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Functional and phenotypic studies of two variants of a human mast cell line with a distinct set of mutations in the *c-kit* proto-oncogene

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SUMMARY

The human mast cell line (HMC)-1 cell line is growth-factor independent because of a constitutive activity of the receptor tyrosine kinase Kit. Such deregulated Kit activity has also been suggested causative in gastrointestinal stromal tumours (GISTs) and mastocytosis. HMC-1 is the only established continuously growing human mast cell line and has therefore been widely employed for *in vitro* studies of human mast cell biology. In this paper we describe two sublines of HMC-1, named HMC-1^{560} and HMC-$1^{560,816}$, with different phenotypes and designated by the locations of specific mutations in the *c-kit* proto-oncogene. Activating mutations in the Kit receptor were characterized using the pyrosequencing^{TM} method. Both sublines have a heterozygous T to G mutation at codon 560 in the juxtamembrane region of the *c-kit* gene causing an amino acid substitution of Gly-560 for Val. In contrast, only HMC-$1^{560,816}$ cells have the *c-kit*^{V816} mutation found in mast cell neoplasms causing an Asp→Val substitution in the intracellular kinase domain. Kit was constitutively phosphorylated on tyrosine residues and associated with phosphatidylinositol 3′-kinase (PI 3-kinase) in both variants of HMC-1, but this did not lead to a constitutive phosphorylation of Akt or extracellular regulated protein kinase (ERK), which are signalling molecules normally activated by the interaction of stem cell factor (SCF) with Kit. The documentation and characterization of two sublines of HMC-1 cells provides both information on the biological consequences of mutations in Kit and recognition of the availability of what in reality are two distinct cultured human mast cell lines.

INTRODUCTION

Cell lines are frequently used in studies of mast cell biology. Only one human mast cell line has been described (HMC-1)[1] thus contrasting with the availability of several growth-factor dependent, or independent, mast cell lines of murine or rat origin. The HMC-1 cell line was established from a patient with mast cell leukaemia, and these cells exhibit many characteristics of tissue mast cells, such as the expression of histamine, tryptase, heparin, and a similar cell surface antigen-profile.[2,3] The major difference between HMC-1 cells and human tissue mast cells is the lack of FcεRI expression, although mast cells developed *in vitro* from human liver, bone marrow or cord blood have a reduced FcεRI expression compared with tissue mast cells.[4–8] Because of the many advantages of using cell lines for studies of cell biology, HMC-1 cells have been widely used in studies of human mast cell function, for example refs 9–19.

One key characteristic of mast cells is the expression of the receptor for stem cell factor (SCF), i.e. Kit, a type III receptor tyrosine kinase encoded by *c-kit*, the cellular homologue of the viral oncogene *v-kit.*[20] Signalling mediated by SCF and Kit is known to be of importance for the control of the proliferation, differentiation, migration, activation, and survival of mast cells.[21,22] Mast cell lines often have a deregulated Kit receptor

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Abbreviations: HMC-1, human mast cell line 1; PI 3-kinase, phosphatidylinositol 3′-kinase; ERK, extracellular regulated protein kinase; SCF, stem cell factor; FcεRI, high affinity IgE-receptor I; GIST, gastrointestinal stromal tumour; GM-CSF, granulocyte–macrophage colony-stimulating factor; CBMC, cord blood derived mast cells; PMA, phorbol-12- myristate-13-acetate.

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signalling due to mutations in the *c-kit* proto-oncogene. These alterations, that convert the receptor into a constitutive active state, have been reported for the RBL-2H3, P-815, C2, and HMC-1 mast cell lines.[23–26] Activating mutations within the *c-kit* gene appear to be causative in most gastrointestinal stromal tumours (GISTs)[27] and some types of mastocytosis,[28–30] the latter of which is a heterogeneous group of disorders characterized by accumulation of mast cells in tissues. Several studies of cells obtained from patients with mastocytosis have described a point mutation in the catalytic domain of Kit (for reviews see Vliagoftis *et al*.[31] and Nilsson *et al*.[32]). This missense mutation (*c-kit*$^{V816)}$ introduces an Asp → Val substitution into the Kit receptor, which makes the receptor constitutively phosphorylated and active.[26,33–35] The V816 mutation is especially common in patients with adult onset sporadic mastocytosis and in patients in which mastocytosis is associated with hematologic disorders such as myelodysplastic or myeloproliferative syndromes.[28–30] Mutations in other regions of Kit are rare in mastocytosis, although there is a report of two mastocytosis patients with a mutation (G560) in the juxtamembrane region between the transmembrane and tyrosine kinase part of Kit.[36] Similarly, many GIST patients have activating mutations located between codon 550–560 in the juxtamembrane part of Kit.[27,37]

In this study we describe two variant sublines of HMC-1, which we designate HMC-1^{560} and HMC-1^{560,816}. Both variants have a point mutation in the juxtamembrane domain (*c-kit*$^{G560)}$ causing an amino acid substitution of Gly-560 for Val but only HMC-1^{560,816} has the Asp-816→Val mutation, which can be found in mast cell neoplasms. The two HMC-1 sublines also exhibit different phenotypic and growth characteristics.

MATERIALS AND METHODS

Reagents

Human recombinant SCF was purchased from Pepro Tech (Rocky Hill, NJ), PIXY321 was provided by Immunex (Seattle, WA) and horseradish peroxidase conjugated donkey anti-rabbit immunoglobulin was purchased from Amersham Pharmacia Biotech (Little Chalfont, UK). The anti-Akt, Phospho-Akt (Ser473) and phospho-p44/42 mitogen-activated protein (MAP) kinase (Thr202/Thr204) antibodies were from New England Biolabs (Beverly, MA). The anti-extracellular protein kinase (ERK)-1 (K-23) antibody was from Santa Cruz Biotechnology (Santa Cruz, CA) and the 4G10 Ab was from Upstate Biotechnology (Lake Placid, NY). Ly 294002 was purchased from Calbiochem (Darmstadt, Germany).

Cell cultures

HMC-1 cells[1,2] were cultured in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 IU/ml penicillin, 50 μg/ml streptomycin, 1·2 mM α-thioglycerol and passaged every 3–4 days.

MO7e cells (a megakaryocytic cell line expressing normal Kit) were cultured as for HMC-1 cells with the addition of 10 ng/ml of PIXY321, a granulocyte–macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-3 fusion protein. Human cord blood derived mast cells (CBMC) were developed *in vitro* as described.[6]

Cytochemistry

Cytocentrifuge preparations of HMC-1 cells were stained with May–Grünwald Giemsa for morphological analysis.

Cell cycle analysis

Cell cycle analysis was performed using Vindelöv's method for DNA measurements.[38] Briefly, stained nuclei were analysed in a flow cytometer (FACScan, Becton Dickinson, Mountain View, CA) and the percentage of cells in each cycle phase was calculated by using the MacCycle software (Phoenix flow systems, San Diego, CA).

Karyotyping

Standard chromosome preparations were made of HMC-1 cells and conventional cytogenetic analysis was carried out on G-banded chromosomes.

Cell growth

Analysis of cell growth was performed by seeding triplicates of 5×10^4 cells/ml in 5 ml supplemented (see above) IMDM medium. Cells were counted every 24 hr using a Coulter Z1 cell counter (Coulter Electronics, Luton, UK).

The proliferation assay was performed by seeding triplicates of 100 μl cells (600 000 cells/ml) in cell culture medium. Ly 294002 was added at the beginning of the experiment. The cultures were incubated over night before addition of [^{3}H]thymidine (0·5 μCi/well; Du Pont, Boston, MA). Incorporation of [^{3}H]thymidine was measured after eight hours in a scintillation counter and expressed as mean counts per minute (c.p.m.).

Cell survival

Cell survival assay was performed by seeding 200 μl cells (600 000 cells/ml) in cell culture medium ± Ly 294002 at 10 or 50 μM. The cells were incubated for 48 h before the rate of survival was analysed using a flow cytometric assay and propidium iodide.

Flow cytometry

The cell surface antigen expression was analysed by indirect immunofluorescence using a FACScan (Becton Dickinson) as described.[2] Monoclonal antibodies against the following cell surface markers were used: CD 13 (WM-15), CD 32 (IV.3), CD 44 (F-10-44-2), CD 54 (My13) from the 4th International Workshop of Human Leukocyte. The monoclonal antibody (mAb) against the α-chain of the FcεRI receptor (29C6)[39] was kindly provided by Drs R. Chizzonite and F. Riske (Hoffmann-La Roche, Nutley, NJ); the antibody against Kit (YB5.B8)[40] was a kind gift from Dr L. Ashman (Hanson Centre for Cancer Research, Adelaide, Australia), the mAbs against CD 49b (P1H5) and CD 49c (P1B5) were kindly provided by Prof K. Rubin (Uppsala University, Uppsala, Sweden), and the antibody against CD 18 (IB4) was a kind gift from Dr C. Lundberg (Pharmacia, Uppsala, Sweden). As negative controls, isotype-matched irrelevant antibodies were used (Dako Glostrup, Denmark).

Cell adhesion assay

For the adhesion assay, CytoMatrix cell adhesion strips (Chemicon International Inc. Temecula, CA) coated with

human collagen type I, human collagen type IV, human fibronectin, human laminin, human vitronectin or human tenascin were used according to the manufacturer's recommendations. Strips coated with BSA were used for determination of background adhesion. HMC-1 cells were resuspended in serum-free IMDM medium and seeded at 50×10^3 cells/well ± 100 nM phorbol 12-myristate 13-acetate (PMA), incubated at 37° for 1 hr in a CO_2 incubator and washed gently three times with PBS. One hundred μl of 0·2% crystal violet (Sigma) in 10% ethanol was then added to each well and incubated for 5 min at room temperature. The stain was removed and the wells were gently washed three times with phosphate-buffered saline (PBS). Cells were lysed by adding 100 μl of 1% sodium dodecyl sulphate (SDS) in PBS to each well, and the plate incubated on a shaker platform at room temperature until all cells were lysed and the stain dissolved. The absorbance was measured at 540 nm and an adhesion index was calculated by dividing the absorbance in wells coated with an extracellular matrix protein with the absorbance from the wells coated with bovine serum albumin (BSA).

Analysis of c-kit *mutation*

The analysis of the *c-kit* 560 and 816 mutation was performed using pyrosequencing™, a real-time pyrophosphate detection technology. Total RNA was prepared from HMC-1^{560}, HMC-$1^{560,816}$ and CBMC using TriPure isolation reagent (Roche, Basel, Switzerland). The RNA was then reverse-transcribed into complementary DNA (cDNA) using a first strand cDNA synthesis kit (Roche) and subjected to PCR amplification by a PCR core kit (Roche). Primers used in this study were obtained from Interactiva (Ulm, Germany). To be able to use the PCR product as a sequencing template, one of each PCR primer pair was covalently coupled to biotin. Primers (5′–3′ orientation) used were, biotin-CTT CCC GAA AGC TCC AG and CCC TGT TCA CTC CTT TGC, for the *c-kit*^{G560} mutation; and biotin-CAG GTG CCA TCC ACT TCA and CTT TCC TCG CCT CCA AGA for the *c-kit*^{V816} mutation. The amplification steps involved denaturation for 5 min at 94° followed by 30–35 cycles of denaturation for 30 s at 94°; annealing for 45 s at 63° for *c-kit*^{G560} at 65° for *c-kit*^{V816}; extension for 30 s at 72°, and a final extension for 7 min at 72°. The PCR products were analysed on a 2% agarose gel, with the expected sizes for the *c-kit*^{G560} and *c-kit*^{V816} PCR products 248 bp and 174 bp, respectively. Biotinylated DNA fragments (20 μl) were attached to streptavidin-modified Dynabeads (Dynabeads™ M-280, Dynal A/S, Skøyen, Norway) according to a standard protocol by incubating at 65° for 10 min (shaking) in high salt (BW) buffer (0·05% Tween; 0·05 mM EDTA; 1 M NaCl; 5 mM Tris-HCl, pH 7·6). The Dynabeads were then captured using a PSQ 96 Sample Prep Tool with 96 magnetic ejectable microcylinders (Pyrosequencing AB, Uppsala, Sweden). This tool was also used for incubation (1 min) of the biotin–streptavidin complex in 0·5 M NaOH before washing in annealing buffer (200 mM Tris-HAc and 50 mM Mg-HAc). Subsequently, the samples were hybridized to 15 pmol of sequencing primers in annealing buffer at 80° for 2 min, followed by cooling to room temp. Sequencing primers (5′–3′ orientation) were AGT ACA GTG GAA GGT TG for the *c-kit*^{G560} mutation and TGT GAT TTT GGT CTA GCC for the *c-kit*^{V816} mutation. Pyrosequencing™ was performed using a SNP Reagent kit (Pyrosequencing AB) containing enzyme and substrate mixture, dATP-S, dCTP, dGTP and dTTP according to the manufacturer's instructions.

Mutation-specific restriction analysis

To analyse the A→T substitution at nt 8213 in *c-kit*, which produces a new *Hin*fI restriction site (GANT-C), restriction endonuclease digestion of a PCR product was performed as described.[29] Briefly, genomic DNA was extracted from peripheral blood mononuclear cells from two patients with mastocytosis, following informed consent, and from HMC-1 cells, and PCR-amplified. The amplified 332-bp single DNA fragment was digested with *Hin*fI and separated on an 8% polyacrylamide gel. Since the V816 mutation creates a new *Hin*fI site, predicted fragments were 257, 51 and 14 bp for the mutated and 271 and 51 for the wild type *c-kit* gene.

Immunoprecipitation and immunoblotting

The procedures of immunoprecipitation, gel electrophoresis, and immunoblotting were performed according to methods described.[41] Briefly, lysates from untreated or SCF-treated cells were immunoprecipitated with a rabbit-polyclonal antibody against human Kit[42] (kindly provided by Dr L. Rönnstrand, Ludwig Institute for Cancer Research, Uppsala, Sweden), collected with 50 μl of protein-A sepharose (Amersham Pharmacia Biotech) and washed in lysis buffer containing protease and phosphatase inhibitors. The immunoprecipitates were separated on 4–12% gradient polyacrylamide gels (Novex, San Diego, CA) and electrophoretically transferred from the gel to nitrocellulose membranes (Amersham Pharmacia Biotech). After blocking, immunoblotting was performed with 4G10 antiphosphotyrosine mAb and antibody recognizing the p85 subunit of phosphatidyl inositol (PI) 3-kinase and horseradish peroxidase-conjugated secondary antibodies (Amersham Pharmacia Biotech). p85 and phosphorylated Kit were visualized using enhanced chemiluminescence detection (LumiGLO, New England Biolabs). Phosphorylation analysis of Akt and ERK was carried out by separating total cell lysates on 4–12% gradient polyacrylamide gels (Novex); followed by transfer to nitrocellulose membranes (Amersham Pharmacia Biotech) and blocking. Blotting of membranes was performed using Abs specific for Akt, Phospho-Akt (Ser473), ERK-1 and phospho-p44/42 mitogen-activated protein (MAP) kinase. Proteins were visualized as described above.

RESULTS

Cell morphology and proliferation rate

When culturing the HMC-1 sublines, we observed morphological and proliferative differences. HMC-1^{560} cells were heterogeneous in size and exhibited some homotypic aggregation (Fig. 1a). They were also more adherent than HMC-$1^{560,816}$ cells, which grew in single cell suspension (Fig. 1b). HMC-$1^{560,816}$ cells were smaller in size and tended to be more homogeneous (Fig. 1b).

To measure cell growth in suspension, HMC-1^{560} and HMC-$1^{560,816}$ cells were counted on days 1–7 after plating. Both HMC-1 variants grew without addition of growth factors. HMC-$1^{560,816}$ cells exhibited a markedly increased proliferation

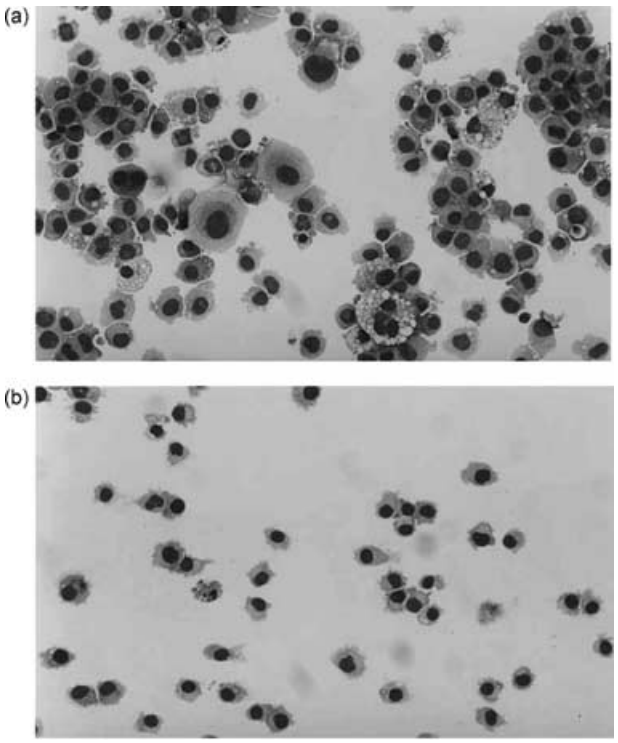


Figure 1. Morphologic analysis of HMC-1 cells. HMC-1^{560} (a) and HMC-$1^{560,816}$ cells (b) were cytocentrifuged and stained with May–Grünwald Giemsa.

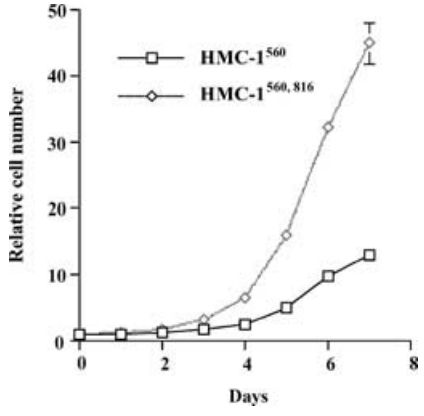


Figure 2. Comparison of rate of proliferation for HMC-1^{560} and HMC-$1^{560,816}$. Cells (5×10^4/ml) were seeded on day 0. Cell numbers on days 1–7 were determined using a Coulter cell counter. Relative cell number is the cell count divided by the number of seeded cells.

rate compared with HMC-1^{560} cells. After 7 days there were approximately three times more cells in the HMC-$1^{560,816}$ cultures compared with the number of cells in the HMC-1^{560} cultures (Fig. 2).

DNA profiles and karyotypes

We continued the characterization of the two HMC-1 sublines by cell cycle analysis. The DNA of HMC-1 cells was stained with propidium iodide and analysed by flow cytometry. HMC-$1^{560,816}$ cells exhibited a normal diploid DNA profile, whereas the analysis of HMC-1^{560} cells indicated an increased DNA content (data not shown). This observation was then confirmed by karyotyping the cells that revealed that among 11 cells 4 were diploid (46 XX) and the remaining 7 were hyperdiploid with chromosome numbers ranging from 53 to 82. In contrast, the karyotype of analysed HMC-$1^{560,816}$ cells were 46 XX (data not shown).

Phenotypic characterization of two HMC-1 sublines

The observed morphological differences between the HMC-1 variants (Fig. 1) was verified by flow cytometer analysis (Fig. 3a) where HMC-1^{560} cells again were heterogeneous in size. Then, in order to characterise the differences in surface antigen expression of HMC-1^{560} and HMC-$1^{560,816}$ cultures, cells were analysed using a panel of mAbs and flow cytometry (Fig. 3b). Both HMC-1^{560} and HMC-$1^{560,816}$ cells displayed surface expression of CD13, CD32, CD44, CD54, and CD117 (Kit). HMC-1^{560} cells had a higher level of expression of the integrins CD18, CD49b, and CD49f. Both variants were negative for expression of the α-chain of the high affinity immunoglobulin E (IgE)-receptor complex.

Adhesion of HMC-1 cells to extracellular matrix proteins

In an adhesion assay, HMC-1^{560} cells, but not HMC-$1^{560,816}$ cells, adhered to collagen I and collagen IV coated surfaces, while both adhered to fibronectin and laminin coated surfaces. After activation with a phorbol ester (PMA) HMC-1^{560}, but not HMC-$1^{560,816}$ cells, adhered to vitronectin and tenascin (data not shown).

c-kit mutations in HMC-1 cells

Kit has been reported to be constitutively activated in HMC-1 cells by two point mutations resulting in amino acid substitutions of Gly-560 for Val and Val-816 for Asp in the intracellular domain.[26] These mutations are thought to cause malignant transformation of cells. However, the mechanism by which Kit is constitutively activated does not appear to be consistent and different mutations are found in distinct tumours/cancers. We thus evaluated the two HMC-1 variants for *c-kit*^{G560} and *c-kit*^{V816} mutations in cDNA. Evaluation of sequencing data was achieved by comparing peak heights in the pyrogram, where samples heterozygous for a point mutation produce half-height peaks for both allelic positions, whereas homozygous samples have a single base peak of full height.[43] Target sequences were derived from the nucleotide sequence and organization of the human *c-kit* gene.[44] Both variants of the HMC-1 cell line exhibited a heterozygous T→G mutation at codon 560 (Fig. 4a,b). This can be seen as a peak of half height for the G nucleotide, in comparison the CBMC wt sequence lack incorporation of G at this position (Fig. 4c). The *c-kit*^{V816} mutation was also found to be heterozygous in HMC-1. However, this A to T substitution was only found in HMC-$1^{560,816}$ cells (Fig. 4d–f).

The 816 mutation results in a new *Hin*fI cleavage site in the *c-kit* gene.[29] This enabled us to confirm the existence of this

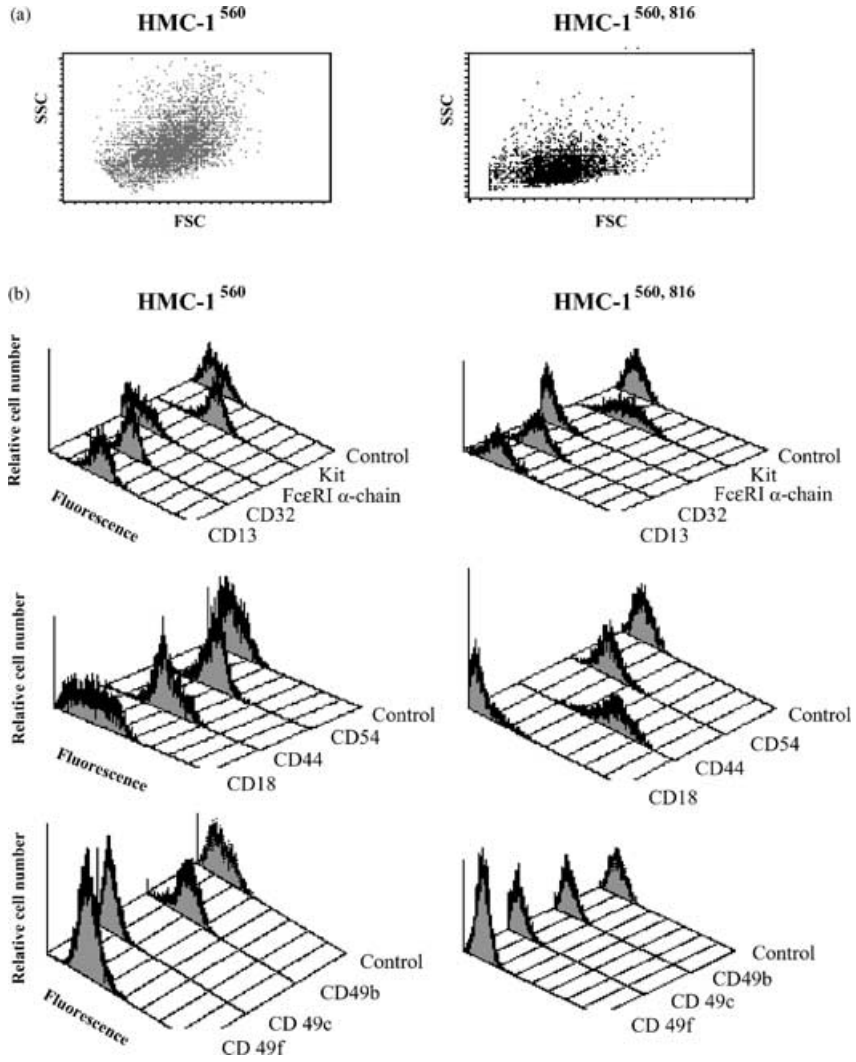


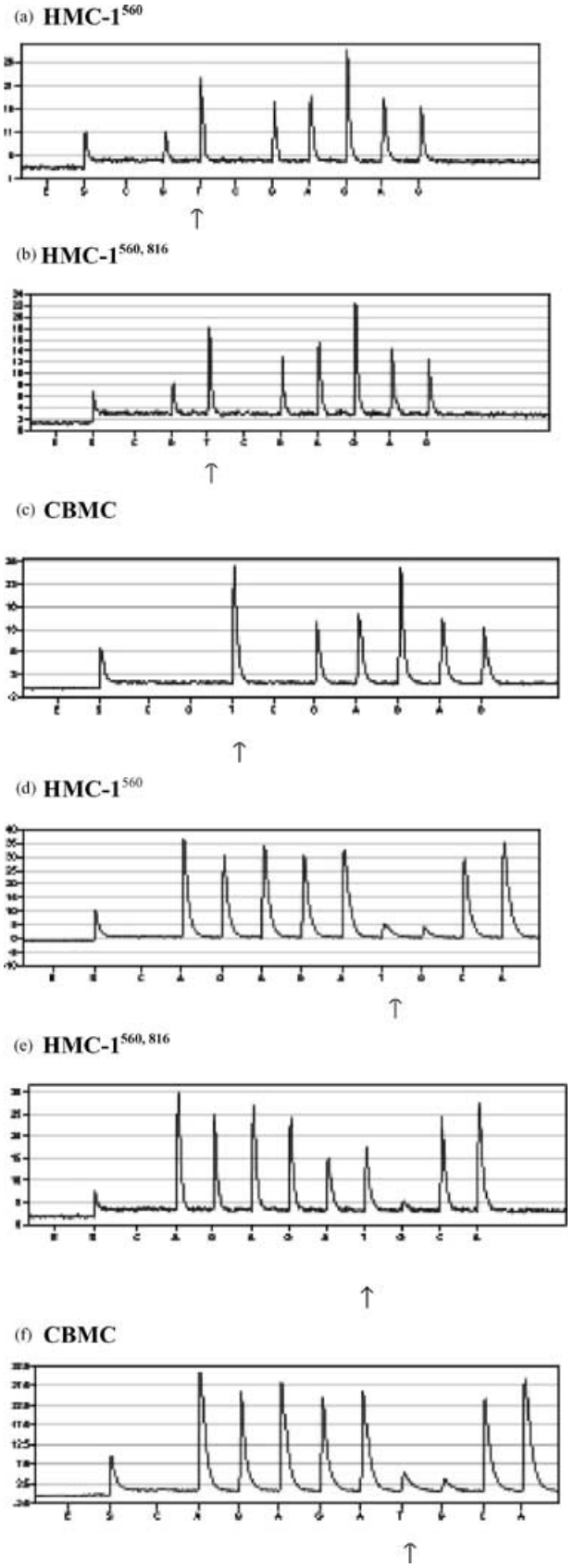
Figure 3. Flow cytometry analysis of expression of cell surface antigens on HMC-1^{560} and HMC-1^{560,816} cells. (a) The forward and light scatter of HMC-1^{560} cells (left) and HMC-1^{560,816} cells (right). Cells were stained as shown in (b) for expression of CD13, CD32, the α-chain of FcεRI, and Kit (first grouping), CD18, CD44 and CD54 (second grouping) and CD49b, CD49c and CD49f (bottom panel). Isotype-matched antibodies were used as controls.

mutation in HMC-1^{560,816}. A 322-bp PCR product of genomic *c-kit* DNA was cleaved with *Hin*fI and digested PCR fragments were separated on a polyacrylamide gel. As predicted from the sequencing data, only HMC-1^{560,816} cells contained the restriction site and hence the point mutation at codon 816. The point mutation in HMC-1^{560,816} cells and a mutation in one of the mastocytosis patients were both found to be heterozygous (Fig. 5).

Constitutive tyrosine phosphorylation of Kit

Ligand binding is known to induce tyrosine phosphorylation of Kit. In order to determine the effect of SCF on mutated and normal Kit, the two HMC-1 variants and MO7e cells were investigated for tyrosine phosphorylation of Kit. Both the mature, glycosylated (145 000 MW) and the immature (125 000 MW) forms of *c-kit* gene products were identified and found to be phosphorylated on tyrosine in HMC-1^{560} and HMC-1^{560,816} cells (Fig. 6). This phosphorylation on tyrosine was constitutive. However, stimulation with SCF increased the baseline phosphorylation. Unstimulated MO7e cells were found to have unphosphorylated receptors but in the presence of SCF the mature 145 000 MW form of Kit was phosphorylated.

Kit phosphorylation is known to activate PI 3-kinase- and MAP kinase pathways, considered to be involved in the regulation of cell survival and proliferation of mast cells. We therefore analysed the association of the p85 subunit of PI 3-kinase to Kit, as well as the phosphorylation status of Akt and ERK in the two HMC-1 variants. Kit association with PI 3-kinase was investigated by immunoprecipitation of Kit followed by SDS–polyacrylamide gel electrophoresis (PAGE) and immunoblotting



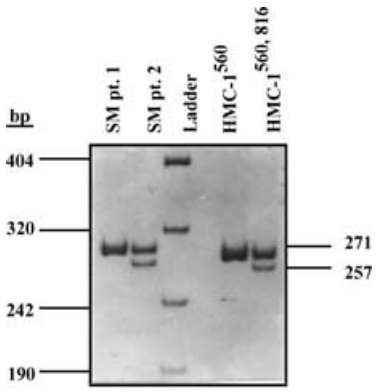


Figure 5. *c-kit* mutations in DNA from HMC-1 variants and patients with systemic mastocytosis (SM). In *c-kit*, the A-to-T substitution at nucleotide 8213 of exon 17 creates a new *Hin*fI restriction site. Genomic DNA from patients with systemic mastocytosis and from HMC-1^{560} and HMC-$1^{560,816}$ cells was extracted and a segment of the DNA including this site was amplified by primers complimentary to the sequence of introns 16 and 17 of the *c-kit* gene. The PCR product was digested with *Hin*fI and separated on polyacrylamide gel. Predicted sizes of digested fragments are 271 and 51 bp for wild type, and 257, 51 and 14 bp for mutated *c-kit*. Samples are: lane 1, patient 1 with indolent mastocytosis; lane 2, patient 2 with mastocytosis with an associated hematologic disorder; lane 3, ladder; lane 4, HMC-1^{560}; and lane 5, HMC-$1^{560\ 816}$.

with anti-p85 antibodies. An identical pattern was found for the two variants of HMC-1. Kit was associated with the p85 subunit in a SCF independent manner (Fig. 7a).

Subsequently, the effect of a PI 3-kinase inhibitor, Ly 294002, was investigated on the proliferation and survival. As shown in Figs 7(b,c), treatment with Ly inhibited proliferation and induced apoptosis in both HMC-1^{560} and HMC-$1^{560,816}$ cells.

We next investigated the phosphorylation of Akt in total cell lysates. Akt is a serine-threonine kinase involved in an anti-apoptotic signalling pathway downstream of PI 3-kinase.[45] Although PI 3-kinase was constitutively associated with Kit, Akt exhibited only minor phosphorylation in the absence of SCF. Addition of SCF induced Akt phosphorylation in both HMC-1^{560} and HMC-$1^{560,816}$ cells (Fig. 8a). Phosphorylation of ERK, in the Ras-Raf-MAP kinase pathway, also showed a similar pattern in HMC-1^{560} and HMC-$1^{560,816}$ cells. Unstimulated cells exhibited only a modest or little phosphorylation mainly on ERK2. However, stimulation with 100 ng/ml SCF for 10 min induced substantial phosphorylation of both ERK1 and ERK2 (Fig. 8b).

Figure 4. Analysis of mutations at codon 560 (a–c) and at codon 816 (d–f). RNA from HMC-1^{560} cells (a,d), HMC-$1^{560,816}$ cells (b,e) and CBMC (c,f) was extracted and used for RT–PCR. The PCR products were analysed for the 560 and 816 point mutations by means of pyrosequencing™. The arrows in a–c indicates the T→G substitution at position 560, and the arrows in d–f indicate the A→T substitution at position 816. E = enzyme, S = substrate.

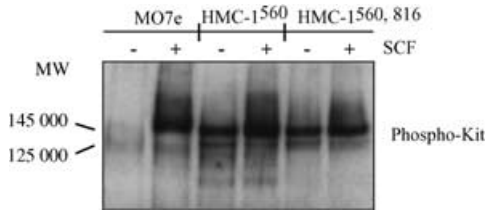


Figure 6. Phosphorylation of Kit in HMC-1 and MO7e cells. Cells were treated with SCF (100 ng/ml) for 15 min at 37° and lysed. Kit was immunoprecipitated with a rabbit anti-human-Kit antibody and protein A-sepharose beads from the cell lysates. Immunoblot was performed with the antiphosphotyrosine mAb 4G10.

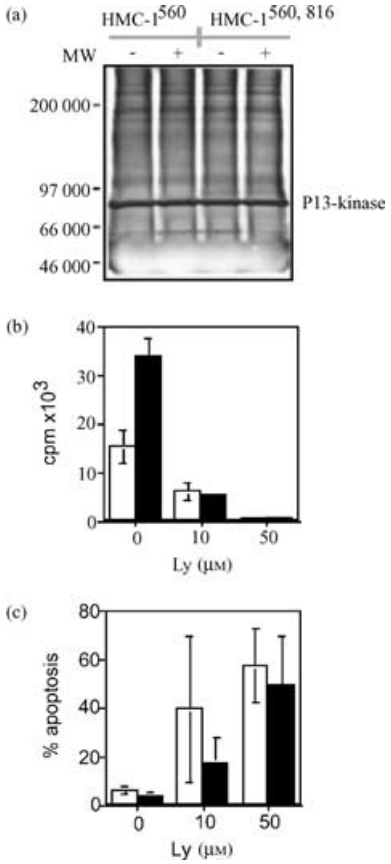


Figure 7. Intracellular signalling downstream of Kit in HMC-1 cells. (a) Kit immunoprecipitates were separated on a gel and analysis of association of c-Kit with PI 3-kinase was performed by immunoblotting with an antibody recognising the p85 regulatory subunit of PI 3-kinase. The effect of the PI3-kinase inhibitor Ly 29400 on HMC-1 proliferation (b) and apoptosis (c) was investigated. Open bars represent HMC-1^{560}; and filled bars HMC-$1^{560,\ 816}$.

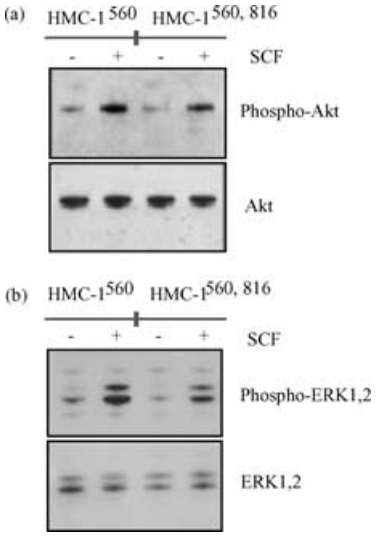


Figure 8. Phosphorylation of Akt and ERK. Cells were treated with SCF (100 ng/ml) for 15 min and lysed. Total cell lysates were analysed for phosphorylation of Akt (a) or ERK (b). Antibodies against total AKT and ERK, respectively, were used for control of loading.

DISCUSSION

The HMC-1 cell line is the only established human mast cell line. The purpose of this study was to characterise the phenotype, the set of disease associated gain-of function mutations in the *c-kit* gene, and the signalling outcome of these mutations in two sublines of the HMC-1 cell line. Based on their *c-kit* mutations, we have decided to designate these two variants as HMC-1^{560} and HMC-$1^{560,816}$. When culturing these cells we observed differences in both morphology (Fig. 1) and cellular growth rate (Fig. 2). The two variants also exhibited different karyotypes, as many HMC-1^{560} cells were found to be hyperdiploid. Such aneuploidy has been observed in virtually all analysed solid cancers (reviewed by Li *et al.*[46]). However, aneuploidy among HMC-1 cells was not observed during the initial establishment and characterization made by Butterfield *et al*. in 1988[1] and therefore may represent an outcome of long-term continuous *in vitro* culture. The pattern of cell surface antigen expression also differed between the two variants. Interestingly, by point mutation analysis using the pyrosequencing^{TM} method it was found that HMC-1^{560} and HMC-$1^{560,816}$ exhibited a divergent set of mutations in the *c-kit* gene. Corresponding mutations in mouse, *c-kit*^{G559} and *c-kit*^{V814} have been reported to activate Kit in a SCF independent manner in transfected cells and may alter Kit substrate specificity.[26,33–35,47] HMC-1^{560} cells were found to be heterozygous for *c-kit*^{G560} but negative for *c-kit*^{V816}. In contrast, HMC-$1^{560,816}$ cells were heterozygous for both mutations (Figs 4 and 5).

These mutations are associated with the development of different diseases. Mutation at codon 560 or alterations in the juxtamembrane region near this codon is mainly associated with GISTs.[27] Similarly, malignant canine mastocytomas often have mutations in the juxtamembrane region of Kit.[25] In contrast, human mast cell disorders are generally negative for mutations in the juxtamembrane region, with the exception of one report of indolent mastocytosis patients with an activating *c-kit* mutation at codon 560.[36] However, the other activating point mutation, *c-kit*^{V816} is common in mastocytosis. It is hypothesized that this mutation is necessary for the persistence or progression of adult onset mastocytosis and a subset of paediatric cases.[28]

Point mutations in the *c-kit* proto-oncogene and the subsequent activation of Kit are thought to change the cell into a more

tumorigenic type with a higher rate of proliferation. As the two variants of HMC-1 cells express different forms of mutated Kit, we investigated the rate of cell growth for the two HMC-1 sublines. We found that HMC-$1^{560,816}$ cells had a higher rate of proliferation than that of HMC-1^{560} cells. This may be due to the presence of the activating V816 mutation in HMC-$1^{560,816}$, as IC-2 and FDCP-1 cells transfected with murine *c-kit*^{V814} also exhibit a higher proliferation rate than cells transfected with murine *c-kit*^{G559}.[33,34] Furthermore, mast cells cultured from patients with indolent or aggressive variants of mastocytosis had a higher rate of proliferation if compared with mast cells from normal subjects.[48] Interestingly, the V816 mutation also appears to be involved in SCF-induced migration of mast cell progenitor cells. We have previously reported that cells from mastocytosis patients having this mutation migrate to a higher extent towards SCF if compared with cells having wild type Kit.[49] In that study we also used the HMC-1 cell lines but referred to them as HMC-1.1 and HMC-1.2, respectively.[49]

Subsequently, we analysed the rate of tyrosine phosphorylation of Kit. Both HMC-1 variants were found to have the same magnitude of constitutive phosphorylation. To determine how this Kit phosphorylation might affect downstream kinase signalling pathways, Kit immunoprecipitates were analysed for association with PI 3-kinase. These experiments revealed that in both HMC-1 variants, the regulatory p85 subunit of PI 3-kinase was constitutively associated with Kit. Thus, there appears to be a loss of normal regulated activity of Kit in HMC-1 cells. Our results are in agreement with Furitsu *et al*. who described that *c-kit*^{560,816} in HMC-1 cells is autophosphorylated and associated with PI3-kinase.[26]

Similar findings have been reported for cells transfected with *c-kit*^{816}. D816V *c-kit* mutant have been described to be constitutively associated with PI3-kinase, leading to higher levels of PI3-kinase activity than unstimulated wild-type *c-kit.*[50] In another study it was shown that transfected MO7e cells had a constitutive activation of Akt, which could be blocked with a PI 3-kinase inhibitor.[51] In the same study they showed that the Ras pathway is not activated in transfected cells. Surprisingly, although Kit is constitutively phosphorylated and associated with p85, neither of the two variants of HMC-1 had a constitutive phosphorylation of Akt or ERK (Fig. 8). Therefore, we speculate that Akt and ERK signalling pathways do not play a major role in the growth factor independent proliferation and survival observed in HMC-1 cells (Fig. 2). This is in agreement with previous observations that *c-kit*^{V816} does not lead to ERK phosphorylation and that ERK activity is not sufficient for induction of mast cell mitogenesis.'[51,52] SCF-induced proliferation has instead been reported to be dependent of PI 3-kinase and Src, which converged to activate Rac1 and JNK.[52]

In summary, these characterizations help us to better understand the mechanism behind the transforming capacity of the juxtamembrane and *c-kit*^{V816} mutations found in GIST and mastocytosis, respectively. We are currently using HMC-1^{560} and HMC-$1^{560,816}$ cells in a study of the impact of Kit inhibitors on deregulated cellular responses due to activating point mutations in different regions of Kit. The availability of two distinct cultured human mast cell lines will also be useful in future studies of mast cell biology.

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