Recombinant Human Stem Cell Factor Mediates Chemotaxis of Small-Cell Lung Cancer Cell Lines Aberrantly Expressing the c-kit Protooncogene

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ABSTRACT

Accumulating evidence suggests that c-kit and its ligand, stem cell factor (SCF), play an important role in the regulation of at least three lineages of stem cell growth and possibly in leukemogenesis, while only limited data are available that suggest possible involvement of c-kit/SCF in the development of human solid tumors such as lung cancer. We have recently reported that c-kit is aberrantly expressed almost exclusively in small-cell lung cancer (SCLC) among various types of solid tumors. The present study revealed that c-kit protein ectopically expressed in SCLC is indistinguishable from that in leukemia cell lines with megakaryocytic characteristics with respect to amount, molecular size, and autophosphorylation status in response to recombinant human SCF. Furthermore, significant chemotactic response as well as moderate in vitro cell growth was induced in SCLC cell lines by the addition of recombinant human SCF, suggesting that c-kit/SCF may play an important biological role in the development of SCLC. Our extensive search for activating mutations naturally occurring in the c-kit gene revealed an amino acid substitution in the transmembrane domain of an SCLC cell line, although the functional consequences of this variant allele are yet to be determined.

INTRODUCTION

Molecular biological studies have revealed that a series of changes in dominant and recessive oncogenes is involved in the pathogenesis of lung cancer (1). Furthermore, certain growth factors such as gastrin-releasing peptide GRP have been suggested to play an important role in the development of lung cancer as well (1). Recent molecular cloning of the SCF gene has revealed that SCF is a pluripotent growth factor for a transmembrane tyrosine kinase receptor encoded by the protooncogene c-kit (2-4). We previously reported that the c-kit mRNA is expressed in SCLC but not in non-small-cell lung cancer. In contrast, SCF is expressed in a broad spectrum of human cancers including all histological types of lung cancer (5, 6). Our recent immunohistological study using anti-c-kit antiserum revealed that c-kit is expressed in very restricted types of human solid tumors as well as in corresponding fetal and adult normal tissues (7). Of particular interest was that no c-kit protein was detected in fetal and adult normal bronchial epithelial cells, indicating that c-kit expression in SCLC is ectopic (7).

Insights into the molecular and biochemical basis for developmental defects associated with the murine white spotting (W) and steel (S) loci have been based on the identification of causative germ line mutations in the c-kit and SCF genes, respectively (8). Furthermore, remarkable advances have been achieved in research on the essential role of c-kit/SCF, especially on the hematopoiesis (2, 3, 9-12). However, studies on the possible involvement of c-kit/SCF in the oncogenesis of human tumors have been restricted to leukemia (13, 14), and very limited data are available for human solid tumors such as SCLC (5, 6, 15).

In this study, we show that c-kit aberrantly expressed in SCLC is autophosphorylated in response to rhSCF, resulting in significant chemotaxis and moderate proliferation of SCLC cells in vitro. These results suggest that ectopic expression of c-kit may confer selective advantage on this highly aggressive cancer. Interestingly, our molecular biological analysis of the c-kit gene also revealed an amino acid substitution within the transmembrane domain in an SCLC cell line.

MATERIALS AND METHODS

Cell Lines, Tumor, and Normal Samples. SCLC cell lines with the prefix ACC-LC were established in the laboratories at Aichi Cancer Center (16).3 and SK-LC-2, an SCLC cell line with no c-kit mRNA, was kindly provided by Dr. L. J. Old. CMK (17) and MEG-015 (18), leukemia cell lines with megakaryocytic characteristics, were kindly provided by Dr. T. Sato and Dr. M. Ogura, respectively. Normal and lung cancer tissues were obtained from surgical specimens. Fetal tissues at 12 weeks of gestation were obtained from an elective abortion.

Western Blot Analysis. Western blot analysis for the detection of the c-kit protein was performed according to the standard methods using rabbit anti-c-kit antiserum (K961), which was raised against synthetic peptide harboring amino acids 961 to 976 of the c-kit protein (7). Briefly, 1×10⁷ cells were lysed in 1 ml of sample buffer (20 mM Tris-HCl (pH 6.8), 2% SDS, 2 mM EDTA, 80 mM dithiothreitol, 10% sucrose, 20 μg/ml bromophenol blue) containing 1 mM of sodium orthovanadate and sheared through a 26-gauge needle. 10 μl aliquots of the lysates were subjected to SDS-PAGE and transferred to Immobilon filters (Millipore, Bedford, MA). Following blocking in phosphate-buffered saline containing 3% bovine serum albumin, the filters were incubated overnight at 4°C with rabbit anti-c-kit antiserum (K961) at 1 μg/ml. Enzymatic color development was performed using a POD immunostain set (Wako, Osaka, Japan) according to the manufacturer’s instructions, which was based on a biotinylated goat anti-rabbit secondary antibody and avidin-biotin peroxidase complex.

Western blot analysis using anti-phosphotyrosine antiserum, kindly provided by Dr. M. Hamaguchi (19), was performed to examine tyrosine phosphorylation before and after SCF stimulation. After a 2-h serum starvation, 10⁶ cells were stimulated for 15 min with 100 ng/ml of rhSCF (2). Lysates were prepared in the presence of 1 mM of sodium orthovanadate, subjected to SDS-PAGE and transferred to Immobilon filter as described above. After blocking in phosphate-buffered saline containing 3% ovalbumin, the filters were incubated overnight at 4°C with rabbit anti-c-kit antiserum (K961) at 1 μg/ml. Enzymatic color development was performed using a POD immunostain set (Wako, Osaka, Japan) according to the manufacturer’s instructions, which was based on a biotinylated goat anti-rabbit secondary antibody and avidin-biotin peroxidase complex.

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Immunoprecipitation Analysis. After a 2-h serum starvation, 5×10⁶ cells (2×10⁷ for MEG-015) were stimulated for 15 min with 100 ng/ml of rhSCF. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: GRP, gastrin-releasing peptide; SCF, stem cell factor; SCLC, small-cell lung cancer; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PCR, polymerase chain reaction; SSCP, single strand conformation polymorphism; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; rhSCF, recombinant human SCF; nt, nucleotide(s); cDNA, complementary DNA; s, sense (primer); as, antisense (primer); KDa, kilodaltons.

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rhSCF and then lysed in 1 ml of lysis buffer (50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.5), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1.5 mM MgCl₂, 1 mM ethyleneglycol bis(β-aminopropyl) ether)-N,N,N',N'-tetraacetic acid) containing 10 µg/ml leupeptin, 10 µg/ml apro- tinin, 1 mM phenylmethylsulfonyl fluoride, 200 µM sodium orthovanadate, and 10 mM sodium fluoride. After centrifugation at 10,000 × g at 4°C for 15 min, supernatants were incubated with anti-c-kit antiserum (K961) at 1 µg/ml for 1 h at 4°C, followed by incubation with protein A-Sepharose beads (Pharmacia-LKB, Uppsala, Sweden) for an additional 30 min at 4°C. After repeated washings, the immunoprecipitates were resuspended in 50 µl of SDS sample buffer (10% glycerol, 80 mM dithiothreitol, 2.0% SDS, 20 mM Tris- HCl (pH 6.8), 0.002% bromophenol blue) and then boiled for 3 min. The eluates were subjected to SDS-PAGE. Immunoblotting analysis using anti-phosphotyrosine antiserum was performed as described above.

Chemotaxis Assay. Cell aggregates of SCLC cell lines were dissociated by mild trypsinization, and single cell suspensions were resuspended at 10⁶ cells/ml in the basal medium consisting of RPMI 1640 supplemented with 10 µg/ml transferrin and 300 nM selenium. "Checkerboard" analysis was performed by the addition of cells (100 µl) into the upper chamber of a Transwell cell culture plate with 8-µm pore size filter (Costar, Cambridge, MA), followed by incubation at 37°C for 5 h in the presence of the specified amount of rhSCF in the upper and lower chambers. Cells migrating to the lower chamber were counted under a phase contrast microscope. A flowcytometric analysis using a monoclonal antibody against extracellular domain of the c-kit protein (17F11; Immunotech, S. A., Marseille, France) was conducted to confirm that the mild trypsinization to obtain single cell suspensions did not alter the levels of c-kit expression (data not shown).

Cell Proliferation Assay. The MTT rapid colorimetric assay was used to measure stimulation of in vitro cell growth of SCLC cell lines as well as leukemia cell lines by the addition of rhSCF. After washing 3 times in RPMI 1640, at least triplicate aliquots of 2 × 10⁴ cells (5 × 10⁴ for ACC-LC-51) were seeded in 96-well flat-bottom plates in the presence of various concentrations of rhSCF in 100 µl of serum-free basal medium, which consisted of RPMI 1640 plus a final concentration of 10 µg/ml transferrin and 300 nM selenium. After a 48-h incubation period, the MTT assay was performed as described previously (20). Each experiment was repeated 3 times, and all values represent the mean ± 2SE of a minimum of 9 wells.

PCR-SSCP and Sequencing Analysis. PCR using random primed cDNAs were performed with c-kit-specific primers in the presence of [32P]dCTP as described previously (21). The PCR products were digested with appropriate restriction enzymes to yield a higher sensitivity due to their smaller size. They were then electrophoretically separated both on a 6% nondenaturing polyacryl- amide gel at 5°C and on a 6% nondenaturing polyacrylamide gel containing 5% glycerol at room temperature. Restriction enzymes used in this study were Apal, Aval, Aul, Banl, Bgfl, MvaI, HindIII, Hinfl and Sry. The sense primers used were: 1s, nt -6-13; 2s, nt 740-760; 3s, nt 1567-1587; and 4s, nt 2228-2248. The antisense primers were: 5as, nt 772-792; 6as, nt 1595-1615; 7as, nt 2248-2268; 8as, nt 2299-2319; and 9as, nt 2977-2997. The nucleotide coordinates were obtained from the sequence of Yarden et al. (22) and Sekido et al. (5). All sense and antisense primers except 1s, which had an Xhol site at the 5' end, had extra from the sequence of Yarden et al. (22) and Sekido et al. (5). All sense and antisense primers except 1s, which had an Xhol site at the 5' end, had extra

RESULTS

Detection of c-kit Protein in SCLC and Leukemia Cell Lines. Expression of the c-kit protein was studied by Western blot analysis using anti-c-kit antiserum raised against its COOH terminus (Fig. 1). Four SCLC cell lines as well as two leukemia cell lines with comparable amounts of c-kit mRNA expressed abundant c-kit protein. The c-kit protein in these cell lines except for ACC-LC-51 had a molecular mass of 145 kDa. The molecular mass of c-kit protein in ACC-LC-51 was 160 kDa. This may be attributable to its higher sensitivity and the fact that the

Fig. 1. Detection of c-kit protein in SCLC and leukemia cell lines. Western blot analysis was performed using anti-c-kit antiserum (K961) raised against COOH terminus of the c-kit protein (7). ACC-LC-51 has c-kit protein with a molecular mass of 160 kDa (arrowhead). The remaining cell lines except SK-LC-2 which does not express any c-kit mRNA (5, 6) show 145-kDa c-kit protein (arrow). KD, kilodaltons.

curser (data not shown). Furthermore, PCR products of c-kit cDNA had identical sizes and normal electrophoretic mobilities in PCR-SSCP analysis, excluding a possibility of alternative splicing or subtle mutations in the glycosylation site (see below). Different molecular masses of c-kit proteins (145, 155, and 160 kDa) have also been reported in mouse brain, spleen, and testis, respectively (24).

Tyrosine Phosphorylation of the c-kit Protein Stimulated with rhSCF. To examine whether SCF can stimulate tyrosine phosphorylation in SCLC cell lines, whole cell lysates prepared from untreated or stimulated cells were subjected to Western blot analysis using an anti-phosphotyrosine antiserum (Fig. 2A). Induction of tyrosine phosphorylation by the addition of rhSCF was readily detectable in all cell lines except MEG-01S and was almost exclusive to the proteins with molecular masses similar to those of c-kit. We note that the degree of phosphorylation varied greatly and did not correlate well with the amount of c-kit in each cell line.

To verify the identity of the phosphorylated protein as c-kit, we conducted immunoprecipitation of c-kit protein followed by immuno blotting using an anti-phosphotyrosine antiserum (Fig. 2B). In ACC-LC-80 and CMK, immunoprecipitated c-kit protein was found to be highly tyrosine-phosphorylated by the addition of rhSCF, while ACC-LC-97 and ACC-LC-51 showed lower levels of autophosphorylation of c-kit. Although no stimulation of c-kit phosphorylation could be detected by Western blotting analysis, MEG-01S also showed weak tyrosine phosphorylation of c-kit protein in this assay. This may be attributable to its higher sensitivity and the fact that the lane for MEG-01S was loaded with four times the lysates of other lanes. In all lines tested in the present study, no constitutive tyrosine phosphorylation of c-kit was observed.

Identification of Chemotactic Response of SCLC Cell Lines to SCF. Tumor cell motility was measured in the presence of various gradients of rhSCF. ACC-LC-80, which showed tyrosine phosphorylation of c-kit by the addition of rhSCF as described above, exhibited a dose-dependent migration through the 8-µm pore filter. Checkerboard analysis further indicated that SCF induced a chemotactic response in a positive gradient of SCF, although a weak response of random motility was also observed (Fig. 3). Two additional SCLC cell lines were tested for chemotactic response to SCF. ACC-LC-76, an SCLC cell line with moderate c-kit phosphorylation, exhibited weak chemotactic response to SCF, while noticeable chemotaxis was not
SCF MEDIATES CHEMOTAXIS OF SCLC EXPRESSING c-kit

**Stimulation of the Proliferation of SCLC and Leukemia Cell Lines by the Addition of rhSCF.** Proliferation of SCLC and leukemia cell lines were examined by the MTT assay (Fig. 4). All three SCLC cell lines as well as CMK showed various degrees of stimulation of cell proliferation, whereas MEG-01S did not show any enhancement. ACC-LC-80 and CMK showed increased cell growth at the range of 10 to 100 ng/ml of rhSCF, and maximal proliferative response was observed at 100 ng/ml. Significant increases in cell number in response to rhSCF were also observed by the trypan blue dye exclusion method (data not shown). ACC-LC-51 and ACC-LC-97 also exhibited a weak yet reproducible response to SCF both at 10 and 100 ng/ml. Proliferative stimulation at 1000 ng/ml of these responders was similar to that at 100 ng/ml by the MTT method (data not shown). Synergistic effects on the cell growth between SCF and other growth factors including GRP (10–50 nm), GM-CSF (10–100 ng/ml), and interleukin 3 (10–100 ng/ml) were also investigated. GM-CSF showed synergistic effects with rhSCF on the proliferation of CMK, whereas neither GRP nor interleukin 3 exhibited such effects on any lines tested in the present study (data not shown).

**PCR-SSCP and Sequence Analysis of the c-kit Gene in SCLC Cell Lines and Tumor Samples.** Since activating mutations in the c-fms gene which is closely related to c-kit have been identified in human tumors, the entire c-kit coding region was examined in 15 SCLC cell lines as well as 13 SCLC primary tumor samples using the PCR-SSCP technique (Fig. 5). Distinct PCR-SSCP patterns were observed only within the fragments amplified with primers 3s and 7as. Of 28 specimens, 23 showed a PCR-SSCP pattern identical to that of case 90, while one specimen (case 53) exhibited a clearly different pattern. Three of the remaining 4 specimens such as case 51 appeared to be heterozygous for those observed in cases 90 and 53 in Mval or AluI digested fragments, suggesting the existence of polymorphism in the c-kit coding region. However, ACC-LC-97 appeared to be distinct from such variations in PCR-SSCP patterns.

To elucidate the nature of these differences in PCR-SSCP patterns, we sequenced representative specimens. As shown in Fig. 6, an A to C transversion at c-kit codon 541 within the transmembrane domain (AGT to CTG, methionine to leucine) was found in ACC-LC-97. Fortunately, genomic DNAs taken from normal lung and primary tumor samples of case 97 from which ACC-LC-97 originated were available for sequence analysis. The primary tumor of case 97 exhibited an identical base substitution to ACC-LC-97, whereas a normal lung showed both normal and distinct alleles, indicating loss of the normal allele during tumor development. These results suggest that the base substitution observed in ACC-LC-97, which results in an amino acid substitution within the transmembrane domain, may be a germ line mutation or a rare polymorphism among normal subjects.

Sequence analysis also revealed a silent nucleotide substitution at codon 546 in the cytoplasmic domain, which corresponded clearly to induced in ACC-LC-51 (data not shown). In contrast, SK-LC-2 with no detectable c-kit did not show any stimulatory effects on cell migration even at 100 ng/ml of rhSCF in the bottom chamber (data not shown). We note that chemotactic migration of a megakaryocytic leukemia cell line (CMK) was also observed by the addition of rhSCF.

![Fig. 2](image-url) Detection of tyrosine phosphorylation of c-kit protein in response to the addition of rhSCF. In A, whole cell lysates prepared from rhSCF-stimulated or untreated cells were analyzed by Western blot analysis using anti-phosphotyrosine antiserum. Enhancement of tyrosine phosphorylation is observed in all cell lines except MEG-01S, which corresponds to the molecular mass of c-kit protein. In B, tyrosine phosphorylation stimulated by rhSCF was confirmed to be c-kit by the immunoprecipitation using an anti-c-kit antiserum followed by the immunoblot analysis using anti-phosphotyrosine antiserum. Although phosphorylation in MEG-01S is detectable in this analysis, we note that 4 times the cell lysate of other lanes is loaded onto the one for MEG-01S. Arrow, 145-kDa c-kit protein; arrowhead, 160-kDa c-kit protein in ACC-LC-51.

![Fig. 3](image-url) Checkerboard analysis of SCF-induced cell motility. Checkerboard analysis was performed in the presence of various amounts of rhSCF in the upper and lower chambers. Diagonals, the number of cells in the lower chamber induced in a series of increasing uniform concentrations of SCF. The lower triangle gives values for migration induced toward a positive gradient. Each experiment was repeated 3 times in duplicate, with representative results. Values are the mean number of migrated cells to the lower chamber.
Cultures, *P < 0.01, when compared with the control and all values represent the mean ± 2SE of at least 9 cell growth of SCLC cell lines as well as leukemia contribute to the development of human solid tumors. Together with 6, two alleles identified were AAA and AAG both of which encode the polymorphism detected by PCR-SSCP analysis. As shown in Fig. 6, two alleles identified were AAA and AAG both of which encode lysine.

DISCUSSION

Accumulating evidence suggests that c-kit and its ligand (SCF) play an important role in the regulation of at least three lineages of stem cell growth and possibly in leukemogenesis (8, 13, 14), but very few studies have been conducted to investigate whether c-kit/SCF may contribute to the development of human solid tumors. Together with our previous reports (5-7), the present study extends our understanding regarding the functional consequences of the ectopic expression of the c-kit gene products in SCLC.

The expression and phosphorylation of the c-kit protein in SCLC cell lines were analyzed and found to be indistinguishable from that in CMK, a leukemia cell line with megakaryocytic characteristics. The c-kit protein in SCLC was autophosphorylated by the addition of rhSCF, resulting in the induction of in vitro chemotactic response of SCLC cells in a positive gradient of rhSCF. In this regard, it has been suggested that SCF may have a role in guiding the migrations of germ cells and melanocyte precursors (7). Direct evidence has been recently provided indicating that SCF can stimulate chemotaxis of the porcine aortic endothelial cells transfected with the c-kit expression construct (25). Together with the production of SCF by various cell types including stromal cells at the primary tumor sites as well as those of the bone marrow, the present study makes an important implication in that ectopic expression of c-kit in SCLC would also enhance the migration of SCLC cells in vivo, resulting in the acquisition of a higher invasive and/or metastatic phenotype. Notably, SCLC is known to metastasize to the bone marrow much more frequently than non-small-cell lung cancer, while c-kit is preferentially expressed in SCLC among various histological types of lung cancer.

We also observed weak stimulation of in vitro proliferation of SCLC cell lines in response to the addition of rhSCF. Since SCF has been shown to interact synergistically with a number of cytokines to augment growth of various types of hematopoietic cells as observed in CMK in this study (2, 3, 9-12), there remains a possibility of discovering an as yet unidentified growth factor(s) which can synergistically stimulate SCLC cell growth. It is also of considerable interest to examine whether a membrane-bound form rather than a soluble form of SCF like the rhSCF has enhanced effects on the SCLC cell growth as reported in certain hematopoietic systems (26).

Our extensive search for c-kit mutations in SCLC revealed an amino acid substitution [CTG (leucine) instead of ATG (methionine)] in the transmembrane domain of c-kit in an SCLC cell line, ACC-LC-97. Similar single amino acid substitutions in the transmembrane domain have been reported in the neu gene as a mechanism for oncogenic activation of a tyrosine kinase receptor (27). Although the amino acid substitution identified in the c-kit transmembrane domain is reminiscent of that in the neu gene, it does not seem to be an activating mutation because the basal level of the phosphorylation in ACC-LC-97 was undetectable by the assay condition used in the current study. Furthermore, the amino acid substitution was also found in the genomic DNA extracted from the normal lung of case 97 the tumor of which yielded the ACC-LC-97 cell line, suggesting that it is either a germ line mutation or polymorphism.

Careful examination of the patient and family members disclosed no sign of piebaldism, a human homologue of W mutant in mice, for which germ line c-kit mutations were recently shown to be responsible (28, 29). Since c-kit in ACC-LC-97 responded weakly but reproducibly to rhSCF with respect to autophosphorylation and proliferative stimulation, this amino acid substitution does not seem to represent a germ line mutation which impairs c-kit function. In addition, our preliminary experiments using genomic DNAs extracted from 75 individuals yielded 6 instances carrying this substitution. Altogether, the amino acid substitution may represent a variation among normal subjects, although further studies using the variant cDNA clone as described in this study with a c-kit expression assay may be required to accurately determine whether this variant allele has functional consequences. In this regard, it is notable that a recently identified c-kit isoform containing an in-frame insertion, Gly-Asn-Asn-Lys, within the extracellular domain exhibited distinct signaling properties when compared to c-kit protein without this insertion (30).

Unpublished observation.
The two independent nucleotide substitutions identified here in the c-kit gene either with or without amino acid substitution will also be useful for the detection of allelic loss in the genomic region surrounding the c-kit locus at 4q11-21 (22). This is because our recent cytogenetic analysis revealed chromosomal abnormalities involving 4q in 37.5% (6 of 16) of our SCLC cell lines as is ACC-LC-97 in this study.

In conclusion, we have shown that c-kit aberrantly expressed in SCLC is autophosphorylated in response to rhSCF, resulting in significant chemotaxis and moderate in vitro proliferation of SCLC cells. These results suggest the possibility that ectopic c-kit expression may confer selective advantage on this fatal cancer. Studies aiming to address whether inhibition of such stimulatory effects via c-kit/SCF can block chemotaxis and/or proliferation of SCLC will be of great interest and may provide new therapeutic approaches to control this aggressive cancer.

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