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Transforming and Differentiation-Inducing Potential of Constitutively Activated c-*kit* Mutant Genes in the IC-2 Murine Interleukin-3-Dependent Mast Cell Line

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Two mutations of c-kit receptor tyrosine kinase (KIT), valine-559 to glycine (G559) and aspartic acid-814 to valine (V814), resulted in its constitutive activation. To examine the transforming and differentiation-inducing potential of the mutant KIT, we used the murine interleukin-3-dependent IC-2 mast cell line as a transfectant. The IC-2 cells contained few basophilic granules and did not express KIT on the surface. The KIT^{G559} or KIT^{V814} gene was introduced into IC-2 cells using a retroviral vector. KIT^{G559} and KIT^{V814} expressed in IC-2 cells were constitutively phosphorylated on tyrosine and demonstrated kinase activity in the absence of stem cell factor, which is a ligand for KIT. IC-2 cells expressing either KIT^{G559} or KIT^{V814} ($IC\text{-}2^{G559}$ or $IC\text{-}2^{V814}$ cells) showed factor-independent growth in suspension culture and produced tumors in nude athymic mice. In addition, $IC\text{-}2^{G559}$ and $IC\text{-}2^{V814}$ cells showed a more mature phenotype compared with the phenotype of the original IC-2 cells, especially after transplantation into nude mice. The number of basophilic granules and the content of histamine increased remarkably. KIT^{G559} and KIT^{V814} also influenced the transcriptional phenotype of mouse mast cell proteases (MMCP) in IC-2 cells. The expression of MMCP-2, MMCP-4, and MMCP-6 was much greater in $IC\text{-}2^{G559}$ and $IC\text{-}2^{V814}$ cells than in the original IC-2 cells. The results indicated that constitutively activated KIT had not only oncogenic activity but also differentiation-inducing activity in mast cells. (Am J Pathol 1996, 148:189–200)

The c-*kit* receptor tyrosine kinase (KIT) belongs to the same receptor tyrosine kinase (RTK) subfamily (type III RTK) as the receptors for platelet-derived growth factor (PDGF) and colony-stimulating factor-1 (CSF-1).[1,2] The type III RTK subfamily is characterized by the presence of five immunoglobulin-like repeats in the extracellular domain and an insert that splits the cytoplasmic kinase domain into adenosine triphosphate (ATP)-binding and phosphotransferase regions.[1–5] The kinase activity of KIT is tightly regulated by its ligand, stem cell factor (SCF), which is also called mast cell growth factor, *kit* ligand, or steel factor.[6–9] KIT-mediated signal transduction plays an important role in the proliferation, survival, and differentiation/maturation of mast cells.[10–16]

We found that KIT was constitutively activated by two point mutations of the c-*kit* proto-oncogene, the substitution of Val-560 to Gly (G560) and that of Asp-816 to Val (V816), in the human mast-cell leukemia cell line HMC-1.[17] The activating mutation in the corresponding Asp codon was also found in the P-815 mouse mastocytoma and the RBL-2H3 rat mast-cell leukemia cell lines.[18,19] When murine c-kit^{G559} or c-kit^{V814} mutants, corresponding to human c-kit^{G560} or c-kit^{V816} genes, were introduced into cells of the murine interleukin (IL)-3-dependent cell lines Ba/F3 (pro-B type) and FDC-P1 (myeloid type),

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the expression of KIT^{G559} or KIT^{V814} with constitutive tyrosine kinase activity not only abrogated the IL-3 requirement of the cells but also caused the cells to become tumorigenic in nude athymic mice.[20] However, the effects of the c-*kit*-activating mutations on the neoplastic growth and phenotypic change of mast cells themselves remain to be determined.

The murine IL-3-dependent mast-cell line IC-2 was derived from cultured mast cells (CMCs).[21] In the original description of the IC-2 cell line by Koyasu et al,[21] the expression of the high affinity immunoglobulin E receptor and the content of Alcian blue-positive granules containing histamine are described. In addition, the profile of surface antigens of the original IC-2 cell line is consistent with that of CMCs described by other investigators.[22–24] The IC-2 cells maintained in our laboratory were dependent on IL-3 as the original IC-2 cells, but there were few Alcian blue-positive granules. Moreover, the IC-2 cells maintained in our laboratory did not express KIT in detectable levels in contrast with normal CMCs. Because of these unique phenotypes, the IC-2 cells appeared useful as a transfectant with which to study the transforming and differentiation-inducing activities of the c-*kit* activating mutations. In this study, we introduced the c-*kit*^{G559} and c-*kit*^{V814} genes into IC-2 cells and examined the transforming activity of the mutant KIT. In addition, we investigated whether the constitutive activation of KIT increased the number of granules and the contents of histamine in IC-2 cells. Furthermore, because various proteases are the most definitive markers of mast cell phenotypes in mice,[25–31] rats,[32,33] and humans,[34,35] we studied how the constitutive activation of KIT influenced the transcriptional phenotypes of mast cell proteases in IC-2 cells.

Materials and Methods

Reagents

Recombinant murine (rm) SCF and rmIL-3 were gifts from the Kirin Brewery Co. Ltd. (Tokyo, Japan). Antiphosphotyrosine antibody,[36,37] a murine monoclonal antibody (MAb) generated against phosphotyramine, was supplied by Dr. B. Drucker (Oregon Health Sciences University, Portland, OR). Rat anti-mouse KIT (ACK2) MAb was given by Dr. S.-I. Nishikawa (Kyoto University, Kyoto, Japan).[38] Rabbit antiserum against a C-terminal peptide corresponding to the last 10 amino acids of the murine KIT was provided by Dr. D. E. Williams (Immunex Corporation, Seattle, WA).[7] G418 sulfate (geneticin) was purchased from GIBCO BRL (Grand Island, NY).

Cells and Mice

Pokeweed mitogen-stimulated spleen cell-conditioned medium (PWM-SCM) was prepared as described by Nakahata et al.[39] CMCs were obtained by culturing spleen cells of C57BL/6-+/+ mice with α-minimal essential medium (α-MEM, ICN Biomedicals, Costa Mesa, CA) containing 10% PWM-SCM and 10% fetal calf serum (FCS, Nippon Bio-Supply Center, Tokyo, Japan) (hereafter called α-MEM$_{PWM\text{-}SCM}$).[40] The IC-2 cell line was provided by Dr. I. Yahara (The Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan)[21] and maintained in α-MEM$_{PWM\text{-}SCM}$. Nude athymic mice of BALB/c-*nu/nu* were purchased from Japan SLC, Inc. (Hamamatsu, Japan) and kept under specific-pathogen-free conditions. All nude athymic mice were female and were 8 weeks of age at the time of cell injection.

Retroviral Transfer

Packaging cells producing pM5Gneo, pM5Gneo-KIT^{WT}, pM5Gneo-KIT^{G559}, or pM5Gneo-KIT^{V814} virus were prepared in our laboratory.[20] For gene transfer, IC-2 cells (10^6) were incubated on irradiated (30 Gy) subconfluent monolayers of virus-producing packaging cells for 48 hours in α-MEM$_{PWM\text{-}SCM}$. Infected cells were selected by culturing in α-MEM$_{PWM\text{-}SCM}$ containing G418 (1.0 mg/ml) for at least 3 weeks. The resulting IC-2 cells, which expressed pM5Gneo, pM5Gneo-KIT^{WT}, pM5Gneo-KIT^{G559}, or pM5Gneo-KIT^{V814} virus, were named IC-2^{VECTOR}, IC-2^{WT}, IC-2^{G559}, or IC-2^{V814} cells, respectively.

Flow Cytometry

Cells were incubated first with the ACK2 MAb at 4 C for 30 minutes, rinsed, then stained with fluorescein isothiocyanate-conjugated rabbit anti-rat immunoglobulin antibody (DAKO A/S, Glostrup, Denmark), and rinsed before analysis on a FACScan (Becton Dickinson, Los Angeles, CA).

Immunoprecipitation and Immunoblotting

Immunoblotting proceeded as described.[36,37,41,42] Briefly, after depleting serum and factors, cells (10^7) were suspended in 1 ml of α-MEM and exposed to 100 ng/ml of rmSCF for 15 minutes at 37°C. The cells were then lysed in lysis buffer (20 mmol/L Tris-HCl, 137 mmol/L NaCl, 10% glycerol, 1% Nonidet P-40, pH 8.0, protease and phosphatase inhibitors) at 4°C for 20 minutes. The extracts were precipitated with

ACK2 MAb and Protein-G Sepharose beads (Pharmacia, Uppsala, Sweden). The immunoprecipitates were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) with a 5 to 20% gradient polyacrylamide. Proteins were electrophoretically transferred from the gel onto a polyvinylidene difluoride membrane (Immobilon, Millipore Corp., Bedford, MA) and immunoblotted with antiphosphotyrosine MAb.

Immune Complex Kinase Assay

The cell lysates were prepared by using lysis buffer, and incubated for 45 minutes at 4°C with rabbit antiserum against a C-terminal peptide corresponding to the last 10 amino acids of the murine KIT and Protein-G Sepharose beads to collect the antigen-antibody complex. After washing, the immune complexes were incubated in kinase buffer (10 mmol/L $MnCl_2$, 20 mmol/L Tris-HCl, pH 7.4) containing 1 μl of γ-[^{32}P]-ATP (DuPont/NEN Research Products, Boston, MA; 10 mCi/ml) for 20 minutes at 25°C and separated by SDS-PAGE with a 5 to 20% gradient polyacrylamide. The gel was dried, and radioactive proteins were detected by autoradiography.[17–19,43]

Cell Proliferation Assay

Cell proliferation was quantified by an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) (Sigma Chemical, St. Louis, MO) rapid colorimetric assay as described.[20,36,41] Briefly, triplicate aliquots of cells (2×10^4) suspended in 100 μl of Cosmedium-001 (Cosmo Bio Co., Tokyo, Japan) were cultured in 96-well microtiter plates for 72 hours at 37°C with various concentrations of rmSCF or rmIL-3. MTT (10 μl of 5 mg/ml solution of MTT in phosphate-buffered saline) was added to all wells for the final 4 hours of the culture. Acid isopropanol (100 μl of 0.04N HCl in isopropanol) was added to all wells and mixed to dissolve the dark blue crystals. The optical density (OD) was then measured on a Multiskan MCC/340MKII (Labsystems, Helsinki, Finland) with a test wavelength of 540 nm and a reference wavelength of 620 nm.

Tumorigenicity Assay

IC-2^{WT}, IC-2^{G559}, or IC-2^{V814} cells were harvested during the exponential phase of growth, washed and resuspended in α-MEM. IC-2^{VECTOR} cells were used as the negative control. Cells (3.0×10^5) were injected subcutaneously into the right and left flanks of nude mice that had received whole body X-ray irradiation (2.5 Gy) 1 day before the injection. Mice were carefully monitored for the development of palpable or visible tumors at the sites of injection. The tumors were measured with a vernier caliper every 5 days. The tumor volume (V) was calculated from the formula, $V = 0.5 \times a \times b^2$, where a and b are the length and width in mm of the tumor mass, respectively.[44]

Morphology

IC-2 cells cultured under various conditions were collected, washed with phosphate-buffered saline, and cytocentrifuged. The specimens were fixed with Carnoy's solution for 30 minutes, then stained with Alcian blue and nuclear fast red. The tumor tissues obtained from nude athymic mice were fixed with Carnoy's solution overnight and embedded in paraffin. The sections (4 μm thick) were stained with hematoxylin and eosin or with Alcian blue and nuclear fast red.

Cells or tissues were fixed for 2 hours at 4°C in 2% glutaraldehyde in 0.1 mol/L cacodylate buffer at pH 7.4 and postfixed for 2 hours at 4°C in 2% osmium tetroxide in 0.1 mol/L cacodylate buffer at pH 7.4. Cells were dehydrated with ethanol and embedded in Quetol 812 (Nissin EM, Tokyo, Japan). Ultrathin sections were cut with a Porter Blum MT II-B ultramicrotome (DuPont Company, Wilmington, DE) and stained with uranyl acetate. A JEOL 1200 EX electron microscope was operated at a voltage of 60 kV.

Purification of Peritoneal Mast Cells

C57BL/6−+/+ mice were anesthetized with ether and killed by exsanguination. After an injection of Tyrode's buffer (3 ml) containing 0.1% gelatin into the peritoneal cavity, the abdomen was gently massaged for 90 seconds. The peritoneal cavity was opened, and the fluid containing peritoneal cells was aspirated using a Pasteur pipette. The peritoneal cells were sedimented at 400 × g for 15 minutes and resuspended in Tyrode's buffer. Peritoneal mast cells (PMCs) were separated from major components of mouse peritoneal cells (macrophages and small lymphocytes) as described by Yurt et al.[45] Peritoneal cells (10^7) suspended in 1 ml of Tyrode's buffer were layered on 2 ml of 22.5% (w/v) metrizamide (density, 1.120 g/ml, Nyegaard & Co., Oslo, Norway) and centrifuged at room temperature for 15 minutes at 400 × g. The cells in the pellet were resuspended in 1 ml Tyrode's buffer. To obtain a PMC suspension of >99%, it was necessary to repeat this step twice.

Histamine Content

IC-2^{VECTOR}, IC-2^{WT}, IC-2^{G559}, or IC-2^{V814} cells were harvested during the exponential phase of growth and suspended in α-MEM (2.0×10^5/ml). The histamine content was determined in 100 μl aliquots of the cell suspensions. The DNA content was determined in 5 ml of cell suspensions containing 10^6 cells. Nude mice were killed 4 weeks after the injection of cells. Tumors were washed with sterile cold saline and divided into six pieces, each of which was weighed. Individual tumor pieces were homogenized in cold perchloric acid (2%) using a polytron homogenizer (Kinematica, Lucerne, Switzerland) to measure histamine content per weight. DNA content per weight was determined in other tumor pieces. The histamine concentrations of the cell suspensions and tumor homogenates were measured by means of high-performance liquid chromatography coupled with fluorometry as described by Yamatodani et al.[46] DNA was extracted from cells and tumor tissues by the standard method. To compare the histamine content between cultured cells and tumor tissues, the histamine content per μg of DNA was calculated.

Northern Blotting Analysis

The cDNA probes for mouse mast cell protease (MMCP)-2, MMCP-4, and MMCP-6, and mast cell carboxypeptidase A (MC-CPA) were prepared in our laboratory.[47,48] To obtain the MMCP-5 cDNA probe, sense (5′-CTACATGGCCTATCTGGAAA-3′, 130 through 149) and antisense (5′-CAGGGCTTCAGCAGGAACTA-3′, 855 through 874) oligonucleotide primers were synthesized by conventional means. Nucleotide numbers were based on the report of McNeil et al.[30] Total RNA (5 μg) obtained from CMCs was used as a template, and the single-strand cDNA was synthesized with a specific antisense primer by reverse transcriptase. The cDNA was amplified by polymerase chain reaction using sense and antisense primers. The products were subcloned into the *Eco*RV site of Bluescript KS (−) plasmid, and the sequence was confirmed as described by Sanger et al.[49]

Total RNA was prepared from IC-2^{VECTOR}, IC-2^{WT}, IC-2^{G559}, or IC-2^{V814} cells cultured at various conditions and from tumor tissues harvested from nude mice using lithium chloride-urea.[50] Northern blots were performed using MMCP-2, MMCP-4, MMCP-5, MMCP-6, MC-CPA, or β-actin cDNA labeled with α-[^{32}P]-deoxycytidine triphosphate (DuPont/NEN Research Products; 10 mCi/ml) as a probe. After hybridization at 42°C, blots were washed to a final stringency of 0.2 × standard saline citrate (150 mmol/L NaCl, 15 mmol/L trisodium citrate, pH 7.4) at 50°C and autoradiographed.

Results

Retroviral Transfer of KIT^{G559} and KIT^{V814}

IC-2 cells were infected with viruses containing KIT^{WT}, KIT^{G559}, or KIT^{V814} cDNA by incubating them on irradiated subconfluent monolayers of virus-producing packaging cells. After selection in a G418-containing medium for 3 weeks, the expression of KIT on the surface of infected cells was examined by flow cytometry using the ACK2 MAb. IC-2^{WT}, IC-2^{G559}, and IC-2^{V814} cells expressed KIT on the surface, whereas IC-2^{VECTOR} cells did not (Figure 1A). Immunoblotting with an antiphosphotyrosine MAb showed that rmSCF significantly increased the tyrosine phosphorylation of KIT, particularly the 145-kd form, in IC-2^{WT} cells (Figure 1B). On the other hand, the 145-kd and 125-kd forms of KIT were phosphorylated on tyrosine in either IC-2^{G559} or IC-2^{V814} cells even in the absence of rmSCF (Figure 1B). Moreover, rmSCF had little or no effect on the level of phosphotyrosines in KIT from either IC-2^{G559} or IC-2^{V814} cells.

We further examined the autokinase activity of KIT in IC-2^{VECTOR}, IC-2^{WT}, IC-2^{G559} and IC-2^{V814} cells. KIT was immunoprecipitated from cell lysates without stimulation by rmSCF and the kinase activity was assayed. Although a detectable level of KIT was not immunoprecipitated from the lysate of IC-2^{VECTOR} cells, comparable amounts were immunoprecipitated from the lysates of IC-2^{WT}, IC-2^{G559}, and IC-2^{V814} cells (data not shown). Tyrosine kinase activity of KIT^{WT} was not detectable without rmSCF stimulation (Figure 1C). KIT^{G559} and KIT^{V814} had moderate and high levels of kinase activity, respectively. (Figure 1C). The *in vitro* evidence of the ligand independent activation of KIT in the mutant forms was more convincingly shown in Figure 1C than in Figure 1B.

Effect of KIT^{G559} and KIT^{V814} Expression on Proliferation

IC-2, IC-2^{VECTOR}, IC-2^{WT}, IC-2^{G559}, or IC-2^{V814} cells were cultured with various concentrations of rmIL-3 or rmSCF for 72 hours and their proliferative potential was measured by means of the MTT colorimetric assay (Figure 2). Like the original IC-2 cells, rmIL-3 induced the dose-dependent proliferation of IC-2^{VECTOR} cells, and rmSCF had no effect. In addition

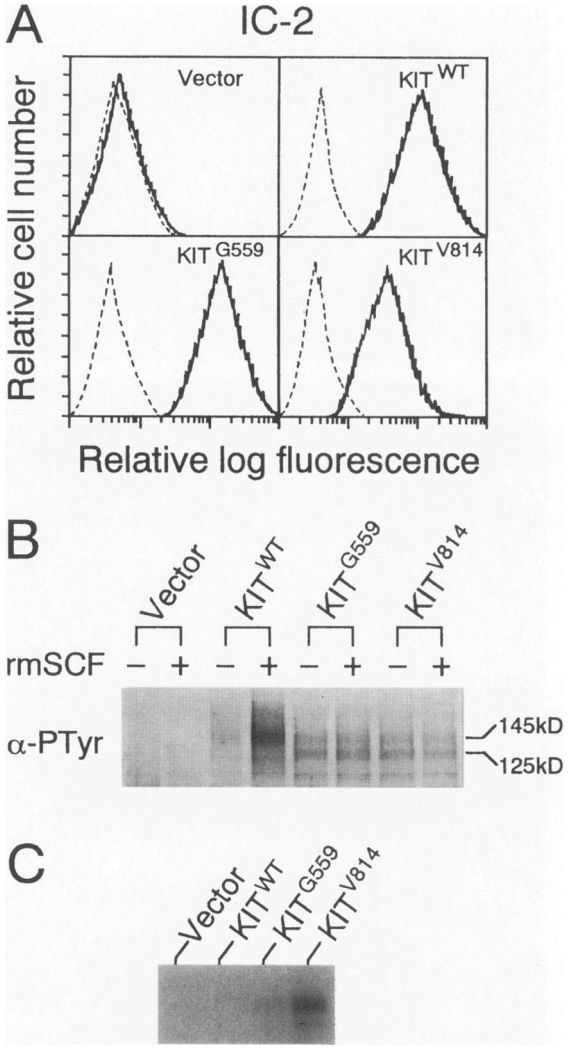

Figure 1. *Constitutive tyrosine phosphorylation and activation of KIT^{G559} and KIT^{V814}.* A: *Flow cytometry of the surface binding of ACK2 MAb to IC-2^{VECTOR}, IC-2^{WT}, IC-2^{G559}, and IC-2^{V814} cells. Cells were incubated with either ACK2 MAb (—) or negative control antibody (- - -).* B: *Constitutive tyrosine phosphorylation of KIT^{G559} and KIT^{V814}. The state of tyrosine phosphorylation of KITs in IC-2^{VECTOR}, IC-2^{WT}, IC-2^{G559}, and IC-2^{V814} cells before and after stimulation with rmSCF was examined by immunoblotting using an antiphosphotyrosine MAb. The mobilities of the mature (~145 kd) and immature (~125 kd) forms of KIT are indicated.* C: *Constitutive activation of KIT^{G559} and KIT^{V814}. KIT was immunoprecipitated from lysates of IC-2^{VECTOR}, IC-2^{WT}, IC-2^{G559}, and IC-2^{V814} cells without rmSCF stimulation, and kinase activity was assayed.*

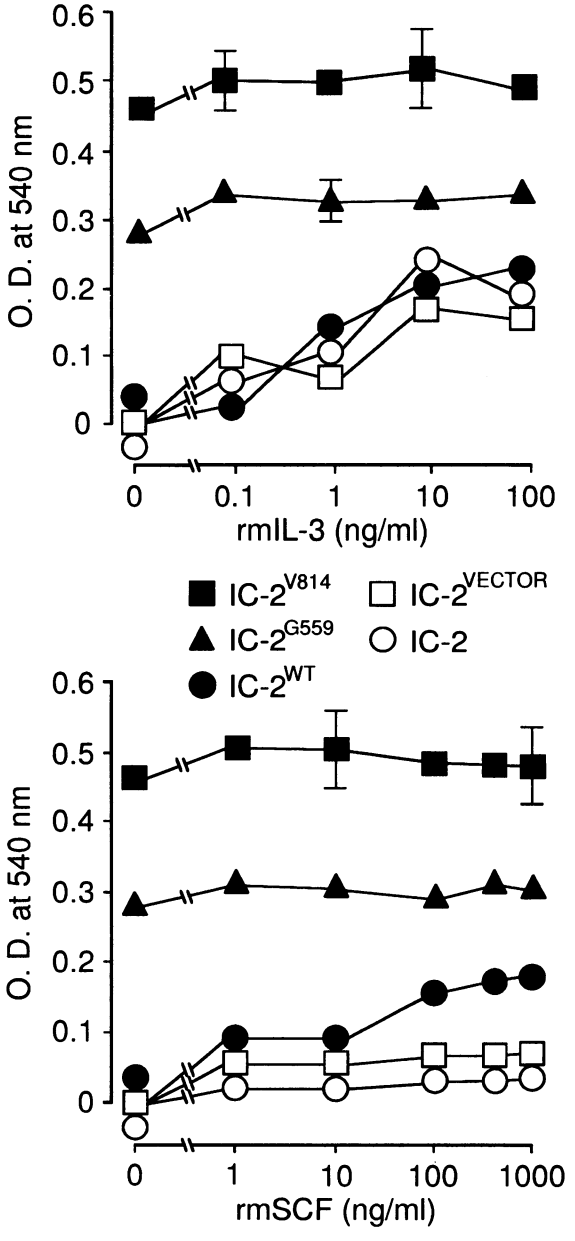

Figure 2. *The factor-independent proliferation of IC-2^{G559} and IC-2^{V814} cells. Proliferation of IC-2^{VECTOR}, IC-2^{WT}, IC-2^{G559}, and IC-2^{V814} cells at various concentrations of rmIL-3* (upper panel) *or rmSCF* (lower panel) *was measured by means of the MTT colorimetric assay. Three independent experiments were performed with comparable results, and those of a representative experiment are shown. Each point represents the mean of triplicate samples. Bars are standard errors; the standard error was sometimes too small to be shown by bars.*

to this response to rmIL-3, IC-2^{WT} cells dose-dependently proliferated in response to rmSCF over the range of 1 to 1000 ng/ml, indicating the functional expression of KIT^{WT}. IC-2^{G559} and IC-2^{V814} cells proliferated in a factor-independent manner, and the addition of either IL-3 or SCF did not affect their growth. The magnitude of proliferation of IC-2^{G559} cells was lower than that of IC-2^{V814} cells.

IC-2^{VECTOR}, IC-2^{WT}, IC-2^{G559}, or IC-2^{V814} cells were subcutaneously injected into the right and left flanks of nude athymic mice. IC-2^{VECTOR} cells did not yield any detectable tumors within the 45-day observation period. In contrast, IC-2^{G559} and IC-2^{V814} cells produced tumors at all injection sites. The growth of tumors derived from IC-2^{V814} was greater than that from IC-2^{G559} cells (Figure 3). All nude mice bearing tumors succumbed to leukemia and died within 8 weeks. In the nude mice injected with IC-2^{WT} cells,

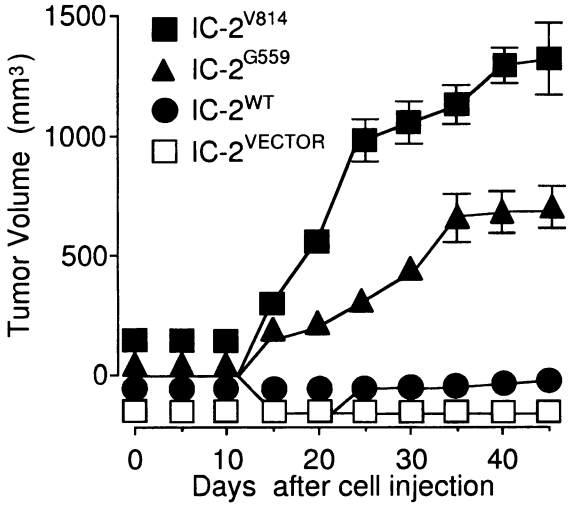

Figure 3. *Development of tumors in nude mice after injection of IC-2^{G559} or IC-2^{V814} cells. The tumor volume was calculated as described in Materials and Methods. Each point represents the mean of the tumor volume of 10 injection sites. Bars are standard errors; the standard error was sometimes too small to be shown by bars.*

no tumors were detectable within 20 days, but small nodules appeared at 3 of the 10 injected sites 25 days after the injection. Leukemia did not develop and all nude mice remained alive within the 8-week observation period.

Phenotypic Change by Constitutive Activation of KIT

Original and IC-2-derived cells were examined morphologically. About two-thirds of the original IC-2 cells were not stained with Alcian blue, and the remaining third was stained weakly (Figure 4A). Less than 1% of the original IC-2 cells were intensely stained with Alcian blue. In contrast, most of the IC-2^{V814} cells were stained with Alcian blue, of which about one-third were intensely stained (Figure 4B). The microscopic features of IC-2^{G559} cells were intermediate between those of the original IC-2 cells and those of the IC-2^{V814} cells (data not shown). The microscopic features of IC-2^{WT} cells stimulated in culture with rmSCF (100 ng/ml) were comparable to those of IC-2^{V814} cells (data not shown). Moreover, the proportion of cells intensely stained with Alcian blue increased when IC-2^{V814} cells were grafted into nude mice (Figure 4C). Typical electron microscopic features of an original IC-2 cell and an IC-2^{V814} cell are shown in Figure 5. There were more electron-dense granules in IC-2^{V814} than in original IC-2 cells.

As another index, we measured the relative histamine content of various IC-2-derived cells under different conditions. As controls, we measured the

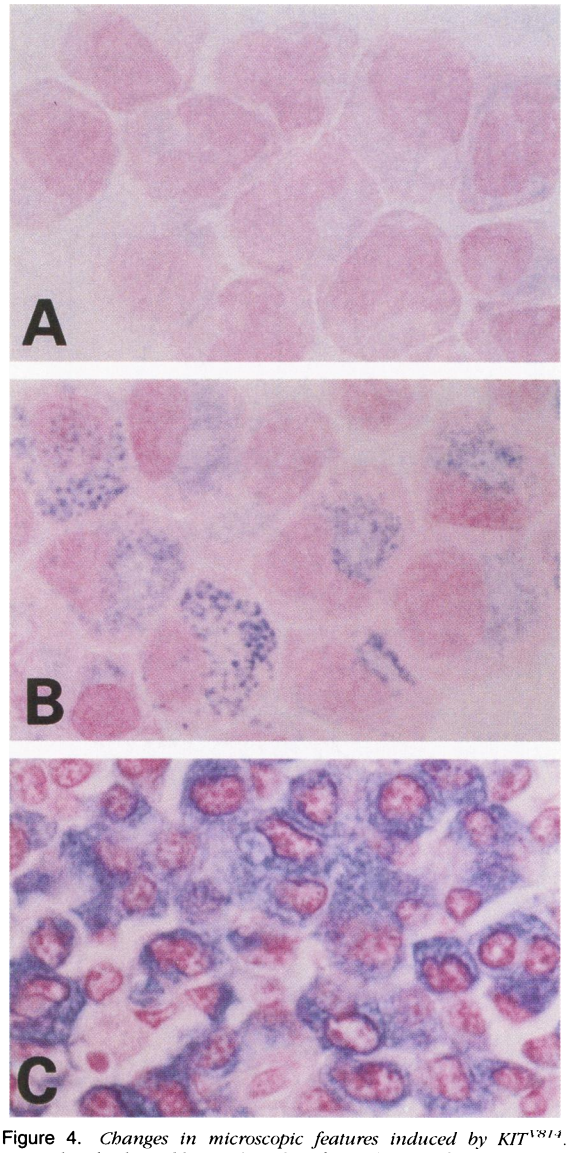

Figure 4. *Changes in microscopic features induced by KIT^{V814}. Stained with Alcian blue and nuclear fast red. Magnification, ×960.* **A:** *Original IC-2 cells growing in suspension culture with rmIL-3 (50 ng/ml).* **B:** *IC-2^{V814} cells growing in suspension culture with neither rmIL-3 nor rmSCF.* **C:** *Histological features of a subcutaneous tumor that developed at the injection site of IC-2^{V814} cells.*

relative histamine contents of CMCs and PMCs. The histamine contents of the original IC-2 and IC-2^{VECTOR} cells were comparable to those of CMCs cultured in the medium containing rmIL-3 alone (Table 1). In medium containing rmSCF alone, the histamine contents of CMCs increased ninefold. The histamine contents of IC-2^{WT} cells also increased in the medium containing rmSCF, but the magnitude of the increase was higher in CMCs than in IC-2^{WT} cells (Table 1). The histamine contents of IC-2^{G559} and IC-2^{V814} cells were comparable to the value obtained from IC-2^{WT} cells that were cultured in the medium containing rmSCF. The histamine contents

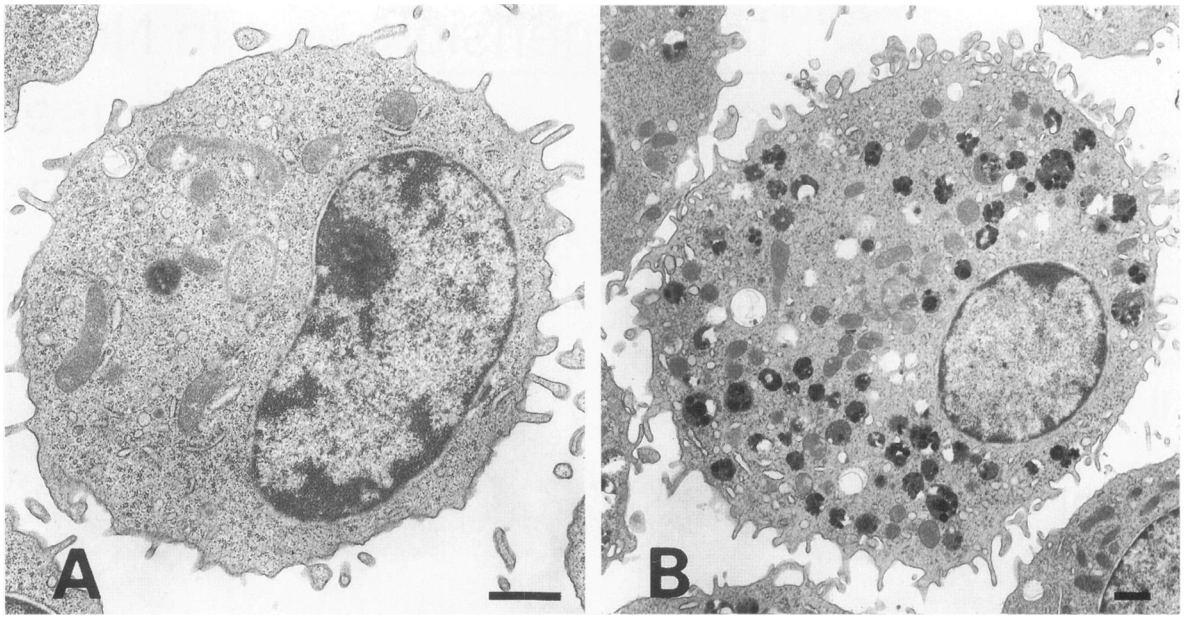

Figure 5. *Changes in electron microscopic features induced by KIT^{V814}. Bar = 1 μm.* **A:** *An original IC-2 cell growing in suspension culture with rmIL-3 (50 ng/ml).* **B:** *An IC-2^{V814} cell growing in suspension culture with neither rmIL-3 nor rmSCF.*

per μg DNA increased more than fivefold when IC-2^{G559} or IC-2^{V814} cells were grafted into nude mice. Because such tumors were mostly composed of mast cells (Figure 4C), the increased histamine levels were attributed to the tumor cells themselves and not to the infiltrated mast cells of host origin.

The transcription of various proteases has been used as a marker to describe mast cell phenotypes; more than eight protease cDNAs have been cloned from mast cells of mice.[27–30] We therefore examined the protease transcriptional phenotypes of various types of IC-2 cells after culture with or without IL-3

Table 1. *Relative Histamine Contents of CMCs, PMCs, and IC-2-Derived Cells under Various Conditions*

Cells	Cytokines added to the culture: IL-3 (50 ng/ml)	Cytokines added to the culture: SCF (100 ng/ml)	Relative histamine contents* (nmol/μg DNA)
Controls			
CMCs	Yes	No	0.112 ± 0.001 (3)
	No	Yes	1.005 ± 0.017 (3)
	Yes	Yes	1.364 ± 0.042 (3)
PMCs	No†	No†	4.837 ± 0.021 (3)
IC-2-derived cells in suspension culture			
IC-2	Yes	No	0.097 ± 0.002 (3)
IC-2^{VECTOR}	Yes	No	0.100 ± 0.013 (3)
IC-2^{WT}	Yes	No	0.282 ± 0.024 (3)
	No	Yes	0.470 ± 0.065 (3)‡
	Yes	Yes	0.484 ± 0.025 (3)‡
IC-2^{G559}	No	No	0.387 ± 0.067 (3)‡
	Yes	No	0.443 ± 0.013 (3)‡
IC-2^{V814}	No	No	0.541 ± 0.003 (3)‡
	Yes	No	0.776 ± 0.011 (3)‡§
IC-2-derived cells in nude mouse			
IC-2^{G559}	No	No	3.346 ± 0.396 (3)‡‖
IC-2^{V814}	No	No	4.668 ± 0.175 (3)‡§

Cells were cultured without cytokines or with rmIL-3 (50 ng/ml) and/or rmSCF (100 ng/ml) for 2 weeks. Tumors of IC-2^{G559} and IC-2^{V814} cells were harvested from nude mice 4 weeks after transplantation.

*Mean ± standard error. The number of samples is shown in parenthesis.

†PMCs were not cultured.

‡$P < 0.05$, when compared with the value of IC-2 cells by the *t*-test.

§$P < 0.01$, when compared with the value of IC-2^{V814} cells cultured in suspension without cytokines.

‖$P < 0.01$, when compared with the value of IC-2^{G559} cells cultured in suspension without cytokines.

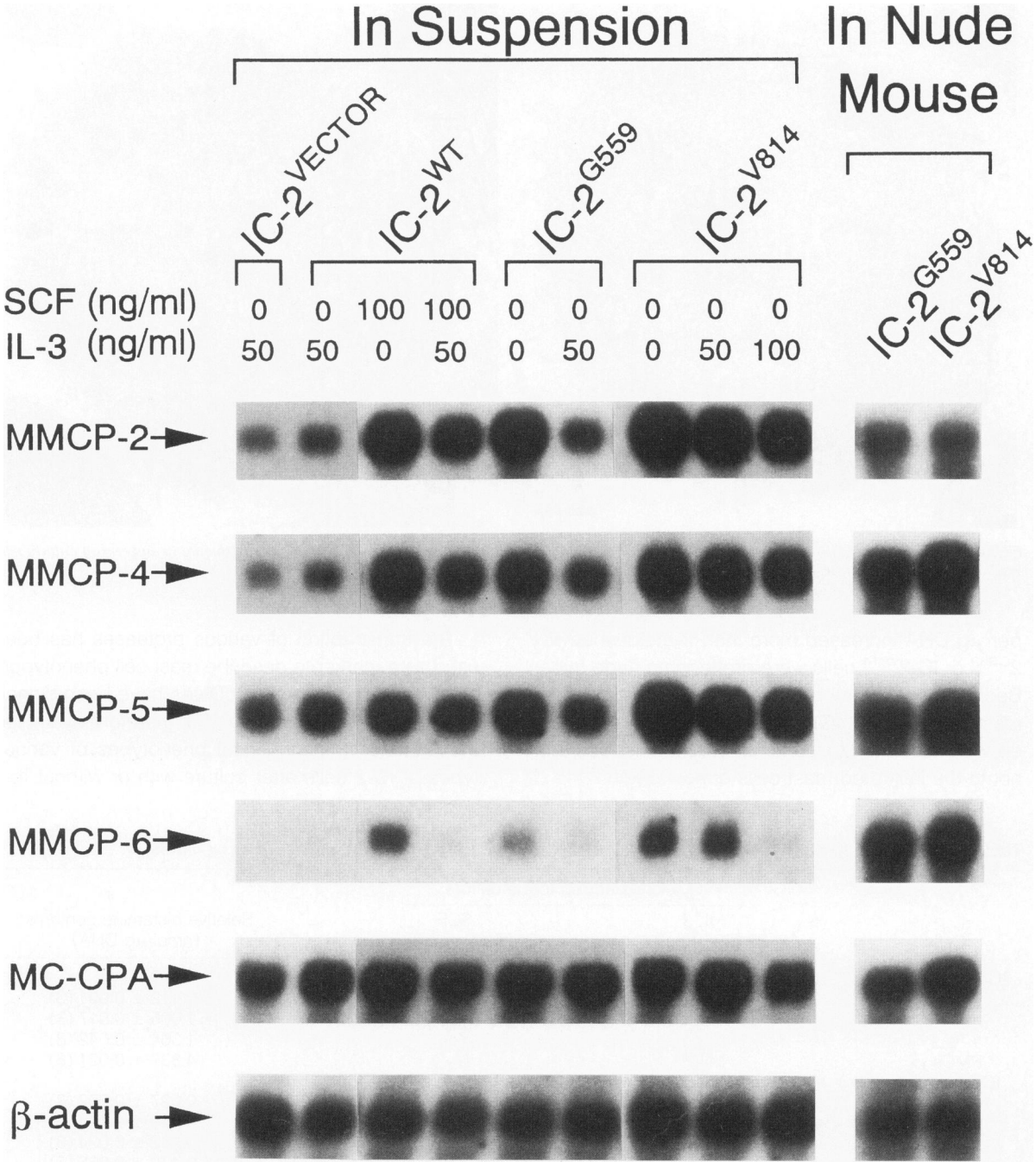

Figure 6. *Expression of MMCP-2, MMCP-4, MMCP-5, MMCP-6, and MC-CPA mRNAs in IC-2 cells under various conditions. Cells were cultured with various concentrations of rmSCF and/or rmIL-3 for 2 weeks after the depletion of α-MEM$_{IWM\text{-}SCM}$, then harvested. Tumors of IC-2^{G559} and IC-2^{V814} cells were removed from nude mice 4 weeks after transplantation. The blot was hybridized with ^{32}P-labeled cDNA probe of MMCP-2, MMCP-4, MMCP-5, MMCP-6, or MC-CPA. The β-actin probe was used to verify that an equal amount of total RNA (20 μg) was loaded in each lane.*

and/or SCF or after grafting into nude mice. The mRNA expression of MMCP-2 and MMCP-4 in IC-2^{WT} cells was comparable to that of IC-2^{VECTOR} cells in the medium containing rmIL-3 alone. IC-2^{WT} cells, but not IC-2^{VECTOR} cells, expressed KIT on the surface, and IC-2^{WT} cells responded to rmSCF by increasing the expression of MMCP-2 and MMCP-4 mRNAs (Figure 6). The transcriptional levels of MMCP-2 and MMCP-4 in nonstimulated IC-2^{G559} and IC-2^{V814} cells were comparable to that of IC-2^{WT} cells stimulated with rmSCF. Stimulation with rmIL-3 decreased the transcriptional levels of MMCP-2 and MMCP-4 mRNAs in IC-2^{G559} and IC-2^{V814} cells. The expression level of MMCP-5 did not change remark-

ably in various IC-2-derived cells (Figure 6). The expression of MMCP-6 mRNA was not detectable in IC-2^{VECTOR} cells, or in IC-2^{WT} cells stimulated by IL-3 alone. MMCP-6 was apparently expressed when IC-2^{WT} cells were stimulated by rmSCF alone. However, when IC-2^{WT} cells were stimulated by both rmIL-3 and rmSCF, MMCP-6 expression dropped to undetectable levels (Figure 6). MMCP-6 expression was detectable in unstimulated IC-2^{V814} cells and to a lesser degree in unstimulated IC-2^{G559} cells. MMCP-6 expression decreased significantly when IC-2^{V814} cells were stimulated with a high concentration of rmIL-3 (ie, 100 ng/ml) (Figure 6). When IC-2^{G559} and IC-2^{V814} cells were injected into nude mice, the expression of MMCP-6 mRNA increased remarkably. MC-CPA expression was not influenced by our experimental conditions (Figure 6).

Discussion

SCF is probably the most important growth factor required for the development and survival of mast cells. Mutant mice and rats possessing a double gene dose of mutant alleles at either SCF or KIT gene are deficient in mast cells.[12,13,51,52] When SCF is injected into rats,[53] baboons,[54] or cynomolgus monkeys,[54] the number of mast cells increases up to 100-fold. However, the number of mast cells drops to preinjection levels after stopping the injection.[53,54] Thus, the enzymatic activity and subsequent signaling of KIT is controlled by the binding of SCF. However, we showed that KIT can be activated in a ligand-independent manner by point mutations of the c-*kit* gene in the neoplastic mast cell lines, HMC-1,[17] P-815,[18] and RBL-2H3.[19] The selective expression of these mutations on neoplastic mast cell lines suggested that c-*kit* activating mutations are involved in the abnormal growth and aberrant differentiation of mast cells.

Because it was rather difficult to obtain normal controls for HMC-1, P-815 and RBL-2H3 cells, in this study we used the IL-3-dependent IC-2 mast cell line to examine the effects of KIT^{G559} and KIT^{V814} on mast cells. The results showed that KIT^{G559} and KIT^{V814} were activated without SCF binding, and that the expression of KIT^{G559} and KIT^{V814} in the IL-3-dependent IC-2 mast cell line resulted in factor-independent growth in suspension culture and tumorigenesis in nude mice. Furthermore, IC-2^{V814} cells grew more rapidly than IC-2^{G559} cells in both suspension culture and nude mice. Because the magnitude of tyrosine phosphorylation and kinase activity was greater in KIT^{V814} than in KIT^{G559}, the activation potential of the mutations appeared to parallel their potential for malignant transformation.

KITs are expressed as mature (145 kd) and immature (125 kd) forms, which presumably represent membrane-bound and cytoplasmic forms, respectively. In this study, mature (145 kd) and immature (125 kd) forms of KIT^{G559} or KIT^{V814} were phosphorylated on tyrosines in the absence of rmSCF stimulation, whereas the phosphorylation was predominantly increased in the mature (145 kd) form of KIT^{WT} after exposure to rmSCF. These results suggested that the activating mutation of c-*kit* gene affects the conversion from the immature to the mature form of KIT. Under physiological conditions, SCF exerts its function through binding to the mature form of KIT, and the immature form of KIT is unlikely to be involved in SCF-induced signal transduction. In the presence of an activating mutation, however, mature and immature forms of KIT appeared to be constitutively activated. This suggested that the immature form of mutant KIT as well as the mature form mediates growth signals that participate in cell transformation.

One of the important findings in this study is that phenotypes of IC-2 cells became more mature in suspension culture upon the expression of the oncogenic KIT^{G559} or KIT^{V814} proteins. Original IC-2 cells contain very few basophilic granules and low levels of histamine, and did not express MMCP-6. However, IC-2^{G559} and IC-2^{V814} cells had an appreciable number of basophilic granules when cultured in medium containing neither IL-3 nor SCF. The content of histamine also increased in IC-2^{G559} and IC-2^{V814} cells. Furthermore, MMCP-6 mRNA transcripts were readily detectable in IC-2^{V814} cells and to a lesser extent, in IC-2^{G559} cells. These more mature phenotypes of IC-2^{G559} and IC-2^{V814} cells were almost identical to those of IC-2^{WT} cells that were cultured in medium containing SCF. These results suggested that KIT^{G559} and KIT^{V814} had differentiation-inducing activity in addition to oncogenic activity in mast cells. Moreover, IC-2^{V814} cells had more mature mast cell phenotypes than IC-2^{G559} cells, suggesting that the magnitude of activating potential in KIT mutations parallels that of the differentiation-inducing potential.

IC-2^{WT} cells expressed MMCP-2 and MMCP-4 mRNAs in medium containing IL-3 alone, and the expression was enhanced in medium containing SCF alone. When IC-2^{WT} cells were cultured in medium containing both IL-3 and SCF, the expression levels of MMCP-2 and MMCP-4 were reduced. The mRNA expression levels of MMCP-2 and MMCP-4 in the IC-2^{G559} and IC-2^{V814} cells, which were cultured in the absence of IL-3 or SCF, were comparable to

those of IC-2^{WT} cells stimulated with SCF. Here again, the constitutive activation of KIT had differentiation-inducing potential in IC-2 cells.

The mRNA expression levels of MMCP-2 and MMCP-4 in IC-2^{G559} and IC-2^{V814} cells were reduced by adding IL-3 to the culture as they were in IC-2^{WT} cells. The effect of IL-3 was also negative upon the mRNA expression of MMCP-6, whereas a higher concentration of IL-3 was required in IC-2^{V814} than in IC-2^{WT} cells. These observations were consistent with the report of Gurish et al,[31] who reported that the transcriptional levels of MMCP-4 and MMCP-6 in CMCs were significantly down-regulated by adding IL-3 to culture media containing SCF. The molecular mechanism underlying the down-regulation of the proteases by IL-3 remains unclear. However, there are the following speculations of Eklund et al,[55] regarding the down-regulation of MMCP-1, MMCP-2, and MMCP-4 by IL-3 and IL-4. 1) IL-3 may induce a dominant *trans*-acting factor(s) that suppresses the transcription of MMCP-2, MMCP-4, and MMCP-6. 2) IL-3 may accelerate degradation of mRNAs of MMCP-2, MMCP-4, and MMCP-6. *In vivo* experiments using parasite-infected mice also suggested the effect of IL-3 on the expression of mast cell-specific proteases. The number of mast cells in the intestinal mucosa increased remarkably after infection with *Trichinella spiralis*. The developing mast cells contained large amounts of MMCP-1 and MMCP-2 mRNAs, but not MMCP-4, MMCP-5, MMCP-6, and MC-CPA mRNAs.[56] The regulation mechanisms of mast cell-specific proteases by T cell-derived factors including IL-3 remain to be clarified.

Although KIT^{G559} and KIT^{V814} had differentiation-inducing activity in IC-2 cells, IC-2^{G559} and IC-2^{V814} cells did not have fully differentiated phenotypes in suspension culture. However, IC-2^{G559} and IC-2^{V814} cells exhibited considerably differentiated mast cell phenotypes after transplantation into nude mice. When IC-2^{G559} and IC-2^{V814} cells were grafted into nude mice, both types of cells had an appreciable number of basophilic granules, and the histamine contents of the resulting tumors were comparable to those of PMCs. Moreover, the expression of MMCP-6 mRNA transcripts in the cells was markedly enhanced in nude mice. These results suggested that IC-2 cells bearing mutant KIT were still capable of acquiring mature mast cell phenotypes in nude mice. However, the factors involved in the maturation of the cells in tissues of nude mice remain to be investigated.

The present results demonstrated that the activating mutation of KIT conferred factor-independent growth and tumorigenicity in the originally IL-3-dependent IC-2 cells. In addition, IC-2 cells with the mutant KIT acquired a more mature phenotype than original IC-2 cells. Generally, malignant transformation and acquisition of the mature phenotype do not occur simultaneously. In this system, however, tumorigenicity and maturation were acquired by IC-2 cells at the same time, indicating the crucial role of mutant KIT-mediated signals for both neoplastic growth and differentiation at least in mast cells.

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