c-Kit–Dependent Growth of Uveal Melanoma Cells: A Potential Therapeutic Target?

Charlotte All-Ericsson,1,2 Leonard Girnita,2,3 Anja Müller-Brunotte,1 Bertha Brodin,3 Stefan Seregard,1 Arne Östman,4 and Olle Larsson3

PURPOSE. This study was conducted to investigate the expression and functional impact of the proto-oncogene c-kit in uveal melanoma.

METHODS. Based on immunohistochemical (IHC) study of paraffin-embedded specimens from 134 uveal melanomas and Western blot analysis on eight fresh-frozen samples the expression of c-kit in uveal melanoma was studied. Furthermore, the phosphorylation of c-kit and the impact of the tyrosine kinase inhibitor STI571 was examined in the three uveal melanoma cell lines OCM-1, OCM-3, and 92-1.

RESULTS. Eighty-four of 134 paraffin-embedded samples and six of eight fresh-frozen samples expressed c-kit. C-kit was strongly expressed and tyrosine phosphorylated in cultured uveal melanoma cells compared with cutaneous melanoma cells. Moreover, in contrast to cutaneous melanoma cell lines c-kit maintained a high phosphorylation level in serum-depleted uveal melanoma cells. No activation-related mutations in exon 11 of the KIT gene were found. On the contrary, expression of the stem cell growth factor (c-kit ligand) was detected in all three uveal melanoma cell lines, suggesting the presence of autocrine (paracrine) stimulation pathways. Treatment of uveal melanoma cell lines with STI571, which blocks c-kit autophosphorylation, resulted in cell death. The IC50 of the inhibitory effects on c-kit phosphorylation and cell proliferation was of equal size and less than 2.5 μM.

CONCLUSIONS. The results confirm that c-kit is vastly expressed in uveal melanoma, suggest that the c-kit molecular pathway may be important in uveal melanoma growth, and point to its use as a target for therapy with STI571. (Invest Ophthalmol Vis Sci. 2004;45:2075-2082) DOI:10.1167/iovs.03-1196

Uveal melanoma is the most common primary intraocular tumor in adults with an annual incidence of 8.4 to 11.7 cases per million in whites.1 Although uveal and cutaneous melanoma are of similar embryologic origin, they differ in biological behavior. Uveal melanomas metastasize hematogenously and preferentially to the liver, whereas cutaneous melanomas spread to regional lymph nodes. The average survival time in uveal melanoma is only 2 to 5 months after detection of liver metastases. Chemotherapy and/or immunotherapy has been used in the treatment of metastatic melanoma, but the results have generally been disappointing, with a median survival time after treatment of 5 to 8 months.2 In contrast to improved survival rates in a variety of cancers where there is a continuing evolution toward early detection and management, the survival rate with uveal melanoma has changed little during the past few decades.3-5 Thus, a deeper understanding of the molecular mechanisms governing uveal melanoma development is necessary to design better and more specific drugs able to block and eventually cure metastatic uveal melanomas.

Very little is known about the role of the c-kit proto-oncogene in uveal melanoma. It encodes a receptor tyrosine kinase (kit) whose ligand is a stem cell factor (SCF, also known as steel factor, kit ligand, and mast cell growth factor).5,6 C-kit is expressed by and is critical in the development of cutaneous melanocytes, mast cells, hematopoietic stem cells, germ cells, and the interstitial cells of Cajal.7 Studies have demonstrated that c-kit is expressed in normal melanocytes, but contradictory results have been published concerning the expression of c-kit in metastasizing cutaneous melanoma.8-10 Data from studies on gastrointestinal stromal tumors (GIST), in a substantial proportion of which there is a mutation in exon 11 leading to ligand-independent c-kit activation, have shown that treatment with STI571, an inhibitor of platelet-derived growth factor receptor (PDGFR), Bcr-Abl, and c-kit tyrosine phosphorylation,11 causes tumor regression.12,13

Recently, it has been shown in our laboratory that c-kit is commonly expressed in uveal melanoma.14 Independently, Mouriaux et al.15 have recently demonstrated that c-kit is expressed in as many as 75% of uveal melanomas, based on an investigation of 57 cases.15 The purpose of the present study was to evaluate the expression and possible functional impact of the proto-oncogene c-kit in uveal melanoma.

METHODS

Antibodies and Chemicals

A mouse monoclonal antibody directed to the human c-kit (CD 117) was purchased from Dako (Carpinteria, CA). The rabbit antibody specific for phosphorylated c-kit (Tyr 705) was purchased from Zymed Laboratories (South San Francisco, CA). Anti-SCF was from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). STI571 was obtained from Novartis (Basel, Switzerland). All other chemicals, unless stated otherwise, were from Sigma-Aldrich (St. Louis, MO).

Tumor Material

Paraffin-embedded blocks from 134 cases (64 females and 70 males) derived from surgically removed primary uveal melanomas were used for immunohistochemistry (IHC). The ages of the patients varied between 24 and 92 years with a mean age of 64 years at diagnosis. Sixty-four patients died of tumor-related causes, 49 patients died of other causes, and 21 were alive at the time of follow-up. The follow-up time was 13 years or more.
Fresh-frozen uveal melanoma tissue samples from eight patients and six samples from cutaneous melanoma tumors were used for Western blot analysis. All parts of the study were conducted in compliance with the Declaration of Helsinki.

Cell Culture
The uveal melanoma cell lines OCM-1, OCM-3, and 92–1 (kindly provided by Martine Jager, Leiden University Medical Center, Leiden, The Netherlands), and the cutaneous melanoma cell lines DBF and BE16 (kindly given to us by Rolf Kiessling, CCK, Karolinska Hospital, Stockholm). All three cell lines were initially derived from primary uveal melanomas. The OCM-1 cells have a spindle cell morphology and were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS). The OCM-3 has epithelioid cell morphology, whereas 92-1 cells have a mixed phenotype. OCM-3 and 92-1 cells were cultured in RPMI 1640 supplemented with 10% FBS and 3 mM L-glutamine.

Cells were grown in monolayers in tissue culture flasks, maintained in a 95% air/5% CO2 atmosphere at 37°C. For experimental purposes the cells were cultured in 35- or 60-mm plastic Petri dishes at a density of 200,000 cells/35-mm dish or 500,000 cells/60-mm dish. The experiments were initiated when cells had reached confluence. For the proliferation assay cells were seeded at a density of 5000 cells/well, and the experiments were started 24 hours after.

Immunoprecipitation and Western Blot Analysis
For immunoprecipitation, cell lysates were obtained using PBSTDS buffer with 4% CHAPS, 1% Triton X-100, 5 g sodium deoxycholate, and 1 g sodium dodecyl sulfate in 100 mL of deionized water) containing the aforementioned protease inhibitors. Samples corresponding to 400 µg total proteins were immunoprecipitated with 15 µL protein G Sepharose (Amersham, Arlington Heights, IL) and 1 µg c-kit antibody. After overnight incubation at 4°C on a rocker platform, the immunoprecipitates were collected by centrifugation in a microcentrifuge at 2500 rpm for 2 minutes. The supernatant was discarded, whereupon the pellet was washed four times with 1 mL PBSTDS. The material was then dissolved in sample buffer for SDS-PAGE. Protein samples (from total cell lysates) were dissolved in a sample buffer containing 0.0625 M Tris-HCl (pH 6.8), 20% glycerol, 2% sodium dodecyl sulfate (SDS), bromophenol blue, and dithiothreitol. Sample amounts of 100 µg total proteins/lane were analyzed by SDS-PAGE PAGE with a 4% stacking gel and a 10% separation gel, essentially according to the protocol of Laemmli. Molecular weight markers (Bio-Rad) were run simultaneously. For Western blot analysis, the proteins were transferred overnight to nitrocellulose membranes (Hybond; Amersham) and then blocked for 1 hour at room temperature in a solution of 5% (wt/vol) skimmed milk powder and 0.02% (wt/vol) Tween 20 or 2% (wt/vol) skimmed milk powder and 2% bovine serum albumin (for detection of phosphorylated proteins) in PBS (pH 7.5). Incubation with the primary antibody, directed to the human c-kit or the antibody specific for phosphorylated c-kit, was performed for 1 hour at room temperature. This was followed by three washes with PBS-Tween and incubation with a biotinylated secondary antibody (Amersham) for 1 hour. After a 45-minute incubation with streptavidin-labeled horseradish peroxidase (Amersham), detection was performed (Hyperfilm-ECL; Amersham). Protein content of cell lysates was determined by a dye-binding assay with a reagent purchased from Bio-Rad (Hercules, CA). Known concentrations of bovine serum albumin were used as a standard.

Cell Proliferation Assay
A cell proliferation kit was purchased from Roche Diagnostic GmbH (Mannheim, Germany). The test is based on a colorimetric change of the yellow tetrazolium salt XTT into orange formazan dye by the respiratory chain of viable cells. Cells seeded at a density of 5000 well in 100 µL medium in a 96-well plate for 24 hours were treated with different drugs in specified concentrations. After 48 hours, cells were incubated according to the manufacturer’s protocol, with an XTT labeling mixture. After 4 hours, the formazan dye was quantified with a scanning multwell spectrophotometer with 495- and 690-nm filters as the reference. The absorbance correlates directly with the number of viable cells. To draw the standard absorbance curve, we used untreated cells seeded at concentrations from 1,000 to 10,000 cells/well with an increasing rate of 1,000 cells/well. All standards and experiments were performed in triplicate.

STI571 Treatment
STI571 was provided by Novartis Inc. Stock solutions of the drug were prepared at 10 mM in distilled sterile water and stored in working aliquots at −80°C. To study the effects of STI571 on the in vitro cell growth, cells were seeded with normal growth medium. After 24 hours, cells were changed to new fresh medium with and without increasing doses of the drug (0.1–10 µM). Cell growth was evaluated after 48 hours. The drug concentrations resulting in 50% inhibition of growth, compared with untreated control cells, were determined (IC50).

Immunohistochemistry
Immunostaining was performed using the standard avidin-biotin complex (ABC) technique (Elite Standard Kit, cat. PK-6100; Vector, Burlingame, CA). Deparaffinized, rehydrated sections were pretreated with microwaves in 0.1 M citrate buffer at pH 6.0. Before immunostaining, the endogenous peroxidase activity was blocked by hydrogen peroxide dissolved in methanol (3% hydrogen peroxide in methanol, 1:5 volume) for 30 minutes. Sections were then rinsed in and incubated with blocking serum (1% bovine serum albumin) for 20 minutes followed by incubation with the primary antibody overnight at 4°C. A biotinylated anti-mouse IgG was used as a secondary antibody and followed by the ABC complex. The peroxide reaction was developed using 3,3-diaminobenzidine tetrahydrochloride (DAB; 0.6 mg/mL with 0.03% hydrogen peroxide) for 6 minutes. Counterstaining was performed with Mayer’s hematoxylin. Tris-buffered saline (pH 7.6) was used for rinsing between the different steps. All the slides were coded and analyzed in a blinded fashion.

After tissue processing, all cells displaying distinct immunoreactivity were considered positive, irrespective of staining intensity. We assigned the results of c-kit staining as negative when no staining was present, low when less than 10%, medium when 10% to 50%, and high when more than 50% of melanoma cells were positive. These specimens were also assessed by an independent observer using the same grading system.

PCR Amplification and Sequencing Analysis
DNA was isolated from frozen uveal melanoma specimens by standard methods. We used a nested polymerase chain reaction (PCR) method to amplify genomic fragments of exon 11 using primers annealing the intronic regions that flank exon 11 of the c-kit gene. Shortly, 500 ng of genomic DNA was amplified using 5’ (CTCTGAAGAGCAATCGATGGT) and 3’ (CTTGAGGAGCCAGGACCAATC) primers in a 35-cycle reaction of 94°C for 30 seconds; 52°C for 45 seconds, and 68°C for 2 minutes. For the nested PCR, the primers 5’ (GAGTCCATGATCTCCATCGG) and 3’ (CAGCTGATCAATTCAAGCTGCC) were used in a 34-cycle PCR of 94°C for 30 seconds, 56°C for 45 seconds, and 70°C for 45 seconds. The PCR products were electrophoresed in a 1% agarose gel containing ethidium bromide, and visualized in a UV camera before sequencing in a DNA sequencer (3100; Applied Biosystems [ABI], Foster City CA) using dye termination chemistry (Big Dye Terminator Kit; ABI).

Statistical Analysis
Survival data without loss to follow-up were obtained, in accordance with the tenets of the Declaration of Helsinki, for all patients with
uveal melanoma from the Swedish National Causes of Death Registry. Time from the date of surgery to death or end of 1996 was considered censored if the patient was alive at the end of 1996 or had died of any cause that was not melanoma related. The log rank test was used to assess survival differences. Calculations were computer based (Statistica, 1999; StatSoft, Tulsa, OK; and MedCalc; MedCalc Software, Mariakerke, Belgium).

RESULTS

We first investigated the expression of c-kit in uveal melanomas using IHC on 134 paraffin-embedded surgical specimens of primary uveal melanoma. A distinct immunoreactivity confined to the plasma membrane-cytoplasm of tumor cells (Fig. 1A) was found in 65% of the cases (84/134). In 48 (36%) of 134 cases, most of the tumor cells expressed c-kit (Fig. 1B). We also investigated all available biopsy specimens from liver metastases of uveal melanoma by IHC. Eleven of 18 were positive for c-kit (data not shown). Fifty (37%) of the primary 134 cases showed no detectable c-kit immunoreactivity. However, this does not preclude the possibility that these cases express c-kit. Probably, they express the receptor but at a level below the detection limit for the IHC assay. This was also supported by Western blot analysis as described in a later section.

FIGURE 1. Expression of c-kit in uveal melanoma. (A) Immunostaining of a paraffin section of uveal melanoma with a c-kit antibody (CD117). (B) Archival paraffin-embedded tumor sections from 134 cases of human uveal melanoma were assayed for CD117. Immunostaining was performed by applying the standard ABC technique. Samples were grouped according to level of staining as percentages of positive cells as shown on the x-axis.
We studied the expression of c-kit on eight fresh-frozen samples from primary uveal melanoma and four fresh-frozen samples from lymph node metastases of cutaneous melanoma by Western blot analysis with an antibody specific for c-kit. As shown in Figure 2A, seven (88%) of the uveal melanoma specimens were positive, whereas c-kit was not or was very weakly expressed in the cutaneous melanoma samples. These data also support the possibility that the c-kit is more widely expressed in uveal melanoma than 63%. Western blot analysis is a more sensitive assay than IHC.

Using the clinical follow-up data of these 134 cases of uveal melanoma, we analyzed whether the expression level of c-kit correlates with survival data. Figure 2B shows a Kaplan-Meier diagram, in which we compared tumors with low and high expression of c-kit. There was no statistical significance between the two groups. We also compared insulin-like growth factor (IGF)-1R-negative tumors with positive ones, but this did not show a statistically significant difference, either (data not shown).

The expression and tyrosine phosphorylation of c-kit was assessed in the three uveal melanoma cell lines OCM-1, OCM-3, and 92-1 as well as in the two cutaneous melanoma cell lines DFB and BE. Two different types of analyses were performed, one being a Western blot analysis using phosphotyrosine antibodies on c-kit purified by immunoprecipitation and the alternative method being direct Western blot using an antibody specific for phosphorylated c-kit.
Both analyses showed phosphorylated c-kit in all uveal melanoma cell lines but hardly any signals for the cutaneous melanoma cells (Fig. 3A, top). Because both types of assays for c-kit phosphorylation worked well, Western blot analysis with antibodies to phosphorylated c-kit was used in the remaining experiments.

The uveal melanoma cells and cutaneous melanoma cells were now serum depleted (after repeated rinsing) for 24 hours and then stimulated with SCF. Whereas the cutaneous melanoma cells did not exhibit any c-kit phosphorylation at all after serum depletion, a substantial c-kit phosphorylation remained in the uveal melanoma cells (Fig. 3A, bottom). These experiments were repeated several times with similar results. In both melanoma cell variants, SCF stimulated phosphorylation of c-kit (Fig. 3A), but this response was comparatively limited (because of the already high activity under serum-free conditions) in uveal melanoma cells.

To search for a possible mechanism behind the c-kit activation of uveal melanoma, we have to consider the presence of point mutations in the KIT gene. A recent study by Pache et al.24 investigated the presence of known KIT point mutations (i.e., in exons 2, 8, 9, 11, 13, and 17) in 10 cases of uveal melanoma. However, all cases were found to be negative. Based on these results and the fact that point mutations in exon 11 represent the clearly most common (80%) mutations of the KIT gene,25 we decided to analyze only exon 11. Exon 11 is located in the cytoplasmic portion of the juxtamembrane domain, and has been shown to harbor a specific mutation implicated in ligand-independent activation of c-kit in gastrointestinal stromal tumors (GIST).20 To evaluate whether this mutation also could be involved in uveal melanoma, we analyzed exon 11 on 15 fresh frozen uveal melanoma samples as well as samples from the uveal melanoma cell lines. We used PCR to amplify genomic fragments of exon 11 of the KIT gene followed by direct sequencing. However, none of the 15 tumors, or the cell lines, exhibited point mutations in exon 11 of the KIT gene (data not shown). These results indicate that serum depletion-resistant c-kit activity in uveal melanoma involves other mechanisms.

One possible mechanism of c-kit activation in uveal melanoma might be autocrine or paracrine stimulation of the receptor. To investigate this possibility, we analyzed the expression of SCF in the three uveal melanoma cell lines compared with cutaneous melanoma cells. Figure 3B shows that all three uveal melanoma cell lines expressed the 31-kDa variant of SCF. The expression level was independent of the absence of serum in culture medium. In contrast, SCF was not detectable in the cutaneous melanoma cells (Fig. 3B).

Fig. 3C, top, shows the Western blot of different doses of STI571 on c-kit phosphorylation of OCM-1. These experiments were made under basal conditions (i.e., the cells were growing in complete medium containing 10% serum). This procedure was used, because it will allow comparison of IC_{50} for c-kit phosphorylation and cell proliferation. The densitometry data for all three cell lines are illustrated in Figure 3C, bottom. IC_{50} ranged from 0.8 to 2.5 μM.

To examine the anti-proliferative effects of STI571 on the uveal melanoma cell lines, OCM-1, OCM-3, and 92-1, we were incubated with different concentrations of the drug for 48 hours, after which the level of cell proliferation was assayed. Figure 4 shows the dose-response of OCM1, OCM-3, and 92-1. There was a drastic cell loss even at low concentrations. The IC_{50} values were calculated to 0.15, 0.5, and 1.25 μM for the OCM3, OCM1, and 92-1 cells, respectively, and to more than 10 μM for BE and DFB. We can conclude that STI571 doses of 2.5 μM or less reduce both c-kit phosphorylation and cell proliferation of uveal melanoma cells 50% or more. In other words, both effects correlate well with each other.

**Discussion**

Protein tyrosine kinases (PTK) play a crucial role in signal transduction, as well as in cellular proliferation, differentiation, and various regulatory mechanisms. Some PTKs expressed by normal melanocytes are encoded by proto-oncogenes like c-kit.27

c-Kit expression has been documented in a wide variety of human malignancies, and the kinase activity has been implicated in the pathophysiology of a number of these tumors, including GISTs, neuroblastoma, and cutaneous melanoma.

In cutaneous melanoma c-kit has been shown to be expressed in epidermal melanocytes but is lost in cells infiltrating the dermis.26 Even if c-kit seems to be downregulated during the development of normal melanocytes to melanoma with metastasizing potential, there is no evidence that c-kit-negative cells feature mutations in the KIT gene or in its promoter.29,30 In contrast, among tumors expressing c-kit, gain-of-function mutations have been found in mastocytosis,31 seminomas,32 the catalytic tyrosine kinase domain, and in GISTs33,34 in the regulatory juxtamembrane domain. Because all these neoplasms arise in tissues with development that is dependent on the activity of the SCF/c-kit axis, aberrant activation of this axis may be of pathogenic impact.

We demonstrated in this study by both IHC and Western blot analysis that c-kit is expressed in a large amount of uveal melanomas. When the results from the 134 IHC cases were correlated to clinical follow-up data there was no significance between c-kit expression and survival. The study of Mouriaux et al.15 did not find any significant correlation, either.

When sequencing 15 uveal melanoma samples, however, we could not find any mutations in them. These data are in concordance with recently published data by Pache et al.25 In contrast, we could detect expression of SCF in all three analyzed uveal melanoma cell lines, but not in cutaneous melanoma cells. These results indicate that c-kit expression and activity in uveal melanoma may involve autocrine and/or paracrine stimulation of the receptor by SCF. Such a mechanism of c-kit activation has been thought to play a role in small cell lung cancer.35 Further analysis to evaluate the potential role of autocrine stimulation of c-kit in uveal melanoma is currently under way.

STI571 is a phenylamino-pyrimidine that selectively inhibits proto-oncogenic and oncogenic forms of ABL, PDGFR, and c-kit tyrosine kinases.11 In the case of chronic myelogenous leukemia, the drug inhibits kinase activity of the BCR-ABL fusion gene product, and in GISTs it inhibits the Kit tyrosine kinase. STI571 is conveniently administered as a once-daily oral medication and is well tolerated.36,37

We found that growth of uveal melanoma cells was relatively sensitive (IC_{50} < 1.25 μM) to STI571. Of particular interest was the fact that the sensitivity of the c-kit kinase was of similar size (IC_{50} < 2.5 μM). This situation was also found in chronic myelogenous leukemia and GIST cell lines38,39 and suggests that growth and survival of uveal melanoma is dependent on c-kit activation and consequently highlights a possible therapeutic benefit of STI571 in patients with uveal melanoma. Because the maximum toler-
Figure 3. c-Kit expression and phosphorylation of uveal melanoma cells. (A. top) Basal expression and phosphorylation of c-kit in the uveal melanoma cell lines compared with cutaneous melanoma cells. Cells were harvested for detection of c-kit expression by Western blot analysis or for determination of tyrosine phosphorylation of c-kit. c-Kit phosphorylation was either performed by immunoprecipitation followed by Western blot analysis (using a phosphotyrosine antibody) of the immunoprecipitates, or directly by Western blot analysis using an antibody specific for phosphorylated c-kit (Tyr 703). (A. bottom) SCF-simulation of c-kit phosphorylation. The three uveal and two cutaneous melanoma cell lines were serum depleted overnight and then stimulated or not with SCF (10 ng/mL) for 10 minutes. The cells were harvested for assay of c-kit phosphorylation using the phospho-c-kit-specific antibody, as just described. Separate Western blot analyses using c-kit antibodies confirmed that there were no essential differences in amount of loaded proteins. (B) The three uveal melanoma cell lines and the BE and DFB lines were growing in the presence or absence of 10% serum for 24 hours. Cells were then harvested for Western blot analysis for SCF expression. Actin was used as loading control. (C. top) OCM-1 cells, growing in complete medium supplemented with 10% serum, were treated with different doses of STI571 for 1 hour, whereupon the effects on c-kit phosphorylation were assessed as described above. (C. bottom) the dose-response effect of STI571 on c-kit phosphorylation of OCM-1, OCM-3, and 92-1, illustrated by densitometry of signals obtained by Western blot analysis using the phospho-c-kit-specific antibody.
ated dose (close to 1000 mg/d) of imatinib mesylate (STI571) in phase I trials corresponded to a blood level of 6 to 10 μM our IC$_{50}$ of 0.15 to 1.25 μM regarding antiproliferative effects on uveal melanoma cells may be relevant for obtaining antitumor effects in patients.

It is interesting to note that Fiorentini et al. reported some tumor-reducing effects of STI571 on uveal melanoma metastases expressing immunohistochemical c-kit in two patients at the terminal stage of disease.

Because STI571 also inhibits the tyrosine kinase of PDGFRα and β, the possibility is raised that it could also interfere with PDGFR and other kinases (e.g., Abl) in uveal melanoma. Actually, a preliminary study in our laboratory has shown expression of PDGFRs in some uveal melanoma cell lines and samples (Müller-Brüntete et al., manuscript in preparation). However, our present observations that the IC$_{50}$ of STI571 for c-kit phosphorylation and proliferation of uveal melanoma cells are in the same range of magnitude (<2.5 μM) suggest that c-kit inhibition is the main cause of the antiproliferative effects on this cell type.

**References**


