Unique Effects of KIT D816V in BaF3 Cells: Induction of Cluster Formation, Histamine Synthesis, and Early Mast Cell Differentiation Antigens


*J Immunol* 2008; 180:5466-5476; doi: 10.4049/jimmunol.180.8.5466
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Unique Effects of KIT D816V in BaF3 Cells: Induction of Cluster Formation, Histamine Synthesis, and Early Mast Cell Differentiation Antigens


Oncogeneic tyrosine kinases (TK) usually convert growth factor-dependent cells to factor independence with autonomous proliferation. However, TK-driven neoplasms often are indolent and characterized by cell differentiation rather than proliferation. A prototype of an indolent TK-driven neoplasm is indolent systemic mastocytosis. We found that the D816V-mutated variant of KIT, a TK detectable in most patients with systemic mastocytosis, induces cluster formation and expression of several mast cell differentiation and adhesion Ags, including microphthalmia transcription factor, IL-4 receptor, histamine, CD63, and ICAM-1 in IL-3-dependent BaF3 cells. By contrast, wild-type KIT did not induce cluster formation or mast cell differentiation Ags. Additionally, KIT D816V, but not wild-type KIT, induced STAT5 activation in BaF3 cells. However, despite these intriguing effects, KIT D816V did not convert BaF3 cells to factor-independent proliferation. Correspondingly, BaF3 cells with conditional expression of KIT D816V did not form tumors in nude mice. Together, the biologic effects of KIT D816V in BaF3 cells match strikingly with the clinical course of indolent systemic mastocytosis and with our recently established transgenic mouse model, in which KIT D816V induces indolent mast cell accumulations but usually does not induce a malignant mast cell disease. Based on all these results, it is hypothesized that KIT D816V as a single hit may be sufficient to cause indolent systemic mastocytosis, whereas additional defects may be required to induce aggressive mast cell disorders.

S
ystemic mastocytosis (SM) is a neoplastic disease of mast cells (MC) and their bone marrow-derived progenitors. Indolent and aggressive variants of the disease have been described. In most adult patients, indolent systemic mastocytosis (ISM) is diagnosed. These patients usually have typical (urticaria pigmentosa-like) skin lesions and may suffer from mediator-related symptoms, but the disease does not behave as an overt malignancy. Rather, despite accumulation of neoplastic MC, these patients have a normal or near-normal life expectancy. Clinically, most patients with ISM would even be overlooked if they would not exhibit the striking cutaneous features of mastocytosis. This is not the case in the rare aggressive variant of mastocytosis (ASM) or (the extremely rare) MC leukemia (MCL). In these patients, malignant MC proliferation is found and the survival usually is short.

The pathologic hallmark and major (World Health Organization) criterion of disease shared by all SM variants including ASM, MCL, and ISM is the focal accumulation (cluster formation) of MC in hematopoietic tissues. The MC infiltration in ASM or MCL is aggressive and leads to organopathy within a short time. A second important feature of SM common to all disease variants is the somatic KIT point mutation D816V, which is not only detectable in ASM or MCL, but also in most patients (>80%) with ISM. A number of previous and more recent data suggest that the KIT mutation D816V leads to autonomous tyrosine kinase (TK) activity and enhanced survival of neoplastic cells. So far, however, little is known about the exact role and biologic consequences of this mutation in the context of SM. Based on cell line data and studies conducted with the murine repression domain of human Kox-1; pTyr, phosphoryltyrosine; IP, immunoprecipitation; HDC, histidine decarboxylase; MMCP5, mouse mast cell protease 5; MITF, microphthalmia transcription factor; LAMP-3, lysosome-associated protein 3; MML, myeloma mastocytic leukemia; IB, immunoblot; RFLP, restriction fragment length polymorphism; qPCR, quantitative PCR.

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equivalent of the mutation (i.e., Kit D814V), the hypothesis was raised that the mutation is directly responsible for the abnormal tumorous proliferation of neoplastic MC (17, 18). However, as mentioned above, patients with ISM do not have malignant disease despite the expression of Kit D816V in their neoplastic MC. Furthermore, we have recently shown that Kit D816V transgenic mice usually develop indolent accumulations (clusters) of MC in internal organs, but only rarely a malignant MC disease (19).

This “paradox” prompted us to revisit the transforming effects of the human KIT mutant D816V. In a first step, we used BaF3 cells since these cells are commonly used to define the “transforming” potential of oncoproteins detectable in myeloid neoplasms. Using BaF3 cells with conditional expression of Kit D816V, we found that this oncprotein does not provide a growth advantage, but induces differentiation. To confirm this finding, a human myeloid cell line (UT7) was engineered to inducibly express Kit D816V. The results of our study show that Kit D816V induces cluster formation and expression of MC differentiation Ags, but that it does not promote proliferation in BaF3 or UT7 cells, which is in line with the biology and indolent clinical course of ISM.

Materials and Methods

Reagents

Imatinib (STI571) and PKC412 were kindly provided by Dr. Elisabeth Buchdunger, Dr. Paul W. Manley, and Dr. Dorianno Fabbro (Novartis Pharma). Stock solutions of PKC412 were prepared by dissolving in DMSO (Merck). Recombinant human stem cell factor (SCF) was purchased from Strathmann Biotech, recombinant murine cytokines (IL-3, IL-6, SCF) from PeproTech, RPMI 1640 medium and FCS from PAA Laboratories, puromycin from Invitrogen, hygromycin from Roche, and doxycycline from Clontech.

Generation of BaF3 cells with inducible expression of Kit D816V

BaF3 cells were grown in RPMI 1640 medium with 10% FCS and 10% WEHI-3B conditioned medium (as a source of murine IL-3) at 37°C and 5% CO2. To generate cell lines with doxycycline-inducible expression of Kit, BaF3 cells expressing the reverse tetr-transactivator (Ton.BaF.1 cells) (20) were co-transfected with pTRE2 vector (Clontech) containing Kit D816V cDNA (or wild-type (wt) Kit cDNA) and pTK-Hyg (Clontech) by electroporation. Electroporation was performed as described (21). In brief, plasmids (10 μg each) were transfected into Ton.BaF.1 cells (1 × 106 cells in 800 μl) using a Gene Pulser (0.35 kV, 960 μF) (Bio-Rad). Stably transfected cells were selected by growing in hygromycin (400 μg/ml) and cloned by limiting dilution. Expression of Kit D816V was induced by addition of doxycycline (1 μg/ml). To investigate the role of STAT5 in Kit D816V-dependent signaling, Ton.Kit.D816V cells were retrovirally transduced with two different dominant negative (dn) STAT5 constructs (STAT5aΔ749 or STAT5bΔ754) (22) or with the empty vector. Retrovirus particles were produced by transient transfection of HEK-293FT cells (Invitrogen) with pMSCV-dnSTAT5-IRESDRFP, pYVS, and pGAG-Pol, using Lipofectamine2000 (Invitrogen) according to published techniques (23). Ton.Kit.D816V cells expressing dnSTAT5 (GFP+) were enriched by FACS sorting on a FACSaria (BD Biosciences).

Generation of UT7 cells with inducible expression of Kit D816V

UT7 (kindly provided by Dr. N. Komatsu, Jichi Medical School, Tochigi, Japan) is a human, GM-CSF-dependent myeloid (CD117+) cell line (24). UT7 cells were grown in RPMI 1640 medium with 10% FCS in the presence of recombinant human GM-CSF (1 ng/ml; R&D Systems) at 37°C and 5% CO2. To generate UT7 cells with inducible expression of Kit D816V, cells were retrovirally transduced with pRevTet-On (Clontech) and selected with puromycin (1 μg/ml). To test for doxycycline-inducible gene expression in this cell line model, UT7-Tet-On cells were transduced with the pRevTRE vector (Clontech) containing a GFP cDNA. In these experiments, substantial GFP expression in the absence of doxycycline (indicating leakiness) was observed, confirming results obtained in other cell lines (25). To generate a UT7 cell line with tight doxycycline-dependent regulation of gene expression, the tetracycline repressor fused to the Kruppel-associated box repression domain (KRAB) of human Kox-1 (tTR- KRAB) fusion protein (plasmid pLV-TRKRB, provided by Dr. D. Trono, Ecole Polytechnique Federale de Lausanne, Switzerland) (26) was expressed in UT7-Tet-On cells. Binding of KRAB (through the (TR moiety of the fusion protein) to the tet response element of pRevTRE resulted in complete silencing of gene expression in the absence of doxycycline (26, 27). UT7-Tet-On-Kit cells (termed UT7-Kit) were then transduced with pRevTRE-KIT D816V and selected with hygromycin (400 μg/ml). Immunoprecipitation experiments using the anti-KIT Ab 1C1 (kindly provided by Dr. H.-J. Bühring, University of Tübingen, Germany) and the anti-phosphotyrosine (pTyr) Ab 4G10 (Upstate Biotechnology) revealed tight doxycycline-dependent expression of Kit D816V with virtually no leakiness in the absence of doxycycline (see Results).

Detection of the Kit D816V mutation by restriction fragment length polymorphism (RFLP)

After isolation of RNA and synthesis of cDNA (see below), a 206-bp fragment was PCR amplified using primers D816V-F (5′-TCTATGTTCG GATCACAAGA-3′) and D816V-R (5′-AGGGGCCTGCTTCTAAA GAG-3′). The PCR product was digested with Hinf1 (New England Biolabs) and analyzed on a 15% polyacrylamide gel. The presence of the D816V mutation creates a new Hinf1 restriction site, resulting in cleavage of the 56-bp fragment (wt Kit) into a 42-bp and a 14-bp fragment (mutated Kit).

Western blotting

Ton.Kit.wt or Ton.Kit.D816V cells (105/ml) were incubated with doxycycline (1 μg/ml) at 37°C for 24 h to induce expression of Kit. Thereafter, SCF (10 ng/ml) was added for 15 min. In inhibition experiments, cells were then incubated with PKC412, imatinib, or control medium for 4 h. Immunoprecipitation (IP) and Western blotting were performed as described (28). For IP, lysates from 106 cells were incubated with anti-KIT Ab 1C1 and protein G-Sepharose beads (Amersham Biosciences) in IP buffer (50 mM Tris (pH 7.4), 150 mM NaCl, 100 mM NaF, and 1% Nonidet P-40) at 4°C overnight. Beads were then washed three times in IP buffer. Lysates as well as immunoprecipitates were separated under reducing conditions by 7.5% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Amersham Biosciences). Immunoblotting was performed using the anti-KIT Ab 1C1 and anti-pTyr Ab 4G10, as well as Abs against phosphorylated or total Akt or ERK (Cell Signaling Technology). In each case, at least three independent Western blot experiments were performed. Protein expression levels were quantified by densitometry using the EASY Win32 software (Herolab).

EMSA

STAT DNA-binding activity was analyzed as described (22). Ton.Kit.wt and Ton.Kit.D816V cells were kept in control medium or were cultured in the presence of doxycycline (1 μg/ml) for 24 h. Then, doxycycline-treated cells were stimulated with SCF (100 ng/ml) for 15 min or were left untreated. Extracts were analyzed using blunt-ended annealed oligonucleotides. For STAT5 analysis, the proximal STAT-binding element (5′-AGGGAACATTCAAAATCTGC-3′) of the bovine β-casein promoter was employed. Binding reactions were performed by incubating 10,000 cpm of radiolabeled probe with cell lysates (20 μg) for 30 min. For supershift reactions of STAT-containing complexes, 2 μg of Abs specific for the C-terminal transactivation domains of STAT1 (M22; Santa Cruz Biotechnology) and STAT5 (C-17; Santa Cruz Biotechnology) was added before EMSA was performed. Samples were separated by electrophoresis through 6% native polyacrylamide gels and analyzed by autoradiography.

Immunostaining and RIA

Immunohistochemistry was performed on paraffin-embedded, formalin-fixed bone marrow biopsy sections using the indirect immunoperoxidase staining technique as described previously (7). Endogenous peroxidase was blocked by methanol/H2O2 before sections were incubated with various Abs. The following Abs were used: anti-tryptase G3 (Chemicon; working dilution 1/5000), anti-Kit (Dako; working dilution 1/500), and anti-Ki67 (Dako; working dilution 1/500). The anti-tryptase Ab G3 was applied on non-pretreated bone marrow sections. For all other stainings, slides were pretreated by microwave oven. After washing, slides were incubated with biotinylated horse anti-mouse or goat anti-rabbit IgG for 30 min, washed, and exposed to avidin-biotin-peroxidase complex for 30 min. A combination of 0.1% 3,3′-diaminobenzidine (DAB) was used as chromogen. Slides were counterstained in Mayer’s hemalum and mounted. Immunocytochemistry was performed on cytospin preparations of BaF3 cells as described (21) using a polyclonal rabbit anti-KIT Ab (Dako). Cytospin slides were incubated with
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the primary Ab for 60 min at room temperature, washed, and thereafter exposed to biotinylated goat-anti-rabbit IgG (BioCarta) for 30 min. As chromogen, streptavidin-alkaline-phosphatase complex (BioCarta) was used. Antibody reactivity was made visible using Neofuchsin (Nichirei). Slides were counterstained in Mayer’s hemalaun and mounted.

In selected experiments, flow cytometry was performed on a FACSscan (BD Biosciences) using an Ab against murine ICAM-1 (CD54, BD Biosciences). Histamine was measured by a commercial RIA (Innomettech).

Gene expression profiling

Preparation of cRNA, hybridization to the murine U74Av2 GeneChip set (Affymetrix), and scanning of arrays were conducted according to the manufacturer’s protocols (http://www.affymetrix.com). Images were analyzed with GeneChip software (Affymetrix, MAS 5.0). Changes in gene expression were calculated as ratio of doxycycline-treated vs medium-treated cells.

Real-time PCR

RNA was isolated using the RNeasy Kit (Qiagen), and cDNA was synthesized using Moloney murine leukemia virus reverse transcriptase (Invitrogen) and random primers (Invitrogen) according to the manufacturer’s instructions. mRNA levels were quantified on a 7900HT Fast Real-Time PCR System (Applied Biosystems) using iTaq SYBR Green Supermix with ROX (Bio-Rad) and were normalized to β-actin. Results were expressed as ∆∆CT values (∆∆CT = (CTmRNA Sample) − (CTβ-actin Sample)) or as “fold of control”. Primer sequences are available on request.

Mice

To determine the transforming capacity of KIT D816V in vivo, a tumor model using nude mice as well as a bone marrow transplantation model using C57BL/6 mice deficient for Rag2 and the common γ-chain, were applied. Mice were housed at the Biomedical Research Institute, Medical University of Vienna. Additionally, we used KIT D816V transgenic mice described recently (19). All animal studies were approved by the local institutional review committee for animal research. Doxycycline (400 μg/ml) was changed every 3 days. Mice were housed at the Biomedical Research Institute, Medical University of Vienna. KIT D816V transgenic mice were established. In most experiments, clone Ton.Kit.D816V.27 cells were used. As control, BaF3 cells with inducible expression of wt KIT after exposure to doxycycline remained viable, and that only a smaller percentage of these cells (10–30%) entered apoptosis (not shown). Expression of KIT D816V did not substitute for IL-3 as growth-enhancing factor in these cells. In the present study, BaF3 cells with doxycycline-inducible expression of KIT D816V (Ton.kit.D816V) were used to characterize the effects of this mutant on growth (Ton.B210 cells with inducible expression of BCR/ABL served as control cells). However, expression of KIT D816V did not substitute for IL-3 as a growth-promoting stimulus in BaF3 cells, contrasting with the effect of BCR/ABL (Fig. 2A). Additionally, KIT D816V did not increase, but it even decreased the proliferation of Ton.kit.D816V cells in the presence of cytokine (IL-3; 3 ng/ml, IL-6; 50 ng/ml, SCF; 50ng/ml) for 48 h as described (30). The percentage of transduced progenitor cells was determined by FACS analysis 48 h after the transduction procedure using the Biotin-Conjugated Mouse Lineage Panel (BD Biosciences) and Abs against kit (PC-Cy5-conjugated; eBioscience) and sca-1 (PC-Cy7 conjugated, clone D7; BD Biosciences). Progenitor cells were defined as lin−, GFP+, sca-1+, and c-kit−. Each mouse received 105 progenitor cells transduced with KIT D816V (five animals) or (as a control) with BCR/ABL (three animals). Two days after transplanation, one animal of each group was sacrificed and engraftment of GFP-positive cells in the bone marrow was verified by FACS analysis.

Results

Generation and characterization of BaF3 cells with doxycycline-inducible expression of KIT D816V (Ton.Kit.D816V)

Three BaF3 cell clones with conditional expression of KIT D816V were established. In most experiments, clone Ton.Kit.D816V.27 was used. As control, BaF3 cells with inducible expression of wt KIT were employed. As shown in Fig. 1A, expression of KIT can be induced by addition of doxycycline (1 μg/ml) in Ton.Kit.D816V cells as well as in Ton.kit.wt cells. The presence of the KIT D816V mutation in Ton.kit.D816V cells was confirmed by RFLP (Fig. 1B). As expected, KIT D816V was found to be constitutively tyrosine phosphorylated, whereas wt KIT was phosphorylated only in the presence of SCF (100 ng/ml) (Fig. 1C). In line with the known pharmacologic response profile of the mutant, the KIT-targeting TK inhibitor PKC412 (31) was found to coun-

FIGURE 1. Establishment of BaF3 cell lines with conditional expression of wt KIT and KIT D816V. A, Ton.Kit.D816V.27 cells and Ton.kit.wt cells were kept in control medium (−Dox) or doxycycline (1 μg/ml (+ Doxy)) for 24 h. After incubation, cells were spun on cytofilm slides and analyzed by indirect immunofluorescence staining technique using an anti-KIT Ab. B, Ton.Kit.D816V cells (clones 3, 6, and 27) and Ton.kit.wt cells (wt) were cultured in the presence of doxycycline (1 μg/ml) for 24 h. Thereafter, the presence or absence of the D816V mutation was determined by RFLP as described in Materials and Methods. C, Ton.kit.wt cells and Ton.Kit.D816V.27 cells were incubated in control medium or doxycycline (Doxy) for 24 h, and then were kept with or without SCF (100 ng/ml) for 15 min at 37°C. After immunoprecipitation (IP) for KIT, lysates were split and subjected to immunoblotting (IB) using anti-p-Tyr (p-tyr) or anti-KIT Abs. The blot shows one representative experiment; identical results were obtained in two other independent experiments. D, Ton.Kit.D816V.27 cells were grown in the absence or presence of doxycycline for 24 h and were then treated with PKC412 (200 nM to 1 μM), imatinib (1 μM), or were kept in control medium for 4 h. Phosphorylation of KIT was determined as described above. One typical experiment (out of three independent experiments with identical results) is shown.

teract phosphorylation of KIT D816V in Ton.kit.D816V cells, whereas imatinib showed no effects (Fig. 1D).

Conditional expression of KIT D816V does not promote growth of BaF3 cells

BaF3 cells are factor-dependent cells that undergo apoptosis on withdrawal of IL-3. Growth-promoting oncogenes such as BCR/ABL can substitute for IL-3 as growth-enhancing factor in these cells. In the present study, BaF3 cells with doxycycline-inducible expression of KIT D816V (Ton.kit.D816V) were used to characterize the effects of this mutant on growth (Ton.B210 cells with inducible expression of BCR/ABL served as control cells). However, expression of KIT D816V did not substitute for IL-3 as a growth-promoting stimulus in BaF3 cells, contrasting with the effect of BCR/ABL (Fig. 2A). Additionally, KIT D816V did not increase, but it even decreased the proliferation of Ton.kit.D816V cells in the presence of cytokine (IL-3; SCF, or a combination of both cytokines (Fig. 2B). The same results were obtained when lower cytokine concentrations (10 ng/ml, 1 ng/ml) were applied. Combined annexin V/propidium iodide staining showed that most cells exposed to doxycycline remained viable, and that only a smaller percentage of these cells (10–30%) entered apoptosis (not shown).
To investigate the effects of KIT D816V on in vivo growth of BaF3 cells, Ton.Kit.D816V cells were injected subcutaneously into nude mice and these mice were kept with drinking water containing doxycycline. As expected from our in vitro results, none of these mice developed tumors (Fig. 2D). As a positive control, BaF3 cells with doxycycline-inducible expression of BCR/ABL (Ton.B210 cells) were also examined (Fig. 2D). In a next step, we sought to confirm these findings using a bone marrow transplantation model. For this purpose, Rag2−/−γc−/− mice were transplanted with KIT D816V-transduced or BCR/ABL-transduced bone marrow. As expected, BCR/ABL-transplanted mice developed a lethal leukemia within 6 wk, with substantial leukemic infiltration of the blood, bone marrow, and spleen (Fig. 2E). In contrast, no GFP+ cells could be detected in hematopoietic tissues in KIT D816V-transplanted mice (Fig. 2E). Finally, KIT D816V transgenic mice developed focal accumulations of MC in various organs including the spleen, but they did not develop aggressive MC disorders, confirming our previous data. Together, these data show that KIT D816V does not induce neoplastic growth of hematopoietic progenitor cells.

Inducible expression of KIT D816V cannot substitute for the growth-promoting effects of GM-CSF in human UT7 cells

To investigate the biologic effects of KIT D816V in human myeloid cells, we established a UT7 cell line with doxycycline-inducible expression of KIT D816V (Fig. 3A). As for BaF3 cells, UT7 cells are strictly cytokine-dependent and undergo apoptosis in the absence of GM-CSF within 2 days (24). Confirming our data obtained with BaF3 cells, conditional expression of KIT D816V in UT7 cells did not promote growth or viability in the absence of GM-CSF (Fig. 3B). Interestingly, expression of KIT D816V was found to slightly decrease the proliferation rate of UT7 cells—a result that was also obtained with Ton.Kit.D816V cells (Fig. 2B). In contrast to KIT D816V, expression of BCR/ABL in UT7 cells was found to confer factor-independent growth. These data provide further evidence that KIT D816V does not promote growth of neoplastic hematopoietic/myeloid cells.

Inducible expression of KIT D816V activates a unique pattern of signal transduction molecules in BaF3 cells

Despite the observation that KIT D816V is not a growth-promoting oncoprotein, we asked whether the mutant induces activation of signal transduction molecules and whether KIT D816V-dependent signaling differs from signaling cascades activated by the wt
Akt and total ERK, respectively. Densitometric quantification showed immunoblotting using Abs against phosphorylated forms (p-) or total 37°C. Phosphorylation of Akt and ERK (p42/44) was determined by cells were incubated with or without SCF (100 ng/ml) for 15 min at the absence or presence of doxycycline (Doxy) for 24 h at 37°C. Then, KIT receptor. As shown in Fig. 4, A and B, Ton.Kit.D816V.27 and Ton.Kit.wt cells were kept in the absence or presence of doxycycline (Dox) for 24 h at 37°C. Then, cells were incubated with or without SCF (100 ng/ml) for 15 min at 37°C. Phosphorylation of Akt and ERK (p42/44) was determined by immunoblotting using Abs against phosphorylated forms (p-) or total Akt and total ERK, respectively. Densitometric quantification showed 68 ± 14-fold induction of pAkt in Ton.Kit.wt cells in the presence of doxycycline and SCF, whereas pAkt was induced 127 ± 51-fold (in the presence of doxycycline) and 129 ± 36-fold (in the presence of doxycycline and SCF) in Ton.Kit.D816V cells (three independent experiments, means ± SD). C, Ton.Kit.wt and Ton.Kit.D816V.27 cells were kept in control medium (0) or were cultured in the presence of doxycycline (1 μg/ml) for 24 h. Then, doxycycline-treated cells were stimulated with SCF (100 ng/ml (Dox) + SCF)) for 15 min or were left untreated (Dox). Extracts were analyzed using blunt-ended annealed oligonucleotides. For supershift reactions of STAT-containing complexes, Abs specific for the C-terminal transactivation domains of STAT1 and STAT5 were added before EMSA was performed. One out of three independent experiments is shown.

**FIGURE 4.** Effects of the D816V mutation on signaling of the KIT receptor. A and B, Ton.Kit.D816V and Ton.Kit.wt cells were kept in the absence or presence of doxycycline (Dox) for 24 h at 37°C. Then, cells were incubated with or without SCF (100 ng/ml) for 15 min at 37°C. Phosphorylation of Akt and ERK (p42/44) was determined by immunoblotting using Abs against phosphorylated forms (p-) or total Akt and total ERK, respectively. Densitometric quantification showed 68 ± 14-fold induction of pAkt in Ton.Kit.wt cells in the presence of doxycycline and SCF, whereas pAkt was induced 127 ± 51-fold (in the presence of doxycycline) and 129 ± 36-fold (in the presence of doxycycline and SCF) in Ton.Kit.D816V cells (three independent experiments, means ± SD). C, Ton.Kit.wt and Ton.Kit.D816V.27 cells were kept in control medium (0) or were cultured in the presence of doxycycline (1 μg/ml) for 24 h. Then, doxycycline-treated cells were stimulated with SCF (100 ng/ml (Dox) + SCF)) for 15 min or were left untreated (Dox). Extracts were analyzed using blunt-ended annealed oligonucleotides. For supershift reactions of STAT-containing complexes, Abs specific for the C-terminal transactivation domains of STAT1 and STAT5 were added before EMSA was performed. One out of three independent experiments is shown.

**KIT D816V** but not wt KIT induces cluster formation in BaF3 cells

The observation that KIT D816V, albeit showing no growth-promoting effects, can induce specific signaling prompted us to screen for biologic effects that the mutant exerts in BaF3 cells. Since SM is associated with focal accumulation of MC in hematopoietic tissues, we first examined whether KIT D816V induces cluster formation. Indeed, KIT D816V was found to induce the formation of large clusters of Ton.Kit.D816V cells (Fig. 5A). Cluster formation was observed within 12 h after the addition of doxycycline. As expected, the KIT D816V-induced cluster formation in Ton.Kit.D816V cells was inhibited dose-dependently by PKC412, but it was not inhibited by imatinib (Fig. 5B). No cluster formation was observed in doxycycline-exposed Ton.Kit.wt cells in the presence of SCF. To characterize the signal transduction pathways involved in KIT D816V-dependent cluster formation, we applied pharmacologic inhibitors of MEK (PD98059) and PI3K (LY294002). In these experiments, we found that inhibition of MEK abolishes clustering of Ton.Kit.D816V cells in the presence of doxycycline, whereas the PI3K-targeting drug LY294002 did not show an inhibitory effect on cluster formation (Fig. 5C). In control experiments, LY294002 inhibited the phosphorylation of Akt, and PD98059 inhibited the phosphorylation of ERK in KIT D816V-expressing BaF3 cells (not shown). To investigate a potential role of STAT5 in KIT D816V-induced cell–cell adhesion, two different dn STAT5 constructs (STAT5aΔ479 or STAT5bΔ754) were expressed in Ton.Kit.D816V cells. However, although expression of these dn STAT5 constructs inhibited KIT D816V-induced phosphorylation of STAT5, no effect on cluster formation in the presence of doxycycline was observed (not shown). These data suggest that KIT D816V induces cluster formation of BaF3 cells through a MEK-dependent pathway.

**KIT D816V induces synthesis of histamine in BaF3 cells**

Since expression of KIT D816V was consistently associated with decreased cell growth, we asked whether this oncoprotein would induce differentiation rather than proliferation in hematopoietic progenitors. To test this hypothesis, we first examined the morphology of Ton.Kit.D816V cells after exposure to doxycycline (with or without SCF or additional cytokines: IL-3, IL-4, IL-6). As assessed by light microscopy, a few doxycycline-exposed Ton.Kit.D816V cells were found to display metachromatic granules when stained with Wright-Giemsa, although the numbers of granules was low and the cells remained in a blast stage of differentiation. In these experiments, we found that KIT D816V induces cluster formation of BaF3 cells through a MEK-dependent pathway.

KIT D816V induces de novo synthesis of histamine in Ton.Kit.D816V cells

Since expression of KIT D816V was consistently associated with decreased cell growth, we asked whether this oncoprotein would induce differentiation rather than proliferation in hematopoietic progenitors. To test this hypothesis, we first examined the morphology of Ton.Kit.D816V cells after exposure to doxycycline (with or without SCF or additional cytokines: IL-3, IL-4, IL-6). As assessed by light microscopy, a few doxycycline-exposed Ton.Kit.D816V cells were found to display metachromatic granules when stained with Wright-Giemsa, although the numbers of granules was low and the cells remained in a blast stage of differentiation. In these experiments, we found that KIT D816V induces cluster formation of BaF3 cells through a MEK-dependent pathway.

Next, we asked whether these cells display any biochemical signs of differentiation. In these experiments, we found that KIT D816V induces de novo synthesis of histamine in Ton.Kit.D816V cells (Fig. 6B). Moreover, KIT D816V was found to promote the expression of HDC mRNA in these cells (Fig. 6C, left panel). As expected, PKC412 (but not imatinib) was found to inhibit KIT D816V-induced synthesis of histamine (not shown). The wt form of KIT (in the presence of SCF) was also found to induce HDC mRNA expression, but the effect was less pronounced compared with KIT D816V (Fig. 6C, right panel). Other oncoproteins such as BCR/ABL (Fig. 6B) or RasG12V (not shown) did not induce synthesis of histamine to the same extent compared with KIT D816V. To characterize signal transduction pathways involved in
KIT D816V-associated MC differentiation, pharmacologic inhibitors of MEK and PI3K as well as dn STAT5 constructs were applied. In these experiments, we found that inhibition of both the Ras/Raf/MEK/ERK pathway (by PD98059) and the PI3K/Akt pathway (by LY294002) decreased the production of histamine in KIT D816V-expressing BaF3 cells (Fig. 6D). In line with these data, PD98059 and LY294002 were both found to counteract KIT D816V-induced expression of HDC (Fig. 6E). Expression of dn STAT5 constructs in Ton.KIT.D816V cells had no significant effect on expression of HDC mRNA (Fig. 6E). Taken together, these data suggest that KIT D816V induces...
expression of HDC and histamine synthesis in BaF3 cells through a signaling network involving MEK and PI3K. Finally, we found that also in UT7 cells, KIT D816V can promote the formation of histamine (not shown).

**KIT D816V induces expression of a number of mast cell differentiation and adhesion-related Ags in BaF3 cells**

We next performed gene chip analysis to define the profile of MC Ags expressed in Ton.Kit.D816V cells in response to KIT D816V. In these experiments, KIT D816V was found to promote expression of a number of important MC adhesion- and MC differentiation-associated Ags including IL4Ra, mouse mast cell protease 5 (MMP5), microphthalmia transcription factor (MITF), ICAM-1, and lysosyme-associated protein 3 (LAMP-3) (Fig. 7A and Table I). In contrast, however, KIT D816V did not induce expression of late-stage MC differentiation Ags such as FcεRI. Other MMP5s (MMP1/9, 2, 4, 6, 7, 8) were not expressed in BaF3 cells. Another interesting observation was that proliferation-associated Ags such as Ki-67 and the transferrin receptor CD71 were found to be down-regulated by KIT D816V (Table I). Up-regulation of these Ags by KIT D816V was confirmed by quantitative PCR (qPCR) (Fig. 7B). KIT D816V-induced up-regulation of ICAM-1 on BaF3 cells was also demonstrable by flow cytometry (Fig. 7C).

Together, our results suggest that KIT D816V regulates a number of genes involved in cell–cell adhesion and MC differentiation, but it down-regulates proliferation-associated Ags.

To compare the effects of KIT D816V in our cell line model with the effects of KIT-induced gene expression in normal (non-neoplastic) MC progenitors, mouse bone marrow was stimulated with SCF (100 ng/ml), and the expression of MC differentiation Ags was quantified by qPCR (Table II). Interestingly, we found that SCF leads to a rapid and substantial up-regulation of MMP5 and also induces the expression of HDC in mouse bone marrow cells within 48 h (Table II). However, other MMP5s (i.e., MMP4 and MMP7) or late-stage MC differentiation Ags including the FcεRI were only expressed at later time points, that is, after seven days of culture in the presence of SCF, when these cells were found to acquire mast cell granules (Table II). These data, that is, gene expression profiles observed in early SCF-driven mast cell progenitors (day 2), are in good agreement with gene expression patterns induced by KIT D816V in our BaF3 cell line model.

**Identification of clinical correlates and signatures of KIT D816V in patients with mastocytosis**

To define the specific consequences and features associated with the transforming effects of KIT D816V in neoplastic cells in a human model, we compared histological, phenotypic and biochemical markers in patients with KIT D816VISM with that in patients suffering from KIT D816V but tryptase-“neoplasm, that is, myelomastocytic leukemia (MML) and tryptase- “AML. In patients suffering from KIT D816VISM, MC were found to form clusters and aggregates in tryptase-stained bone marrow sections, whereas no MC clusters were found in patients with D816V-MML (Fig. 8A). In MC clusters, the tryptase-“ neoplastic cells were found to coexpress several MC-differentiation Ags including HDC and CD63, whereas the proliferation-associated Ag Ki67 was not detectable (Fig. 8A and Table III). We also were unable to

<table>
<thead>
<tr>
<th>Table I. KIT D816V-induced regulation of mRNA expression (selected genes) in BaF3 cells determined by GeneChip analysis</th>
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</thead>
<tbody>
<tr>
<td><strong>Experiment 1</strong></td>
</tr>
<tr>
<td>Up-regulated gene (-fold increase)</td>
</tr>
<tr>
<td>HDC</td>
</tr>
<tr>
<td>MMP5</td>
</tr>
<tr>
<td>IL-4Ra/CD124</td>
</tr>
<tr>
<td>ICAM-1/CD54</td>
</tr>
<tr>
<td>CD53</td>
</tr>
<tr>
<td>LAMP-3/CD63</td>
</tr>
<tr>
<td>CD164</td>
</tr>
<tr>
<td>IL-6</td>
</tr>
<tr>
<td>IL-10</td>
</tr>
<tr>
<td>IL-15</td>
</tr>
<tr>
<td>IL-15Ra</td>
</tr>
<tr>
<td>IL-12Rb1</td>
</tr>
<tr>
<td>IL-17R</td>
</tr>
<tr>
<td>STAT1</td>
</tr>
<tr>
<td>SOCS-1</td>
</tr>
<tr>
<td>SOCS-3</td>
</tr>
<tr>
<td>MITF</td>
</tr>
<tr>
<td>PSG</td>
</tr>
<tr>
<td>Down-regulated gene (-fold decrease)</td>
</tr>
<tr>
<td>CD71</td>
</tr>
<tr>
<td>Ki67</td>
</tr>
</tbody>
</table>

* U74Av2 GeneChip analysis was performed on Ton. Kit. D816V-27 cells exposed to control medium or doxycycline for 24 h. Gene expression levels were determined in two independent experiments (1 and 2). SOCS, Suppressor of cytokine signaling; PSG, proteoglycan secretory granule.
was noted (Fig. 8).

Next, we analyzed serum total tryptase levels in our patients over time. Tryptase levels are known to correlate with the body burden of neoplastic MC in SM and thus can be employed as an indirect marker of MC proliferation (32–34). In these analyses, we found that patients with KIT D816V SM display remarkably high serum tryptase levels for many years, even if serum tryptase levels are very high such as in smoldering SM (Fig. 8B). By contrast, in patients with tryptase-negative acute myeloid leukemia or MC leukemia, a more or less rapid increase in tryptase was noted (Fig. 8B). Taken together, these data show that KIT D816V SM is characterized by differentiation and cluster formation, but not proliferation of MC, contrasting with other tryptase-positive neoplasms.

**Discussion**

The tyrosine kinase receptor KIT and its ligand SCF are critically involved in the regulation of differentiation and maturation of MC (35–37). The KIT mutation D816V is detectable in most patients with SM (11–13) and is associated with ligand-independent autonomous kinase activity (16). Therefore, this mutation has been implicated in malignant growth of MC and in the pathogenesis of SM (16–18). However, the mutation occurs not only in aggressive SM but also in most patients (>80%) with ISM, a disease variant without progression and with normal life expectancy (13, 14). This prompted us to revisit the oncogenic potential of this KIT mutation by using BaF3 cells with inducible expression of KIT D816V as well as KIT D816V-transgenic mice. The results of our study show that KIT D816V promotes cluster formation and MC differentiation in BaF3 cells as well as in transgenic mice without inducing a substantial proliferation in MC. Based on these data we think that the effects of this KIT mutation can explain the clinical picture and course of ISM, but cannot explain the clinical course of aggressive SM or MCL.

So far, most studies analyzing mutated KIT in the context of mastocytosis in cell line systems (mostly BaF3) have employed murine KIT D814V (17, 18) or have used human KIT D816V in combination with other oncogenes (38, 39). In several studies, BaF3 cells that had been preselected for growth advantage were used (40, 41). In this study we thought that such cell line models would not be an optimal tool for the evaluation of growth-promoting effects of the KIT mutant. Therefore, we generated BaF3 cell lines with doxycycline-inducible expression of KIT D816V. The precommitted cell line BaF3 was used because 1) SM is known to arise from uncommitted hematopoietic progenitors, 2) BaF3 cells lack oncogenic hits and undergo apoptosis in the absence of IL-3, 3) BaF3 is a suitable and established model to investigate effects of oncogenes expressed by myeloid neoplasms (20, 21, 29, 42, 43), and 4) despite the fact that IL-3 is a major MC differentiation factor in the mouse system, BaF3 cells (with or without IL-3) do not exhibit MC-related Ags by gene chip profiling (precommitted progenitor).

The first results obtained with these newly generated Ton.Kit cell lines were somehow disappointing in that doxycycline-induced expression of KIT D816V was not associated with enhanced growth regardless of the condition tested. In fact, KIT D816V neither promoted cell growth in the presence of IL-3 nor SCF nor did the mutant substitute for IL-3 as a growth-promoting factor.

### Table II. Comparison of SCF-induced effects in mouse bone marrow cells with KIT D816V-induced effects in BaF3 cells on expression of differentiation-associated Ags

<table>
<thead>
<tr>
<th>Gene Product</th>
<th>Mouse Bone Marrow (day 2)</th>
<th>Mouse Bone Marrow (day 7)</th>
<th>Ton.Kit.D816V (day 2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HDC</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MMCP5</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>MMCP4</td>
<td>NE</td>
<td>+</td>
<td>NE</td>
</tr>
<tr>
<td>MMCP7</td>
<td>–</td>
<td>+</td>
<td>NE</td>
</tr>
<tr>
<td>MMCP8</td>
<td>–</td>
<td>+</td>
<td>NE</td>
</tr>
<tr>
<td>FceRα</td>
<td>–</td>
<td>+</td>
<td>NE</td>
</tr>
</tbody>
</table>

* Results represent relative mRNA expression levels quantified by real-time PCR.

### Table III. Expression of differentiation- and proliferation-associated antigens in neoplastic mast cells in patients with systemic mastocytosis

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Normal MC</th>
<th>MC in ISM</th>
<th>Rapidly Proliferating MCL</th>
<th>Tryptase + AML</th>
</tr>
</thead>
<tbody>
<tr>
<td>KIT</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+/-</td>
</tr>
<tr>
<td>HDC</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Histamine</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>LAMP-3/CD63</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+/-</td>
</tr>
<tr>
<td>IgER</td>
<td>+</td>
<td>+/-</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Proliferation associated</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+ +/-</td>
</tr>
<tr>
<td>Ki67</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+/-</td>
</tr>
</tbody>
</table>

*IgER, High-affinity immunoglobulin E receptor (*FceRI); Tf-R, transferrin receptor.*
contrast, wt KIT induced the proliferation of Ton.Kit.wt cells in the presence of SCF. The principal message of our paper, that KIT D816V induces differentiation rather than proliferation, is in line with the data of Ferrao et al. (44), but are in contrast to other previous observations made with cell lines stably expressing KIT D816V or expressing the murine equivalent KIT D814V (17, 18, 40, 41). With regard to cell lines stably expressing KIT mutants, the discrepancy may best be explained by additional growth-promoting factors or cooperating oncogenes (most of these cells were selected for growth advantage). In case of KIT D814V, the difference may also be explained by (species-specific) differences in the aggressiveness or transforming potential of these two mutants. The failure of BaF3 cells to grow better in the presence of KIT D816V in inducible cell line models might also explain why such cell line models had not been established and presented so far.

In a next step, we were interested to learn why KIT D816V is unable to promote growth of BaF3 cells and as to whether the mutant would induce other critical functions or features specific for MC development (and relevant for the pathogenesis of SM). As a first step, we were able to demonstrate that KIT D816V induces massive cluster formation in BaF3 cells in suspension as well as synthesis of histamine. Additionally, we were able to demonstrate by gene expression profiling and qPCR that the KIT D816V-induced histamine synthesis and cluster formation are accompanied by expression of several MC differentiation Ags including MITF, HDC, MMCP5, CD63, and IL-4R, as well as expression of several adhesion molecules including CD54/ICAM-1, CD53, and CD63. These observations are of particular interest, as in SM the major diagnostic feature (and World Health Organization criterion) common to all disease variants is cluster formation of MC (7–10). So far, it remains unknown, however, which of these adhesion molecules are most critical determinants for the abnormal adhesion and MC cluster formation in SM. In fact, all these molecules including CD63 and CD54/ICAM-1 are well known to be expressed on the surface of neoplastic MC in SM (45, 46) and, in most cases, neoplastic MC also display respective counterreceptors.

Another interesting observation was that KIT D816V down-regulates expression of several proliferation-linked Ags such as Ki67 or the transferrin receptor (CD71). This observation is in line with our immunohistochemical data obtained in patients with ISM in whom neoplastic MC did not express Ki67 and did not show signs of proliferation (mitosis). The down-regulation of proliferation-associated Ags may also explain the failure of the mutant to induce growth. Alternatively, the failure of KIT D816V to induce growth in BaF3 cells could be associated with induction of STAT1. The most likely scenario is that several of these factors act together to prevent a proliferative response. Lastly, enhanced differentiation/maturation is in general associated with a decrease in proliferation in normal and neoplastic cells, so that one could speculate that the KIT D816V-induced differentiation is closely linked with the decrease in proliferation. Whatever the explanation is, it appears that KIT D816V induces MC differentiation and cluster formation without inducing proliferation. To test this hypothesis in vivo, we injected BaF3 cells exhibiting KIT D816V into nude mice. However, no tumor formation was observed, whereas the BCR/ABL oncogene was found to induce BaF3 tumor formation in mice. The finding that KIT D816V is not a growth-promoting oncoprotein is also supported by a recently described mouse model (19). In this study, Zappulla et al. found that transgenic mice that express KIT D816V under the control of the chymase promoter do not develop aggressive mast cell neoplasms, but they develop indolent mast cell disorders after prolonged periods (19).

So far, little is known about signaling molecules and signal transduction pathways specifically involved in KIT D816V-induced differentiation or clustering (39). In the present study, we found that both KIT D816V as well as wt KIT (in the presence of SCF) induce activation of several signaling molecules including P3K, Akt, and ERK. A remarkable observation was that KIT D816V but not wt KIT induces phosphorylation of STAT5 as well as STAT1 in BaF3 cells. This is of particular interest since STAT5 has recently been described as a major regulator of MC growth and development (47, 48). In fact, STAT5-deficient mice exhibit MC deficiency similar to KIT-deficient mice (36). Additionally, neoplastic MC in SM show expression of constitutively phosphorylated STAT5 (49, 50). Based on these observations, it is tempting to speculate that STAT5 is a critical downstream molecule and regulator of KIT D816V-dependent functions of neoplastic MC in SM, and thus a potential new interesting target in this disease. An interesting aspect was that STAT5 was activated by KIT D816V but was not induced by wt KIT in BaF3 cells. This difference may have several explanations. First, KIT D816V may activate STAT5 preferentially in human cells but not (or less potently) in murine cells. Another possibility could be that wt STAT5 induces STAT5 activation in very immature stem cells but not in a precommitted progenitor such as BaF3. Lastly, the difference between wt KIT and KIT D816V in their capacity to activate STAT5 may be a BaF3-specific phenomenon. Whatever the reason is for the differential effects of the KIT variants in BaF3 cells, it recently has been described that primary neoplastic MC in patients with SM display activated STAT5 (49, 50), which was recently also confirmed in our studies (data not shown).

To study the potential role of signaling molecules in KIT D816V-induced histamine formation (differentiation) and clustering in BaF3 cells, pharmacologic inhibitors of MEK (PD98059) and of PI3K (LY294002) were applied. In these experiments, KIT D816V-dependent synthesis of histamine was suppressed by both inhibitors, whereas cluster formation was selectively inhibited by PD98059. The latter observation was quite unexpected as both wt KIT and KIT D816V induced the phosphorylation of ERK in our BaF3 cell line model, whereas only KIT D816V (but not wt KIT) induced cluster formation. One possible explanation may be that not only MEK activation, but also other signaling molecules (triggered by KIT D816V but not wt KIT) are required for the induction of cluster formation. An alternative hypothesis would be that MEK downstream signaling pathways differ when comparing KIT D816V-expressing and wt KIT-expressing cells. A role for STAT5 in KIT D816V-induced cluster formation of BaF3 cells could be excluded using a dominant STAT5 construct.

The observation that KIT D816V as a single “hit” is not sufficient to convert a premalignant cell into a malignant cell with major proliferative capacity, but is sufficient to induce MC differentiation and MC cluster formation in hematopoietic progenitors far enough to reach the biological and clinical picture of indolent mastocytosis, is a remarkable finding. To a degree this observation seems to contradict the established “multihit” theory of cancer development (51). On the other hand, ISM behaves as a “pre-neoplasm” rather than a malignant disease, and would simply be overlooked in most cases if patients would not exhibit the striking cutaneous features of mastocytosis (9). An important unresolved question in this regard is how the massive burden of MC in the tissues in patients with ISM can be explained in the absence of MC proliferation. Based on our data, this “paradox” is best explained by a constant differentiation of MC from a pool of uncommitted KIT D816V-bearing progenitors, which, in contrast to their progeny, may undergo (constant) proliferation. An alternative explanation would be an extremely long half-life of neoplastic MC, leading to their accumulation over time (52).
In summary, our data show that the SM-specific mutant KIT D816V induces MC differentiation and MC cluster formation without promoting MC proliferation in BaF3 cells as well as in mice. Based on these data, the effects of this mutant can explain the pathology and biologic behavior of the neoplastic clone in ISM, whereas additional (proliferation-enhancing) defects/shits may be responsible for the clinical picture and adverse course in patients with ASM or MCL.

Acknowledgments
We thank Dr. N. Komatsu (Jichi Medical School, Tochigi, Japan) for providing the pLV-tTR-KRAB plasmid.

Disclosures
The authors have no financial conflicts of interest.

References


