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Absence of Immature Mast Cells in the Skin of *Ws/Ws* Rats with a Small Deletion at Tyrosine Kinase Domain of the c-*kit* Gene

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Ws/Ws rats have a small deletion at the tyrosine kinase domain of the c-kit gene, and practically no mast cells were detectable when the tissues were stained with alcian blue. Because alcian blue stains proteoglycans, there is a possibility that immature mast cells that do not contain a sufficient amount of proteoglycans are not detectable by this method. We examined this possibility by using other markers of mast cells. The histamine content in the skin of Ws/Ws rats was 0.3% that of control normal (+/+) rats. Because the number of alcian blue–positive mast cells in the skin of Ws/Ws rats was also 0.3% that of +/+ rats, histamine in the skin seemed to be concentrated to alcian blue–positive mast cells. Mast cells in the skin of +/+ rats express messenger RNA of FcεRI β-subunit and c-kit protein. Because c-kit messenger RNA was normally expressed at least in the brain of Ws/Ws rats despite the small deletion, we examined the expression of FcεRI β-subunit and c-kit messenger RNA in the skin and stomach of Ws/Ws rats by reverse transcriptase modification of polymerase chain reaction. Expression of either FcεRI β-subunit or c-kit messenger RNA in the skin and stomach of Ws/Ws rats was estimated to be less than 1% that of +/+ rats. Moreover no FcεRI β-subunit-expressing and no c-kit-expressing cells were detectable in the skin of Ws/Ws rats by in situ hybridization histochemistry. The present result suggests the absence of immature mast cells in tissues of Ws/Ws rats. (Am J Pathol 1993, 142:1001–1007)

We have found that black-eyed white mutant rats are deficient in erythrocytes and mast cells.[1] The mutant allele of the rat was designated as white spotting (*Ws*).[1,2] Because the mechanism of mast cell deficiency in homozygous *Ws/Ws* rats was attributed to a defect of precursor cells but not to the cells supporting the differentiation of mast cells, the *Ws* locus of rats was comparable with the *W* locus rather than *Sl* locus of mice.[1–4] The *W* locus of mice had been shown to be identical with the c-*kit* protooncogene coding a tyrosine kinase receptor (c-kit receptor),[5–8] and the *Sl* locus had been shown to code a ligand for the receptor encoded by the c-*kit* locus.[9–12] Therefore, we compared c-*kit* complementary (c)DNA of *Ws/Ws* rats with that of control normal (+/+) rats and found a deletion of 12 bases in the c-*kit* cDNA of *Ws/Ws* rats.[2] Four amino acids encoded by deleted 12 bases were located at two amino acids downstream from the tyrosine autophosphorylation site in the c-*kit* kinase and were conserved not only in mouse and human c-*kit* kinases but also in mouse and human c-*fms* kinases (i.e., receptors of colony stimulating factor-1).[2]

In the previous report, we evaluated the mast cell deficiency of *Ws/Ws* rats by examining tissues stained with alcian blue.[1] Because alcian blue stains proteoglycans, there is a possibility that immature mast cells that do not contain a sufficient amount of proteoglycans are not detectable by this method. Histamine is another substance that is classically known as a content of mast cells. We investigated histamine content in various tissues of W/W^v mice that also lack alcian blue–positive mast cells.[13] When compared with histamine content of control +/+ tissues, the skin, heart, and lung of W/W^v mice contained negligible amounts of histamine, but the brain and stom-

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ach contained a substantial amount of histamine. We attributed the high proportion of histamine content in the brain and stomach of W/W^v mice to that of +/+ mice to the presence of histamine-producing cells other than mast cells; i.e., histaminergic neurons in the brain and histamine-producing enterochromaffin-like cells in the stomach.[13]

Proteoglycan and histamine are present within granules of mast cells. On the other hand, many physiologically important molecules are expressed on the surface of mast cells. Among them the high affinity receptor of IgE (FcεRI) is the best characterized molecule. The immunological specificity of mast cells in an immediate hypersensitivity reaction is determined by IgE molecules bound to the FcεRI.[14] FcεRI is composed of four peptides, an IgE-binding α chain, a β chain, and two disulfide-linked γ chains.[14,15] Because FcεRI gene expression has been reported to occur in the early step of mast cell differentiation,[16] there is a possibility that alcian blue–negative immature mast cells may express messenger (m)RNA of FcεRI in tissues of *Ws/Ws* rats. Basophilic leukocytes are another cell type that has basophilic granules containing proteoglycan and histamine. Basophilic leukocytes also express mRNA of FcεRI[16] but do not express the c-*kit* protein.[17–19] Although the c-*kit* protein of *Ws/Ws* rats seemed to be defective, an appreciable amount of c-*kit* mRNA is expressed at least in the brain of *Ws/Ws* rats.[2] There is a possibility that alcian blue–negative immature mast cells may be identified by using c-*kit* mRNA as a marker.

The purpose of the present study is to characterize some biochemical and histological features of *Ws/Ws* rats to increase the usefulness of this animal model. We compared the histamine content of various tissues between *Ws/Ws*, *Ws/+*, and +/+ rats. Moreover, we compared the amount of mRNA of FcεRI β-subunit and c-*kit* protein between *Ws/Ws* and +/+ rats by using reverse transcription modification of polymerase chain reaction (PCR) and *in situ* hybridization histochemistry.

Materials and Methods

Rats

The origin and the breeding procedure used have been described.[1,2] Homozygous *Ws/Ws* rats were not obtained in the genetic background of the BN strain, in which the original heterozygous rat with coat color dilution and white spots was found.[1] Therefore, spotted BN-*Ws/+* rats were crossed with normal (+/+) rats of the Donryu strain. The resulting F_1 rats with white spots (F_1-*Ws/+*) were mated together; *Ws/Ws*, *Ws/+*, and +/+ rats of F_2 generation were obtained. Genotypes of rats were easily identified by their coat color.

Histamine Content

Rats of *Ws/Ws*, *Ws/+*, and +/+ genotypes were killed by exsanguination under ether anesthesia. Twenty μl of saline was injected into the peritoneal cavity, and the abdomen was gently massaged for 60 seconds. The peritoneal cavity was opened and the fluid containing peritoneal cells collected. The cell suspension was centrifuged at 150*g* for 10 minutes at 4 C, and the pellet was used as a sample. Tissues were harvested quickly and stored at −80 C. Histamine was measured using high performance liquid chromatography fluorometry according to Yamatodani et al.[13] Briefly, each sample was homogenized in 10× volume of 3% perchloric acid with a Polytron homogenizer (Kinematica, Lucerne, Switzerland) operated at maximal speed in an ice bath. The homogenate was centrifuged at 10,000*g* for 20 minutes at 4 C, and 50 μl of the supernatants was applied to high performance liquid chromatography fluorometry. Histamine contents were expressed as nmol/g wet weight.

Preparation of cDNA for Southern Blot

Total RNA was obtained from the skin, stomach fundus, forestomach, and whole brain of +/+ and *Ws/Ws* rats by acid guanidinium thiocyanate-phenol-chloroform extraction.[20] Various amounts of total RNA (5, 0.5, 0.05, 0.005 μg) were reverse transcribed in 20 μl of reaction mixture containing 400 units of Molony murine leukemia virus reverse transcriptase and 50 pmol anti-sense primer for FcεRI β-subunit, c-*kit* and β-actin. Then 1 μl of each reaction product was amplified in a 25-μl PCR mixture containing 0.125 units of Taq DNA polymerase and 12.5 pmol each of specific primers (sense and anti-sense) for rat FcεRI β-subunit, c-*kit* or β-actin. Oligonucleotides used for the reverse transcription and PCR were as following: the rat FcεRI β-subunit sense primer TGCTCTCCCAAACCCACAAG and anti-sense primer ATCGACTCTCAAGACTATAC (87–106 and 817–836, respectively);[21] the rat c-*kit* sense primer GGCCCACCCTGGTCATTACAGAAT and anti-sense primer TTCCTTGATCATCTTGTAGAACTT (2041–2064 and 2679–2703, respectively;[2] and the rat β-actin sense primer TAAA-

GACCTCTATGCCAACAC and anti-sense primer TAAAGCCATGCCAAATGTCTC (2759–2770 and 3203–3223, respectively).[22]

Southern Blot Analysis

Ten μl of the PCR products were electrophoresed in agarose gel. Membrane-transferred DNA was hybridized with random-primed probes labeled with digoxigenin dUTP as described.[23] Hybridized filters were washed, and signals were detected using Nucleic Acid Detection Kit (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer's instructions. For generation of the rat FcεRI β-subunit probe, a HinfI-digested 393-bp fragment of rat FcεRI β-subunit cDNA (107–499)[21] was used, and for generation of the rat c-*kit* probe, a BanI-digested 713-bp fragment of rat c-*kit* cDNA (1843–2555)[2] was used.

Histology and In Situ *Hybridization*

Skin pieces and glandular stomachs of +/+ and *Ws/Ws* rats were fixed with 4% paraformaldehyde in phosphate buffer (0.1 mol/L, pH 7.2) for overnight and embedded in paraffin. Sections of the glandular stomach was stained with alcian blue and nuclear fast red. Serial sections (3-μ thick) of the skin were cut, and sections of odd numbers were used for *in situ* hybridization and those of even numbers were stained with alcian blue. For *in situ* hybridization, a 1.2-kb fragment of rat c-*kit* cDNA (356–1552)[2] and a 1-kb fragment of rat FcεRI β-subunit cDNA (1–965)[21] were subcloned into Bluescript pKS (–) plasmid. Complementary RNA probes were prepared by using DIG RNA Labeling Kit (Boehringer Mannheim) according to the manufacturer's instructions. The procedure of hybridization has been described.[24] Signals were detected by color reaction.

Results

Histamine content of various tissues was compared among +/+, *Ws*/+,and *Ws/Ws* rats. In all tissues examined, the histamine content was greatest in +/+ rats, intermediate in *Ws*/+ rats, and smallest in *Ws/Ws* rats (Table 1). The histamine content in the skin of +/+, *Ws*/+, and *Ws/Ws* rats was approximately proportional to the number of mast cells, which was counted in the skin sections stained with alcian blue (i.e., 317/cm in +/+ rats, 167/cm in *Ws*/+ rats, and 1/cm in *Ws/Ws* rats).[1] When the histamine content was compared among tissues of +/+ rats, that of peritoneal cells was 43,144 times as much as that of the brain. The difference among tissues was much smaller in *Ws/Ws* rats than in +/+ rats. Among tissues of *Ws/Ws* rats, the histamine content of the stomach fundus was remarkably greater than the values found in other examined tissues. However, no alcian blue–positive mast cells were detectable in the stomach fundus of *Ws/Ws* rats. When the proportion of histamine content of *Ws/Ws* rats to that of +/+ was calculated, the proportion was greatest in the brain and smallest in the peritoneal cells (Table 2). From the viewpoint of this proportion, tissues may be classified into three groups: 1) the proportion was high in the brain and stomach fundus; 2) the proportion was intermediate in the spleen, kidney, liver, lung, and heart; and 3) the proportion was low in the forestomach, skin, and peritoneal cells.

In the next experiment, we estimated the relative content of mRNA of FcεRI β-subunit, c-*kit* protein, or β-actin in the skin of +/+ and *Ws/Ws* rats. β-actin

Table 1. *Histamine Contents in Various Tissues of* Ws/Ws *and +/+ Rats*

Tissues	Histamine contents (nmoles/g wet wt, mean ± SE)		
	+/+	*Ws*/+	*Ws/Ws*
Peritoneal cells	22866.35 ± 2394.84* (5)	383.38 ± 140.40 (5)	1.66 ± 0.41* (10)
Stomach fundus	558.20 ± 62.55* (9)	329.14 ± 34.17 (7)	274.78 ± 25.85 (10)
Skin	187.42 ± 14.13* (10)	57.86 ± 8.00 (7)	0.48 ± 0.14* (10)
Forestomach	38.76 ± 4.39* (9)	7.13 ± 0.76 (7)	0.22 ± 0.05* (7)
Lung	25.11 ± 3.40* (8)	4.06 ± 0.95 (6)	0.87 ± 0.11* (10)
Heart	20.87 ± 3.47* (5)	1.01 ± 0.12 (7)	0.58 ± 0.05* (8)
Spleen	11.73 ± 1.65* (9)	5.89 ± 0.69 (6)	2.23 ± 0.26* (9)
Liver	10.51 ± 1.17* (10)	3.07 ± 0.31 (8)	0.43 ± 0.05* (7)
Kidney	4.33 ± 0.41* (10)	1.74 ± 0.35 (8)	0.34 ± 0.04* (7)
Brain	0.53 ± 0.04* (9)	0.34 ± 0.04 (7)	0.31 ± 0.02 (5)

Numbers of animals are shown in the parentheses.
* $P < 0.05$, when compared with the value of respective tissues of *Ws*/+ rats by *t*-test.

Table 2. *Proportion of Histamine Content in Tissues of* Ws/Ws *Rats to that of +/+ Rats*

Tissues	Proportion of *Ws/Ws* rats to that of +/+ rats (%)
Brain	58.5
Stomach fundus	49.2
Spleen	19.0
Kidney	7.85
Liver	4.09
Lung	3.46
Heart	2.78
Forestomach	0.56
Skin	0.26
Peritoneal cell	0.01

cDNA was used to confirm the semiquantative nature of the present experiment. Total RNA was isolated from the skin of +/+ and *Ws/Ws* rats, and various amounts of samples (5, 0.5, 0.05, 0.005 μg) were reverse transcribed into cDNA and then amplified by using PCR. The cDNA was detected by Southern hybridization using FcεRI β-subunit or c-*kit* probe. Faint signals of FcεRI β-subunit and c-*kit* protein were detectable when 5 μg of total RNA extracted from the skin of *Ws/Ws* rats used as the starting material (Figure 1). However, these signals were significantly weaker than the signals obtained by using 0.05 μg total RNA extracted from the skin of +/+ rats. Because this experiment was performed in semiquantative condition, the amounts of mRNA of FcεRI β-subunit and c-*kit* protein in the skin of *Ws/Ws* rats were estimated to be less than 1% those of +/+ rats.

Cell types that expressed mRNA of FcεRI β-subunit or c-*kit* protein were investigated by *in situ* hybridization histochemistry. Serial sections of the skin of +/+ and *Ws/Ws* rats were cut; sections of odd numbers were used for *in situ* hybridization and sections of even numbers were stained with alcian blue and nuclear fast red. In the skin of +/+ rats, cells expressing mRNA of FcεRI β-subunit or c-*kit* protein were detected. These cells were considered to be mast cells because in most cases they were stained with alcian blue in the adjacent sections (Figure 2). In the skin of *Ws/Ws* rats, cells expressing mRNA of FcεRI β-subunit or c-*kit* protein were not detectable.

The proportion of histamine content of *Ws/Ws* rats to that of +/+ rats was high in the brain and stomach fundus. We extended the estimation of FcεRI β-subunit and c-*kit* mRNA to the brain and stomach fundus. The forestomach was used as a control because the proportion of histamine content of *Ws/Ws* rats to that of +/+ rats was low in the forestomach, and the value was comparable to that of the skin (Table 2). The expression of FcεRI β-subunit mRNA was weak in the brain of +/+ rats and was not detectable in the brain of *Ws/Ws* rats. In contrast, the expression of c-*kit* mRNA was remarkably high in the brain of both +/+ and *Ws/Ws*

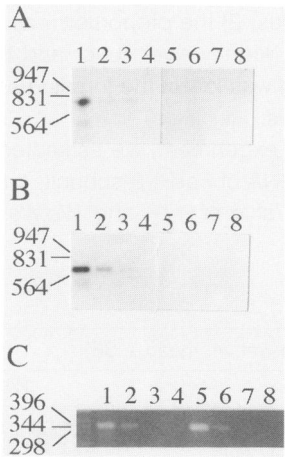

Figure 1. *Expression of FcεRI β-subunit,* c-kit, *or β-actin mRNA in the skin of +/+ and* Ws/Ws *rats. Total RNA from the skin of +/+ or* Ws/Ws *rats was reverse transcribed with FcεRI β-subunit,* c-kit, *and β-actin primers and then PCR-amplified with FcεRI β-subunit* (A), c-kit (B), *or β-actin* (C) *primer for 20 cycles. PCR products from +/+ rat RNA* (lanes 1 *to* 4) *and* Ws/Ws *rat RNA* (lanes 5 *to* 8) *were separated by electrophoresis. Amounts of RNA used for the reverse transcription were 5 μg* (lanes 1 *and* 5), *0.5 μg* (lanes 2 *and* 6), *0.05 μg* (lanes 3 *and* 7), *and 0.005 μg* (lanes 4 *and* 8), *respectively. DNA was transferred to nylon membrane and hybridized with each digoxigenin-labeled probe* (A *and* B) *or was directly stained with ethidium bromide on the gel* (C).

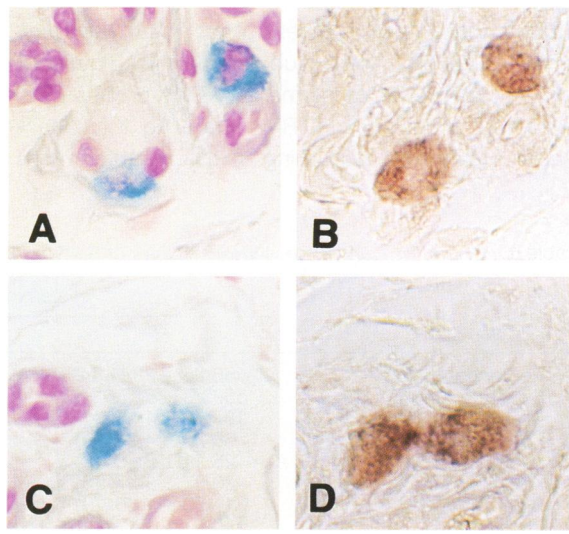

Figure 2. *Cells expressing FcεRI β-subunit or* c-kit *mRNA in the skin of +/+ rats. A section was stained with alcian blue and nuclear fast red* (A) *and the adjacent section was hybridized with cRNA probe for FcεRI β-subunit* (B). *Another section was stained with alcian blue and nuclear fast red* (C) *and the adjacent section was hybridized with cRNA probe for* c-kit (D). *Magnification* × 726.

rats. No significant difference was observed between +/+ and *Ws/Ws* rats (Figure 3). The expression of FcεRI β-subunit mRNA in the stomach fundus and forestomach of *Ws/Ws* rats was less than 1% that of +/+ rats (Figure 3). The expression of c-*kit* mRNA was apparently detectable in the stomach fundus and forestomach of +/+ rats but not detectable in those tissues of *Ws/Ws* rats (Figure 3).

Discussion

Histamine content in the skin of *Ws/Ws* rats was 0.3% that of +/+ rats. When the number of mast cells in the skin of *Ws/Ws* rats was counted by staining them with alcian blue, the number was also 0.3% that of +/+ rats.[1] Therefore, histamine in the skin seemed to be concentrated to alcian blue–positive mast cells. The possibility that alcian blue–negative immature mast cells contain a significant amount of histamine is not plausible. Histamine content in the peritoneal cells and the forestomach also seemed to be concentrated to mast cells. A large amount of histamine was detectable in the stomach fundus of *Ws/Ws* rats. Because no alcian blue–positive mast cells were observed in the stomach fundus of *Ws/Ws* rats, histamine is considered to be contained by enterochromaffinlike cells as in the case of *W/W^v* mice.[13] Histamine secreted by enterochromaffinlike cells stimulates the hydrochloric acid secretion from parietal cells.[25] However, more than a half of histamine in the gastric fundus of +/+ rats seemed to be contained by mast cells. Thus *Ws/Ws* rats may be useful for studying the mechanism of histamine-induced hydrochloric acid secretion, because most histamine is contained by enterochromaffinlike cells in the stomach fundus of *Ws/Ws* rats. Furthermore, *Ws/Ws* rats may be more suitable than *W/W^v* mice for such an experiment due to their bigger size.

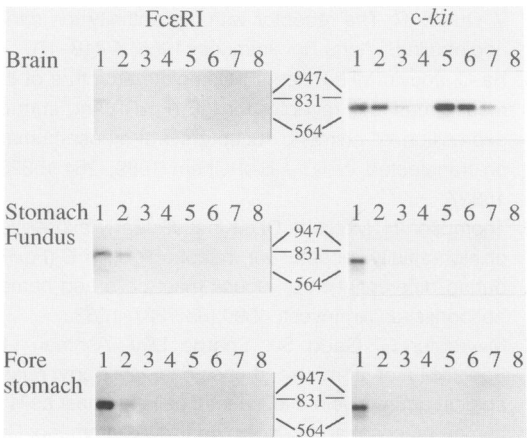

Figure 3. *Expression of FcεRI β-subunit or* c-kit *mRNA in the brain, stomach fundus, and forestomach of +/+ and* Ws/Ws *rats. Total RNA from the brain, stomach fundus, or forestomach of +/+ or* Ws/Ws *rats was reverse transcribed with FcεRI β-subunit and* c-kit *primers and then PCR-amplified with FcεRI β-subunit or* c-kit *primers for 20 cycles. PCR products from +/+ rat RNA* **(lanes 1** *to* **4)** *and* Ws/Ws *rat RNA* **(lanes 5** *to* **8)** *were separated by electrophoresis. Amounts of RNA used for the reverse transcription were 5 μg* **(lanes 1** *and* **5)**, *0.5 μg* **(lanes 2** *and* **6)**, *0.05 μg* **(lanes 3** *and* **7)**, *and 0.005 μg* **(lanes 4** *and* **8)**, *respectively. DNA was transferred to nylon membrane and hybridized with each digoxigenin-labeled probe.*

The proportion of histamine content of *Ws/Ws* rats to that of +/+ rats was 59% in the brain, indicating more than half of histamine is contained by cells other than mast cells in the brain. Cell bodies of histaminergic neurons are concentrated in the tuberomammillary nucleus of the posterior hypothalamus and extend efferent fibers to almost all parts of the brain.[26] The turnover of neuronal histamine is faster than that of mast cell histamine, and histamine in the neuron was depleted completely by the treatment of α-fluoromethylhistidine, a suicide inhibitor of histidine decarboxylase, histamine synthesizing enzyme.[27] Because the brain histamine in *Ws/Ws* rats represents only non-mast-cell histamine, *Ws/Ws* rats may be a useful tool for studying the role of histamine derived from neurons.[28] The proportion of histamine content of *Ws/Ws* rats to that of +/+ rats was intermediate in the spleen, kidney, liver, lung, and heart. Types of histamine-containing cells other than mast cells remain to be clarified in these tissues.

Expression of either FcεRI β-subunit or c-*kit* mRNA in the skin of *Ws/Ws* rats was estimated to be less than 1% that of +/+ rats. Although this value is semiquantitative in nature, it is well consistent with the relative histamine content and the relative number of alcian blue–positive mast cells in the skin of *Ws/Ws* rats. Moreover, cells in the skin of +/+ rats expressing either FcεRI β-subunit or c-*kit* mRNA were stained with alcian blue, and no such cells were detectable in the skin of *Ws/Ws* rats. These results suggested that levels of FcεRI β-subunit and c-*kit* expression in alcian blue–negative immature mast cells were not significantly high.

Expression of FcεRI β-subunit mRNA was weak in the brain of +/+ rats and not detectable in the brain of *Ws/Ws* rats. This is consistent with the low histamine content in the brain of both +/+ and *Ws/Ws* rats. FcεRI-expressing and histamine-containing mature mast cells appeared to be few in the brain of +/+ rats and not present in the brain of *Ws/Ws* rats. In contrast, the expression of c-*kit* mRNA was remarkable in the brain of both +/+ and *Ws/Ws* rats. Moreover, no significant difference was observed between +/+ and *Ws/Ws* rats, suggest-

ing that c-*kit* mRNA was expressed by cells other than mast cells. In fact, Hirota et al[24] has demonstrated the expression of c-*kit* mRNA in neurons located at the cerebellum, hippocampus, and olfactory bulb of rats by *in situ* hybridization histochemistry. Despite high histamine content in the stomach fundus of *Ws/Ws* rats, neither FcεRI β-subunit nor c-*kit* mRNA was detectable. This clearly indicates that histamine detected in the stomach fundus of *Ws/Ws* rats is contained by cells other than mast cells.

The skin and forestomach of *Ws/Ws* rats practically lacked histamine-containing, FcεRI-expressing and c-*kit*-expressing cells. The lack of histamine-containing cells is consistent with the result of *W/W^v* mice.[13] Probably, FcεRI-expressing and c-*kit*-expressing cells are also deficient in the skin and forestomach of *W/W^v* mice, which have been used successfully for studying the physiological roles of mast cells.[29–32] The present result may increase the usefulness of *Ws/Ws* rats as a mast-cell-deficient animal.

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