Chronically KIT-Stimulated Clonally-Derived Human Mast Cells Show Heterogeneity in Different Tissue Microenvironments

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Human mast cell precursors arise in the bone marrow and circulate to different tissue microenvironments, where they develop distinct phenotypes that may be characterized by differential expression of the serine protease, chymase. The growth and development of mast cells is stimulated by mast cell growth factor, which is also known as kit ligand because its obligate receptor is KIT, the protein product of the *c-KIT* proto-oncogene. The *in vivo* influence of the KIT-kit ligand axis on the phenotype of human mast cells has not been determined. We used immunohistochemistry to detect *in situ* expression of tryptase and chymase by mast cells of a patient with urticaria pigmentosa and aggressive systemic mastocytosis, whose pathologic mast cells are clonally derived and chronically stimulated by KIT because they all contain the same point mutation causing constitutive activation of KIT. Mast cells in both spleen and skin expressed tryptase, but only in the skin did a majority of mast cells express chymase. We conclude that chronic stimulation of the KIT-kit ligand axis does not irrevocably commit mast cells to a chymase-positive or chymase-negative phenotype. These findings suggest that factors other than kit ligand predominate in determining mast cell phenotype. ***Key words: mast cell heterogeneity/chymase/c-KIT/mastocytosis/mutation/oncogene. J Invest Dermatol 108:792–796, 1997***

Mast cells are derived from pluripotent CD34+ progenitor cells that originate in the bone marrow and circulate at low levels in the peripheral blood (Kirshenbaum *et al*, 1991, 1992). Mast cells proliferate and develop in different tissue microenvironments under the influence of a variety of factors (Aloe and Levi-Montalcini, 1977; Nakano *et al*, 1985; Tsai *et al*, 1991; Kirshenbaum *et al*, 1992; Longley *et al*, 1993). One of these factors is mast cell growth factor, which is also known as kit ligand because it is the obligate ligand for KIT, the protein product of the *c-KIT* proto-oncogene (Anderson *et al*, 1990; Huang *et al*, 1990; Zsebo *et al*, 1990; Mitsui *et al*, 1993). The KIT protein is a type III receptor tyrosine kinase expressed by mast cells and mast cell progenitors, and it stimulates mast cell growth, development, and differentiated functions including secretion (Anderson *et al*, 1990; Zsebo *et al*, 1990; Tsai *et al*, 1991; Columbo *et al*, 1992; Mitsui *et al*, 1993; Sperr *et al*, 1993).

Mast cells of humans and animals exhibit heterogeneity of a number of phenotypic characteristics that may be correlated with their anatomic location and functional state (Galli, 1990; Longley *et al*, 1995). These characteristics include their response to various histochemical stains (Maximow, 1905; Hardy and Westbrook, 1985; Enerbäck, 1986), the ultrastructural features of their cytoplasmic granules (Craig *et al*, 1988; Weidner and Austen, 1990), and the types of neutral proteases present within those granules (Irani *et al*, 1986). Two neutral proteases found in mast cell granules, tryptase and chymase, appear to be useful markers of different human mast cell subtypes (Craig *et al*, 1986; Irani *et al*, 1986). Essentially all mature human mast cells produce tryptase (Irani *et al*, 1986; Schwartz *et al*, 1987; Craig *et al*, 1988), but only a subpopulation of mast cells contain chymase (Irani *et al*, 1986; Craig *et al*, 1988). Human mast cells containing only tryptase have been called MC^{T}, and those containing both enzymes (chymase and tryptase) have been designated MC^{CT} (Irani *et al*, 1986). Whether kit ligand and other substances present in the microenvironment act on cells that are already committed to a particular phenotype or play a role in commitment to a particular phenotype (if such a commitment exists) has not been definitively determined.

We recently identified a clonal proliferation of mast cells present in both the skin and spleen of a patient with adult-onset urticaria pigmentosa and systemic mastocytosis (Longley *et al*, 1996). In the study reported herein, we show that although the neoplastic mast cells in this patient all contain a point mutation of the *c-KIT* proto-oncogene that is known to cause constitutive activation of KIT, the mast cells show heterogeneity in their expression of chymase in the different tissue microenvironments of skin and spleen. This demonstrates that human clonally derived mast cells can exhibit heterogeneity *in situ* with respect to serine protease production, and it shows that chronic stimulation via the KIT-kit

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Abbreviations: MC^{CT}, human mast cells containing chymase and tryptase; MC^{T}, human mast cells containing tryptase but not chymase; RT, reverse transcriptase.

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ligand axis does not cause permanent commitment to a particular mast cell phenotype.

MATERIALS AND METHODS

Immunohistochemistry Fresh tissue samples of skin and spleen were obtained from a 47-y-old man with a 7-y history of progressive urticaria pigmentosa and a 5-y history of progressive splenomegaly with hypersplenism. Samples of spleen and skin were snap-frozen in OCT embedding compound (Tissue-Tek, Miles Inc., Elkhart, IN) and liquid nitrogen, and 4-μm thick frozen sections cut and mounted on Probe-on glass slides (Fisher Scientific, Pittsburgh, PA). Immunoalkaline phosphatase staining for tryptase and chymase were performed using the Fisher Biotech Microprobe System and the APAAP detection system (Dako, Carpinteria, CA) using nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate as chromagen and nuclear Fast Red as a counterstain. The primary anti-tryptase and anti-chymase antibodies were murine monoclonal IgG1 antibodies purified from ascites (Chemicon International Inc., Temecula, CA), and used at 1 μg per ml. Incubation was for 1 h at room temperature.

Transmission Electron Microscopy Electron microscopy was performed on splenic tissue extracted from paraffin. Appropriate areas were excised and placed at 57°C for 30 min in xylene. The tissue was transferred to fresh xylene at room temperature (×2), rehydrated through graded alcohols from 100% ethanol to 50% ethanol, and washed in 0.1 M cacodylate buffer, pH 7.4, before being placed in 2.5% glutaraldehyde overnight at 4°C. The tissue was further processed by post-fixation in 2% osmium, dehydrated, and embedded in Taab Epon 812 (Marivac Ltd., Halifax, Nova Scotia, Canada). Ultrathin sections were cut on a LKB Ultrotome III (LKB Instruments, Rockville, MD), post-stained with uranyl acetate and bismuth subnitrate, and viewed with a Hitachi 7000 electron microscope (Hitachi Instruments, Inc., Elmsford, NY).

Sequence Analysis RNA was extracted from six 20-μm thick cryostat sections of frozen spleen and skin using guanidium (Longley *et al*, 1991). The RNA was treated with heparinase, 1 unit RNA per μg (Sigma Chemical Company, St. Louis, MO) for 2 h at room temperature (Tsai *et al*, 1995) before being reverse transcribed using random hexamers (2.5 pmol/μl) and M-MuLV reverse transcriptase (RT) (Boehringer Mannheim, Indianapolis, IN). Polymerase chain reaction (PCR) was then performed with AmphiTaq (Perkin-Elmer, Norwalk, CT) using primers corresponding to bases 2013–2035 (5′ GCCCACCCTGGTCATTACAGAAT) and 2594–2575 (5′ AAAATCCCATAGGACCAGAC) of c-*KIT* (Yarden *et al*, 1987) for 40 cycles for 1 min denaturation, 1 min annealing at 52°C, and 1 min extension at 72°C. Sequencing was carried out in the W. M. Keck Biotechnology Resource Laboratory at Yale University using individual cDNAs subcloned from multiple independent RT-PCRs as well as using gel-purified amplimers. Direct amplimer sequencing of genomic DNA derived from the patient by buccal wash was performed after PCR amplification of a portion of exon 17 of c-*KIT*, using primers flanking position 2468 (5′ TGTATTCACAGAGACTTGGC; 5′ GTTTCCTTTA-ACCACATAAT corresponding to bases 2383–2402 and 2505–2486) (Longley *et al*, 1991).

RESULTS

Splenic Mast Cells Express Tryptase but not Chymase Routine histology showed mast cells infiltrating the dermis in the skin samples and infiltrating the periarteriolar areas of the spleen, where there was extensive fibrosis with many of the mast cells assuming a spindled morphology **(Fig 1)**. Increased numbers of mast cells were seen scattered about the red pulp as well. Immunoalkaline phosphatase staining showed tryptase but not chymase expressed by most of the mast cells in the spleen **(Fig 2)**. Both immunoreactive chymase and tryptase, however, were associated with the mast cells infiltrating the skin **(Fig 3)**. Both tryptase and chymase were also present extracellularly in the vicinity of mast cells, perhaps reflecting mast cell degranulation and release of granule proteases. Essentially all of the mast cells infiltrating the skin and spleen also express KIT (data not shown).

Splenic Mast Cells Show Ultrastructural Features of "Tryptase Only" Mast Cells Splenic tissue that had been fixed in formalin and embedded in paraffin was the only material available for electron microscopic study in this case. Mast cells and mast cell granules were easily identified, and the majority of the granules showed scrolls associated with an amorphous, variably electron-dense matrix **(Fig 4)**. These findings are consistent with the less than optimal preservation of mast cell granules containing only tryptase, or with the presence of granules containing both tryptase and chymase. Many of the mast cells, however, showed a spectrum of granule substructures including both small and large granules and rope-like configurations, characteristic of MC^{T} granule solubilization and partial degranulation (Mirowski *et al*, 1990).

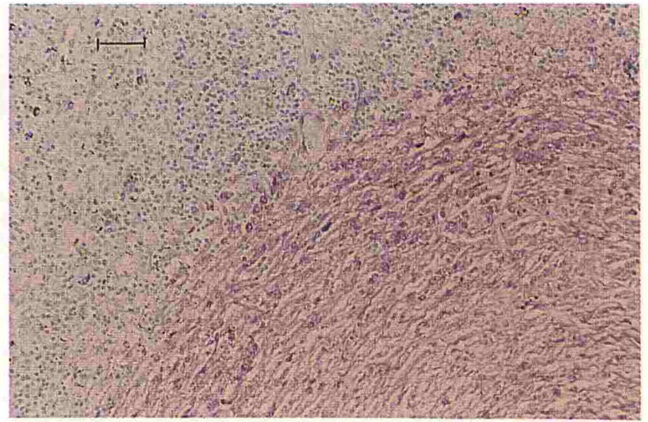

Figure 1. Mast cells infiltrate the spleen. Giemsa stain shows that most of the spindle- and polygonal-shaped cells within the fibrotic area of the spleen are mast cells. *Scale bar*, 50 μm.

Copy DNA Sequencing Shows Activating c-KIT Mutations in Skin and Spleen Sequencing of individual RT-PCR–derived cDNAs, subcloned independently from both skin and spleen, identified a transition point mutation (A → T) in exon 17 at position 2468, as well as the normal (nonmutated, wild type) sequence **(Fig 5)**. Sequencing of RT-PCR amplimers showed approximately equal representation of A and T at this position. Because these are RT-PCR–derived products, and because the vast majority of the cells expressing KIT in these samples are mast cells, it appears the mast cells in this patient contain one mutated and one wild type c-*KIT* allele. Sequencing of genomic DNA amplified from non-mast cell leukocytes and oral epithelial cells showed a normal (nonmutated, wild type) sequence (data not shown). These results indicate that the mutation is a somatic mutation, specifically expressed by mast cells in this patient. Since mast cells at both anatomic sites express the same mutation, they must all be derived from the same clone of chronically KIT-stimulated cells.

DISCUSSION

Our immunohistochemical studies identify both chymase and tryptase expression by essentially all of the mast cells in this skin lesion of mastocytosis, confirming a previous study showing a MC^{CT} phenotype for mast cells in cutaneous mastocytosis (Irani *et al*, 1990). In the spleen, however, the vast majority of mast cells had a MC^{T} phenotype by immunohistochemistry expressing only tryptase. There are several ultrastructural features that support this immunohistochemical phenotype of the splenic cells. First, there was at least focal preservation of well-formed scrolls within granules, consistent with the MC^{T} phenotype. Second, whereas MC^{CT} type granules tend to show less heterogeneity during solubilization, the splenic mast cells showed a spectrum of degranulation within individual cells, typical of MC^{T} type granules (Mirowski *et al*, 1990). Third, the rope-like configurations observed in the splenic mast cells of this patient are usually associated with degranulation of MC^{T} type mast cells (Mirowski *et al*, 1990). Arguably, the amorphous granule subcompartments observed in the majority of the cells could represent chymase-containing granule domains rather than suboptimally preserved tryptase domains, but the mast cells were chymase-negative by light microscopy and showed other ultrastructural characteristics of MC^{T} cells, including the presence of rope-like granules and heterogeneity of granule solubilization.

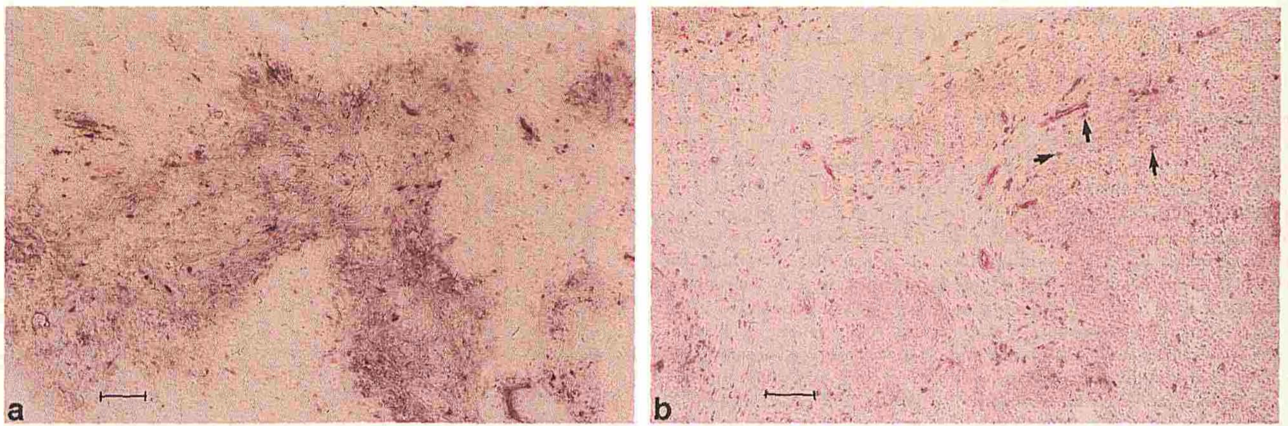

Figure 2. Most splenic mast cells express tryptase but not chymase. *(a)* Immunoalkaline phosphatase staining identifies immunoreactive tryptase in the majority of the mast cells of the spleen (nuclear fast red counterstain; *scale bar*, 100 μm). Note the association of the blue-gray reaction product with the spindled mast cells. *(b)* Staining for immunoreactive chymase shows only a few scattered positive mast cells, several of which are marked with ➡ (nuclear fast red counterstain; *scale bar*, 100 μm).

Therefore, the ultrastructural morphology of these cells supports the interpretation that MC^T were present in the splenic infiltrate.

Tryptase and chymase are both serine endoproteases; the former has specificity for trypsin-like substrates (Schwartz *et al*, 1981), and the latter has specificity for chymotrypsin-like substrates (Schechter *et al*, 1986). Most anatomic sites contain a mixture of MC^T and MC^{CT}, but MC^{CT} predominate in the skin, breast parenchyma, axillary lymph nodes, and bowel submucosa, and MC^T predominate in the lung and bowel mucosa (Craig *et al*, 1988; Weidner and Austen, 1990). Mast cells are rare in normal splenic tissue, and we are not aware of reports of a predominant phenotype associated with that organ. Because our results are derived from the study of splenic tissue derived from a single patient, they may not be relevant to other mastocytosis patients with splenic involvement or to normal splenic mast cells. In addition to chymase, MC^{CT} contain proteases that are not found in MC^T, and these two types of mast cells show differential activation by various biologic mechanisms and differential inhibition by drugs such as cromolyn (Galli, 1990; Schwartz, 1993). It has been suggested, therefore, that the two different mast cell subtypes may have significantly different functional roles and responses to therapeutic intervention.

In general, mast cell heterogeneity could represent the presence of separate sublineages or subclones of mast cells that have different phenotypic characteristics and that migrate to different anatomic locations. Alternatively, heterogeneity could be secondary to the influence of the microenvironment on mast cell phenotype. Studies of murine mast cells support the preeminence of microenvironmental influences on mast cell phenotype (Galli *et al*, 1982; Nakano *et al*, 1985; Galli, 1990; Tsai *et al*, 1991). These studies show that, depending on their localization *in vivo*, clonally derived mast cells and/or their progeny are able to acquire characteristics of both "connective tissue type mast cells" or "mucosal type mast cells," two murine mast cell types that are analogous but not identical to human MC^{CT} and MC^T, respectively, when injected into mast cell-deficient mice (Galli *et al*, 1982; Nakano *et al*, 1985; Galli, 1990; Tsai *et al*, 1991). Kit ligand is produced by fibroblasts, endothelial cells, and keratinocytes and circulates at relatively high levels and therefore is present in most tissues (Flanagan and Leder, 1990; Langley *et al*, 1993; Longley *et al*, 1993; Weiss *et al*, 1995). Injection of pharmacologic doses of kit ligand into rodents induces the accumulation of both types of mast cells in an anatomic distribution similar to that observed for mast cells in normal rodents, also suggesting that kit ligand stimulation does not preferentially influence the development of different mature mast cell phenotypes. Our data do not allow us to resolve this "nature" *versus* "nurture" quandary with respect to mast cell heterogeneity in humans because it is possible that a separate subclone of mast cells lacking chymase expression migrated preferentially to the spleen. Our observations do offer insight, however, into the effects of

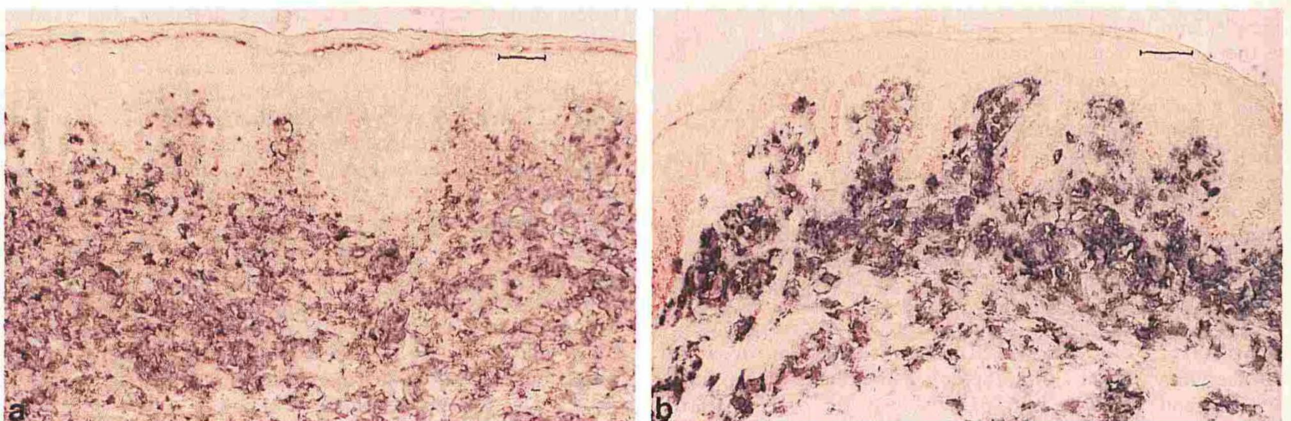

Figure 3. Dermal mast cells express both tryptase and chymase. *(a)* Blue-gray alkaline phosphatase reaction product indicates that most of the mast cells infiltrating the skin express immunoreactive tryptase (nuclear fast red counterstain; *scale bar*, 50 μm). *(b)* A similar pattern of immunoreactive chymase indicates that most of the cutaneous mast cells also express this protein (nuclear fast red counterstain; *scale bar*, 50 μm).

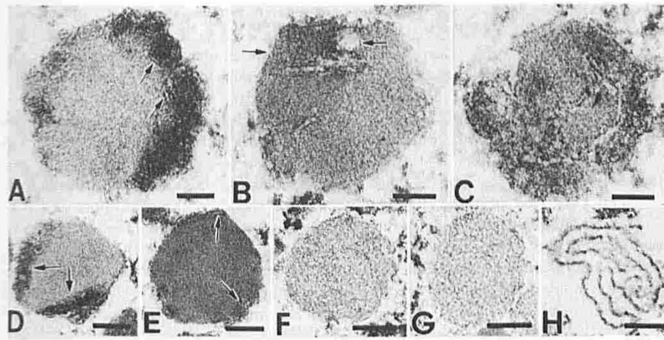

Figure 4. Splenic mast cells contain granules with ultrastructural features of "tryptase only" granules. Electron microscopy of mast cell granule morphology from involved spleen. The predominant granule phenotypes consisted of scrolls cut in transverse plane [*(A)* →], and longitudinal plane [*(B)* and *(D)*, →] juxtaposed with amorphous, variably electron-dense matrix. Occasional granules are largely composed of both poorly formed and well-formed scrolls *(C)*. A spectrum of granule substructures ranging from smaller granules with discrete scroll (→) and amorphous regions [*(D)* and *(E)*] to larger granules with less distinct electron-lucent matrices [*(F)* and *(G)*] and rope-like configurations *(H)* suggest solubilization and degranulation. *Scale bars, (A)–(C)* 0.1 μm; *(D)*, *(E)*, and *(H)* 0.2 μm; and *(F)* and *(G)* 0.4 μm.

chronic *in vivo* stimulation of human mast cells via the KIT-kit ligand axis.

We are unaware of any other reports describing the phenotype of human clonally derived mast cells in different tissue microenvironments. Our identification of a somatic point mutation of c-*KIT* in this patient (Longley *et al*, 1996) provided us with a convenient marker for his neoplastic mast cells and allowed us to document heterogeneity of chymase expression by mast cells of the same clone in different anatomic locations. The point mutation in this patient has been shown to cause constitutive phosphorylation and activation of KIT and to induce ligand-independent proliferation of cells *in vitro* (Furitsu *et al*, 1993; Tsujimura *et al*, 1994). Furthermore, the constitutive activation of KIT resulting from this mutation can transform a murine pro-B type Ba/F3 cell line so that it behaves aggressively *in vivo* (Tsujimura *et al*, 1994). Given the known ability of activated KIT to stimulate mast cell proliferation and our demonstration of its *in vivo* association with mast cells, it appears likely that this mutation caused mastocytosis in this patient and that these mast cells are chronically stimulated via KIT.

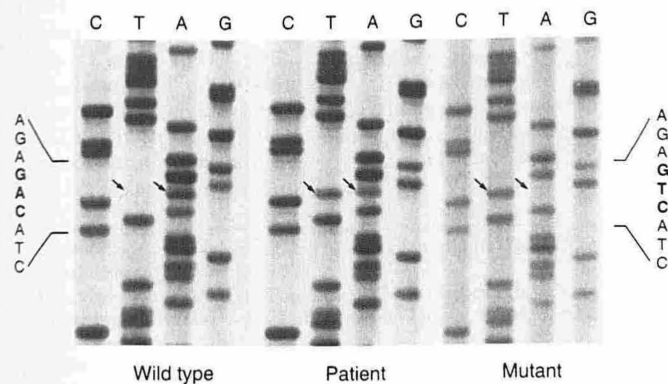

Figure 5. Mast cells are heterozygous for an activating c-*KIT* mutation. Comparison of cDNA sequences (bases 2464–2472) showing wild type c-*KIT* (*left* four lanes) and mutated c-*KIT* (*right* four lanes). Note A → T transition mutation at base 2468 in the mutated c-*KIT* sequence. The *middle* four lanes show both A and T at position 2468, indicating products of normal and mutated alleles present in approximately equal amounts.

The influence of the KIT-kit ligand axis on human mast cell heterogeneity has not been determined *in vivo* (Geissler *et al*, 1988; Anderson *et al*, 1990; Huang *et al*, 1990; Williams *et al*, 1990; Zsebo *et al* 1990). Human mast cells and their CD34+ precursors express KIT, and mast cells develop *in vitro* from kit ligand-stimulated CD34+ bone marrow and peripheral blood cells (Kirshenbaum *et al* 1991, 1992; Mitsui *et al*, 1993). *In vitro*, these kit ligand-stimulated cultured human mast cells express tryptase but do not express chymase unless other factors are introduced into the culture system (Kirshenbaum *et al*, 1992; Mitsui *et al*, 1993), suggesting that KIT activation may be permissive but not directive with regard to mast cell phenotype. In our patient, both the dermal MC^{CT} and the splenic MC^{T} express constitutively activated KIT protein but differentially express chymase, enabling us to conclude that *in vivo* chronic stimulation by KIT did not irrevocably commit human mast cells to one or the other lineage in this case. Thus, although the kit ligand-KIT axis may stimulate proliferation of mast cells and allow their differentiation, it appears unlikely that it directs that differentiation along a particular pathway.

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