RAPID COMMUNICATION

A Point Mutation in the Catalytic Domain of c-kit Induces Growth Factor Independence, Tumorigenicity, and Differentiation of Mast Cells

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The murine W and Steel loci encode the Kit receptor tyrosine kinase and its ligand, Steel factor, respectively. Loss of function mutations at either the W or Steel loci lead to a variety of pleiotropic developmental defects, including mast cell deficiency and severe macrocytic anemia. In addition to these loss-of-function mutations, gain-of-function mutations in c-kit, leading to constitutive activation of the Kit receptor, have also been identified in both rodent and human mastocytomas. In this study, we have examined the transforming potential and biologic effects of a point mutation that results in substitution of the aspartic acid at codon 814 in the cytoplasmic kinase domain to tyrosine (D814Y) by introducing either wild-type (Kit) or mutant Kit(D814Y) (KDY) cDNA into an interleukin-3-dependent mast cell line IC2. Stimulation of cells expressing the wild-type Kit receptor (IC2/Kit) with Steel factor in vitro resulted in a short-term growth response, whereas IC2/KDY cells were capable of sustained proliferation in a ligand-independent manner. In addition, expression of KDY resulted in the oncogenic transformation of IC2 cells, as determined by colony formation in vitro in the absence of exogenous growth factors and the formation of mastocytomas in vivo in syngeneic DBA/2 mice. Surprisingly, KDY expression in IC2 cells triggered dramatic changes in cell size and the extent of granulation. In addition, KDY induced the expression of mouse mast cell protease-4 (MMP-4) and MMP-6. In contrast, neither of these molecular or cellular changes was observed in IC2/Kit cells treated with Steel factor. These results show that the D814Y mutation in the cytoplasmic kinase domain of the Kit receptor induces ligand-independent mast cell growth in vitro, tumorigenicity in vivo, and mast cell differentiation.

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endogenous Kit receptor. Expression of this activated Kit RTK in IC2 cells resulted in ligand-independent cell growth, conferred tumorigenic potential in syngeneic hosts, and induced the expression of various genes associated with mast cell differentiation. These studies show that this activating Kit mutation, first identified in mouse and human mastocytoma cell lines, confers quantitative and qualitative changes that are not observed in mast cells after activation of the wild-type Kit receptor by Steel factor.

MATERIALS AND METHODS

Site-directed mutagenesis. The D814Y substitution was created by overlap extension using the polymerase chain reaction (PCR). Two pairs of primers (Kit4: TATTGTGAAACCTCGCTTGCGCA/ANTI-D814Y: ATTCTCTAGATGTACGCTGACT; and D814Y: CTA-GCCAGATACATCGGAATKit: AGCGGAAAGCCTTGGGACT) and pBluescript KS’-Kit containing the 3.7-kb mouse c-kit cDNA as a template were used for the first round of PCR. The two products were gel purified, and equal amounts were then used as templates for amplification with the Kit1 and Kit4 primers. The final PCR products were digested with the restriction enzymes ApaI and AatII. The resulting DNA fragment, which contained the D814Y substitution, was subcloned into pBluescript KS’-Kit, digested with ApaI and AatII, and named KDY.

Cells and growth conditions. The interleukin-3 (IL-3)-dependent immature mast cell line IC2 (generously provided by Dr Ichiro Yahara, The Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan) was established from a long-term culture of DBA/2 mouse splenocytes in the presence of IL-3. The IC2 cells were cultured in IC2 mix RPMI 1640 containing 10% bovine calf serum (JBCS), 5 × 10^{-5} M L-D-threomethanol [L-β-ME], and 1% X63 cell supernatants as IL-3 source.

Construction of Kit retroviral vectors and generation of cell lines expressing Kit or Kit-DY. Various murine c-kit DNAs were subcloned into the LXSN retroviral vector. The LXSN-Kit and LXSN-KDY constructs were generated by first adding a NotI site to the multiple cloning site of the LXSN vector, followed by cloning the XhoI-NotI Kit or KDY coding region fragments into XhoI-NotI-digested LXSN DNA. The BOSC 23 ecotropic packaging cell line was transiently transduced by means of the Stratagene transfection kit (Stratagene, La Jolla, CA), with either LXSN, LXSN-Kit, or LXSN-KDY, as described. Ten hours after transfection, fresh media was added. Viral supernatants containing virus were collected 48 hours later, filtered, and used to infect either NIH/3T3 cells (for titering) or IC2 cells. Infected 3T3 cells were selected by growth in 6-well plates (Nunc). Colonies were counted after 14 days.

RESULTS

Retroviral-mediated transfer and expression of the Kit and KGY receptor cDNAs in IC2 mast cells. To study the effects of the D814Y mutation in the Kit receptor on mast cells, we constructed retroviral vectors expressing either the wild-type or mutant forms of the Kit receptor, as described in the Materials and Methods. High titers of helper-free retroviral vector stocks were generated by transiently transfecting LXSN, LXSN-Kit, or LXSN-KDY DNAs into the ecotropic virus packaging cell line, BOSC 23. Viral supernatants were used to infect IC2 cells, an immature mast cell line. Forty-eight hours later, the infected cells were subjected to selection in G418 for 1 week. To eliminate clonal variation, pools of resistant cells derived from each infection, named IC2/Neo, IC2/Kit, and IC2/KDY, were used for further

Tumorigenic assay. IC2, IC2/Kit, and IC2/KDY cells (5 × 10^5) were injected intravenously (IV) into syngeneic DBA/2 mice. Two months later, the mice were killed and the livers were examined for tumor foci.

Flow cytometry. IC2, IC2/Kit, and IC2/KDY cells were incubated with ACK2 (monoclonal antibody directed against the extracellular domain of Kit and generously provided by Dr S. Nishikawa, Kyoto University, Kyoto, Japan) at 4°C for 30 minutes. The cells were then rinsed and stained with fluorescein isothiocyanate (FITC)-conjugated rabbit antirat Ig serum (Cedarlane, Hornby, Ontario, Canada). Cells were rinsed and analyzed on a FACSscan flow cytometer (Becton Dickinson, San Jose, CA). To analyze cell size and granulation, the cells were washed and resuspended in PBS at 10^7 cells per milliliter. These cells were then directly analyzed by FACSscan for forward scatter to monitor cell size and for light scatter intensity to monitor the extent of intracellular granulation.

Differential analyses. For assessment of biochemical characteristics, IC2, IC2/Kit, and IC2/KDY cells were analyzed in air-dried cytospinifuge preparations stained with alcian blue and safranin. Differential expression of secretory granule proteases in IC2, IC2/Kit, and IC2/KDY cells were analyzed by Northern blot. Total cellular RNA was extracted by acid guanidinium thiocyanate-phenol-chloroform extraction and fractionated by electrophoresis in 1.5% formaldehyde-agarose gels for 16 hours at 50 V. The separated RNAs were transferred to Hybond membranes (Amersham International plc, Amersham, UK) and hybridized with radiolabeled cDNAs for mouse actin or the mouse mast cell protease-4 (MMCP-4), MMCP-5, or MMCP-6. MMCP-4, MMCP-5, and MMCP-6 species-specific probes were generated by reverse transcriptase-PCR (RT-PCR) with mRNA extracted from IC2/KDY cells and three pairs of primers (MMCP4-5': AGACTTGGCCCATTTCTTCGTGC and MMCP4-3': GCCATGTGAAGGCGGAAATGTTGT; MMCP5-3': GTATACAGGGAGACTCTGGA and MMCP5-3': CCTGACTATTCCAGTCT; and MMCP5-5': TATGTCCGTAGGACTTTAAG). The PCR products were then gel purified and labeled by random-priming (Boehringer Mannheim, Indianapolis, IN) with α-[32P]dCTP (+3,000 Ci/mmol; DuPont/New England Nuclear, Boston, MA). Hybridizations were performed at 42°C for 16 hours in 50% formamide, 0.75 mol/L NaCl, 75 mmol/L sodium citrate, 1% sodium dodecyl sulfate (SDS), 5 mmol/L EDTA, 50 mmol/L sodium phosphate, 2× Denhardt's buffer, and 100 μg/mL denatured, single-stranded herring sperm DNA (Sigma Chemical Co, St Louis, MO). The RNA blots were washed at 55°C in 30 mmol/L NaCl, 0.1% SDS, and 1 mmol/L sodium phosphate, pH 7.0, and autoradiography was performed with NEF-496 autoradiography film (DuPont/New England Nuclear) for 14 to 24 hours. The blot was stripped by an incubation with boiling 0.1% SDS for 5 minutes.
ACTIVATING MUTATION IN c-Kit RECEPTOR

study. Ectopic expression of Kit or KDY was assessed by indirect immunofluorescence staining with monoclonal antibody directed against the extracellular domain of the Kit receptor, as described in the Materials and Methods, followed by FACS analysis (Fig 1). Unlike bone marrow-derived mast cells, IC2 cells, as well as IC2 cells infected with the control neo vector (IC2/Neo), do not express the Kit receptor. In contrast, both IC2/Kit and IC2/KDY cells expressed high levels of the Kit receptor on their cell surface, with a somewhat higher expression of wild-type Kit in the pool of G418 IC2/Kit cells. The Kit receptors on both IC2/Kit and IC2/KDY cells bound FITC-conjugated Steel factor and were internalized at a similar rate upon ligand binding (data not shown). Moreover, the mutant KDY receptors isolated from control or Steel factor-stimulated cells were constitutively phosphorylated on identical tyrosine residues, consistent with the notion that this receptor is fully activated in the absence of ligand (data not shown).

**KDY promotes ligand-independent cell growth.** The biologic consequences of expressing either Kit or KDY in IC2 cells were next examined by comparing the proliferative response of IC2/Neo, IC2/Kit, and IC2/KDY cells under different growth conditions. Both IC2/Neo and IC2/Kit cells were strictly dependent on IL-3 for their growth (Fig 2). Expression of the wild-type Kit receptor in IC2 cells enabled these cells to proliferate in the presence of Steel factor in a concentration-dependent manner (data not shown). In contrast, IC2/KDY cells proliferated at the same rate in the absence of exogenous IL-3 or Steel factor (Fig 2). Moreover, IC2/KDY cells were capable of long-term growth (more than 4 weeks; data not shown) in the absence of any exogenous growth factors, indicating that the expression of KDY enabled on IC2 cells to grow indefinitely in the absence of added growth factors.

**KDY mediates IC2 cell transformation and tumorigenicity.** To determine whether ectopic expression of either the wild-type Kit or the activating KDY receptor would result in the transformation of mast cells, we first determined whether the IC2/KDY cells could form colonies in vitro. As shown in Fig 3, IC2/Neo and IC2/Kit cells were unable to form colonies in semisolid medium, even in the presence of Steel factor, whereas IC2/KDY cells formed Steel factor-independent colonies. In the presence of IL-3, all three cell pools formed colonies, with IC2/KDY having the highest plating efficiency, IC2/Neo the lowest, and IC2/Kit intermediate.

We next injected IC2/Neo, IC2/Kit, or IC2/KDY cells into syngeneic DBA/2 mice (10 mice/cell line), as described in the Materials and Methods, to determine whether the KDY receptor could also confer tumorigenic potential on IC2 cells. Two months later, the mice were killed and examined for mastocytomas. All of the mice injected with IC2/KDY cells developed mastocytomas in the liver, whereas only 40% of the mice injected with IC2/Kit cells did so, with much fewer tumor nodules observed in the livers of these mice. No mice injected with IC2/Neo cells developed tumors (Fig 4). To determine whether these cells had also homed to a hematopoietic organ, bone marrow cells from the injected mice were cultured in IC2 mix without exogenous growth factors with a media change every 3 days. After 2 weeks of cultivation, only cells derived from the mice injected with IC2/KDY cells had grown into a homogeneous cell population, resembling the injected IC2/KDY cells (data not shown). Thus, expression of KDY in IC2 cells is sufficient to confer tumorigenicity on these cells, suggesting that the ability of P815 mastocytoma cells that harbor the original KDY mutation to form tumors in syngeneic mice results, at least in part, from the activating D814Y mutation in the Kit receptor.

**KDY promotes IC2 cell differentiation.** Activation of the Kit receptor by its ligand Steel factor in bone marrow-derived mast cells (BMMC) results in the induction of mast cell differentiation, as judged by an increase in cell size, the
extreme of granulation, transition from alcian blue/ safranin" to alcian blue and safranin double-positive, and changes in the expression of various mast cell proteases. To determine whether the activated KDY receptor might also mediate mast cell differentiation in the absence of ligand, we first examined the cell size, extent of granulation and alcian blue/ safranin staining of the infected IC2 cells. As shown in Fig 5A, ectopic expression of KDY in IC2 cells triggered dramatic changes in cell size and the extent of alcian blue" granulation; however, safranin staining remained negative. These morphologic alterations were measured by flow cytometry by examining forward (cell size) and side (granulation) scatter in cells expressing either the wild-type Kit or KDY receptor. As shown in Fig 5B, analysis of side light scatter clearly showed the effect of KDY expression on granulation content (Fig 5C). In contrast, cells expressing the wild-type Kit receptor did not exhibit these changes in cell size and granulation (Fig 5A, B, and C).

Genetic, biologic, and expression analysis has shown that the Kit receptor and its ligand Steel factor are crucial for the development of mast cells. In addition, activation of the Kit signaling pathway by Steel factor in BMMC results in the induction of mast cell differentiation. To examine the patterns of gene expression in IC2 cells expressing Kit or KDY, we generated MMCP-4-, MMCP-5-, and MMCP-6-specific probes by RT-PCR, as described in the Materials and Methods. These probes were then used to detect MMCP-4, MMCP-5, and MMCP-6 transcripts in IC2/Neo, IC2/Kit, and IC2/KDY cells. MMCP-5 transcripts were observed in all cell types tested, and the levels were not affected by expression of Kit or KDY (Fig 6). MMCP-6 transcripts were expressed at low level in IC2 cells, and the level increased as the result of KDY expression (Fig 6). MMCP-4 transcripts were not detectable in IC2/Neo or IC2/Kit cells, but were specifically and abundantly expressed in IC2/KDY cells (Fig 6).

**DISCUSSION**

Loss-of-function mutations at the W locus in mice affect hematopoiesis, gametogenesis, melanogenesis, neural crest migration, and the development of the interstitial cells of Cajal in the gut. However, our understanding of gain-of-function mutations in c-kit and their role in the development of hematologic malignancies is limited. In this report, we have investigated the effects of the oncogenically activated allele of c-kit, D814Y, on the growth and neoplastic transformation of an immature mast cell line, IC2. Expression of the mutant KDY receptor in IC2 cells enabled these cells to proliferate in suspension and to form colonies in semisolid medium in a ligand-independent manner and to give rise to mastocytomas in syngeneic DBA/2 mice. These results indicate that the constitutively activated KDY receptor possesses transforming activity and that its expression is sufficient to confer tumorigenic potential on IC2 cells.

Although both IL-3 and Steel factor are capable of inducing proliferation and differentiation of bone marrow progenitor cells into mast cells (MC), the patterns of gene transcription induced by IL-3 and Steel factor are quite distinct. Mouse bone marrow cells cultured in medium containing IL-3 differentiate into morphologically immature MC that express transcripts encoding MMCP-5 and MMCP-6, but not MMCP-4. The latter gene encodes a protease expressed in terminally differentiated connective tissue-type MC (CTMC). However, after cultivation in the presence of Steel factor, mast cells express high levels of mRNAs that encode mast cell carboxypeptidase A (MC-CPA), MMCP-4, MMCP-5, MMCP-6, and the high-affinity IgE receptor (FceRIα), a phenotype that is indistinguishable from that of
in vivo differentiated mouse CTMC. Moreover, cultivation in the presence of both IL-3 and Steel factor BMMC fails to express MMCP-4, suggesting that IL-3 acts as an antagonist to Steel factor-mediated mast cell differentiation.\(^{37}\) We analyzed the effects of the wild-type and mutant Kit receptors on the differentiation of IC2 cells. The wild-type Kit receptor failed to induce expression of MMCP-4 and MMCP-6 in the presence of IL-3 and Steel factor. In contrast, the KDY receptor also induced IC2 cell differentiation even in the presence of IL-3, as determined by the appearance of morphologic changes and the induction of RNA transcripts corresponding to the mast cell proteases MMCP-4 and MMCP-6. Together, these experiments suggest that the D814Y mutation not only results in ligand-independent Kit signaling but also results in changes in gene expression that is not seen in cells expressing the wild-type Kit receptor exposed to Steel factor. These observations raise the intriguing possibility that the D814Y mutation in KDY qualitatively affects the intracellular molecular events that lie downstream of Kit.

It is of interest to note that the D814Y mutation in KDY and the mutation described in the RET RTK in patients with

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Fig 4. Tumor formation in syngeneic mice. From left to right, ICZ/Kit, ICZ/Kit-DY, and ICZ/Neo cells \((5 \times 10^5)\) were injected intravenously into syngeneic DBA/2 mice. Two months later, the mice were killed and examined for tumor foci in the liver.

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Fig 5. Induction of cell size and granulation by KDY. (A) Alcian blue/safranin staining of ICZ/Neo (a), ICZ/Kit (b), and IC2/KDY (c) cells was performed as described. All three cell lines remain safranin negative. IC2/KDY contains the highest alcian blue \(^*\) granulation. (B) Semiquantitative analysis of the granulation content. ICZ/Neo (a), ICZ/Kit (b), and IC2/KDY (c) were analyzed for light scatter with FACscan. (C) Histogram analysis of the side scatter data. The profiles from ICZ/Neo (gray shadowed) were overlayed with ICZ/Kit (solid line) and IC2/KDY (broken line).
multiple endocrine neoplasia type 2B (MEN2B) are closely linked in subdomains VII and VIII of the kinase domain, respectively. Structural analysis of both protein kinase A and the insulin receptor has suggested that these domains are involved in substrate(s) recognition and binding. Consistent with this conclusion, the MEN2B mutation has been shown to affect RET substrate specificity. The mutations in both RET\textsuperscript{MEN2B} and KDY change the consensus residue in this region from an RTK-type amino acid to an amino acid usually found at the identical position in nonreceptor tyrosine kinases (NRTKs). RTKs normally associate with group III SH2 domain-containing proteins. In contrast, NRTKs interact with signaling molecules containing a group I SH2 domain. Thus, the mutant KDY receptor, such as the RET\textsuperscript{MEN2B} receptor, may adapt the conformation of NRTKs rather than RTKs. Because RTKs and NRTKs interact with different signaling proteins, such conformational changes may cause an alteration in substrate specificity. Indeed, we have recently shown that the D814Y mutation markedly affects substrate specificities based on both the in vivo patterns of protein tyrosine phosphorylation and in vitro analysis of peptide substrate specificity (Piao et al, manuscript submitted).

In summary, we have shown that mutation in the cytoplasmic kinase domain of the Kit receptor that results in its constitutive activation leads to the induction of transformation and differentiation in the IC2 mast cell line. In contrast, the wild-type Kit receptor failed to support long-term proliferation or to induce differentiation of IC2 cells in the presence of Steel factor. These data provide biologic evidence that the mutant KDY receptor may deliver both quantitatively and qualitatively different signals to IC2 cells. Further clarification of the mechanism of activation of this activating mutation and identification of novel substrates that specifically interact with the KDY receptor would provide insight into the molecular mechanisms underlying malignant transformation by this oncogenic variant of the Kit RTK.

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