolase phosphorylation) in our transfectants is increased 4–11-fold over controls. This increase in in vitro kinase activity, which mirrored the 4–10-fold increase in protein levels, reflects an increase in Src protein levels without a significant increase in specific kinase activity (kinase activity/mg protein). This is the predicted result as long as Src protein levels do not exceed the capacity of c-Src kinase, the enzyme believed to be responsible for inactivating c-Src through Carboxyl terminus phosphorylation (36), and as long as Src is not being activated by some other mechanism (i.e., through receptor tyrosine kinase interactions; Ref. 28).

Our findings could explain why overexpression of normal c-Src, unlike v-Src or mutationally activated c-Src that display higher levels of specific kinase activity (37, 38), might affect tumor growth but not metastasis. This explanation is consistent with the observation (4) that the elevation of c-Src kinase activity in advanced human colon cancers is not solely due to increases in c-Src protein levels but rather involves other mechanisms of kinase activation. For example, induction of metastasis might depend on the interaction of c-Src with additional activated oncogenes (28, 39) shown to be necessary for transformation, which may not be present in the poorly metastatic cancer cells we chose to study.

The effect of c-Src on tumor growth was also assessed by in vitro analysis of [3H]thymidine uptake and cell counts in culture. Although the KM12 cell lines grew faster than the SW 480 cell lines in vitro, there was no difference in growth attributable to c-Src overexpression within the time frames analyzed. The difference between the KM12 and SW 480 cell lines seen in vitro was, however, reflected by the growth of tumors inoculated s.c. The positive effect of c-Src overexpression on in vivo tumor growth without an effect on in vitro proliferation rates suggests that c-Src may be inducing a local growth factor in vivo, affecting tumor growth. Potential candidates for further study might include VEGF and other factors potentially influenced by c-Src overexpression.

Collectively, these data suggest that elevated normal c-Src expression may be necessary but insufficient for development of the metastatic process. If metastatic potential is directly related to c-Src, it is likely that c-Src must not only be overexpressed but also highly activated, as is the case when v-Src transfection alone enhances metastatic potential. Studies using v-Src (11, 37) have demon-
strated that this protein has a significantly larger number of downstream targets than wild-type c-Src. Whereas we were able to achieve significant and stable increases in c-Src levels in colon cancer cells, the specific kinase activity (activity/mg protein) of the transfected c-Src was not significantly affected. Because c-Src did have a clear, positive effect on tumor growth in vivo, however, a role for this protein in tumor initiation and maintenance is supported. These findings suggest that additional events are required for colon cancer cells to become highly metastatic. Additional studies of the mechanisms that underlie the activation of c-Src and its substrates in metastatic tumors are indicated.

Materials and Methods

Cell Culture. Two sets of previously tested human colon cancer cell line metastatic variants were selected for study. The KM12 cell line was kindly provided by I. Fidler (M. D. Anderson Cancer Center, Houston, TX). KM12 C, a poorly metastatic cell line, was derived from a Dukes’ B human colon cancer cell line, which produces few, if any, liver metastatic foci on intrasplenic inoculation in a well-described nude mouse assay for metastatic potential, whereas two cell line derivatives of it, KM12 SM and KM12 L4A, produce liver metastases at a high level (34). We and others have previously characterized the metastatic behavior of these cell lines (2, 29, 30, 40, 41). The SW 480 human colon cancer cell line (American Type Culture Collection, Gaithersburg, MD) has also been well studied, and its behavior in the nude mouse intrasplenic injection assay is predictable (31–33). The SW 480 cell line was derived from a primary Dukes’ C colon cancer and is poorly metastatic to the liver in the nude mouse.

All cell lines were grown as monolayers in plastic Petri dishes (Falcon, Lincoln Park, PA) with standard culture medium (RPMI-1640; Life Technologies, Inc., Grand Island, NY) supplemented with 10% fetal bovine serum (Life Technologies, Inc.). All cells were cultured at 37°C in a 5% humidified CO2 incubator. At 70–80% confluence, tumor cell monolayers were harvested within 4 min into medium with 10% fetal bovine serum using a solution of trypsin (0.05%/EDTA (0.53 mg; Life Technologies, Inc.).

Construction of c-Src Expression Vectors. Wild-type cDNA for human c-Src was released from the Bluescript KS plasmid using the HindII and Ncol restriction enzymes (Life Technologies, Inc.) and cloned into the HindIII/XbaI sites in the pcDNA 3.1 (+) expression plasmid (Invitrogen, San Diego, CA). One μg of the KS plasmid was digested with Ncol and 1 μg of the pcDNA 3.1 (+) plasmid was digested with XbaI for 1 hr at 37°C. Blunt ends were constructed by filling in overhanging 5’ ends with Klenow fragment for 30 min at 37°C. The DNA fragments were gel-purified using a Qiagen gel extraction kit (Qiagen, Chatsworth, CA). Further digestion was performed using HindIII, and the appropriate bands were purified. The c-Src insert and pcDNA expression plasmid were then ligated overnight with T4 DNA ligase, and the recombinant plasmids were amplified and purified using INViF® Escherichia coli. The structure of the resulting expression plasmid containing the c-Src fragment was confirmed by sequencing the c-DNA insert and junctional regions.

Transfection. A helium-driven gene gun (Agracetus, Inc., Middleton, WI) was used for DNA transfections using methodologies described previously (42). The c-Src expression vector was used to transfect the poorly metastatic KM12 C and SW 480 cell lines. Twenty-four h after transfection, cells were incubated in complete medium with 800 μg/ml G418 antibiotic (Life Technologies, Inc.). After a period of approximately 3 weeks, multiple colonies of G418-resistant cells appeared and were harvested individually. Colonies were then expanded and tested by Western blot for c-Src expression. For the KM12 C transfectants, the majority of colonies tested produced no detectable c-Src protein over basal levels observed in wild-type cells. One of the neomycin-resistant colonies was confirmed to produce no detectable c-Src message in Northern analysis and was used as a control cell line for the colony (Src-4) found to overexpress c-Src nearly 10-fold over the wild-type KM12 C cell line. This control cell line was also tested to confirm the persistent lack of c-Src expression before, during, and after passage through the nude mouse. Analysis of tumors derived from the control cell line growing in the nude mouse showed little c-Src expression in vivo (Fig. 2A, panel E). Multiple SW 480 transfectants produced up to 4-fold more c-Src than mock controls transfected with empty vector. One clone, SW 480.7, was se-
ected for further study. For simplicity, KM12 C controls were labeled C and c-Src transfectants were labeled C*. SW480 controls were labeled 480 and c-Src transfectants 480.

Antibodies, Immune Complex Assays, and Immunoblotting. Anti-Src Ab (mAb 327), a gift from J. Brugge, was used for kinase assays and anti-Src Ab for use in immunoblotting was purchased from Upstate Biotechnology, Inc. Sheep antinouse immunoglobulin was purchased from Amersham Life Science. Anti-Yes Abs were purchased from Santa Cruz Biotechnology, Inc.

Cells lysates were clarified by microcentrifugation for 15 min at 4°C, immunoprecipitated with anti-src Ab (mAb 327), and then washed three times with radioimmunoprecipitation assay buffer and three times in Tris buffer [40 mM Tris (pH 7.4)]. The samples were resuspended in 30 μl of kinase reaction buffer [20 mM Tris (pH 7.4), 20 mM magnesium chloride, and 10 μM of sodium orthovanadate] containing 20 μCl [γ-32P]ATP/sample and sonicated (1 μg/sample) as exogenous substrate. The samples were incubated for 15 min at room temperature, washed two times with radioimmunoprecipitation assay buffer [10 mM Tris (pH 7.4), 150 mM NaCl, 10% glycerol, 5 mM EDTA, 1% Triton-X 100, 0.5% deoxycholate, 1 mM phenylmethylsulfonyl fluoride 1 mM sodium orthovanadate, 10 μM 1 μl leupeptin, and 10 μg/ml aprotinin], resuspended in electrophoresis sample buffer, and boiled for 5 min. The sample was centrifuged briefly at 16,000 x g, and the supernatant was loaded onto a SDS-10% polyacrylamide gel and electrophoresed and autoradiographed.

Immunoblotting was performed as described previously (28). At the end of the incubation period, the filters were washed and incubated with the appropriate horseradish peroxidase-conjugated secondary Ab for 1 h at room temperature, and the filters were then washed and exposed with the enhanced chemiluminescence detection system (Amersham).

Kinase assays and blots were quantified by laser densitometry or by phosphorimager analysis.

Immunohistochemistry. Formalin-fixed paraffin-embedded tissue sections 4-mm thick were stained using anti-Src and antiphosphotyrosine Abs (each at a dilution of 1:100). After deparaffinization, microwave antigen retrieval was performed. For the immunohistochemical staining, we used the avidin-biotin peroxidase technique at room temperature (ABC Kit; Vector Laboratories, Burlingame, CA). The primary Ab was incubated on each section at a 1:100 dilution for 1 h at room temperature. Washing, the horse antirat IgG secondary biotinylated Ab (1:200) was applied onto the sections for 30 min. The avidin-biotin peroxidase reaction was performed in the presence of the chromogen 3.3'-diaminobenzidine supplemented with hydrogen peroxide. Positive controls on human colon carcinoma were performed concurrently, and the negative controls omitted the correspondent primary Ab.

Intrasplenic Metastasis Assay and s.c. Growth Assay. This assay has been described previously in detail (34). Briefly, 1 x 10⁶ tumor cells were injected in a volume of 0.2 ml of normal saline over the period of 1 min in fully anesthetized animals. Liver metastatic foci were enumerated at necropsy approximately 2 months after inoculation. Splenic tumors were also enumerated and weighed.

Northern Analyses. Northern analyses were performed as described previously (43).

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