Mice lacking histidine decarboxylase exhibit abnormal mast cells

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Abstract Histidine decarboxylase (HDC) synthesizes histamine from histidine in mammals. To evaluate the role of histamine, we generated HDC-deficient mice using a gene targeting method. The mice showed a histamine deficiency and lacked histamine-synthesizing activity from histidine. These HDC-deficient mice are viable and fertile but exhibit a decrease in the numbers of mast cells while the remaining mast cells show an altered morphology and reduced granular content. The amounts of mast cell granular proteases were tremendously reduced. The HDC-deficient mice provide a unique and promising model for studying the role of histamine in a broad range of normal and disease processes.

Key words: Mast cell; Rodent; Knockout; Histamine

1. Introduction

Histamine is synthesized by a unique enzymatic reaction with L-histidine decarboxylase (HDC). In the pathological skin condition, histamine is involved in the induction of itching, flaring and edema [1,2]. In addition, various studies have suggested a close relationship between wound healing [3], embryogenesis [4], hematopoiesis [5] and malignant growth [6,7] with histamine production. From these points, establishing knockout mice with defective histamine synthesis was thought to be important, and therefore, we introduced a mutation into the *HDC* gene that resulted in mice without HDC activity.

The biological role of histamine has been extensively studied with pharmacological approaches using specific receptor agonists and antagonists [8] or using histamine synthesis inhibitors [9]. Due to the overlapping and sometimes antagonistic function of the receptors in the presence of endogenous histamine, receptor-blocking alone cannot achieve complete elimination of the histamine system. And also, histamine receptors might cryptically bind to substances other than histamine. Although the histamine synthesis blocker α-fluoromethyl histidine (α-FMH) significantly decreases the level of histamine in various organs, it is difficult to achieve complete and long lasting elimination of histamine in vivo in this manner. Therefore, we concentrated on the elimination of histamine biosynthesis by an ES cell-mediated gene targeting approach.

2. Materials and methods

2.1. Generation of HDC gene knockout mice

We designed the *HDC* targeting construct to replace a ~2.4 kb fragment extending from intron 5 to exon 9 with a PGK promoter-*neo*^{r} cassette. PCR positive ES clones were confirmed for proper homologous recombination by Southern blot in two steps. First we checked whether there was a homologous recombination-based insertion at the 3′ homology arm in the candidate cell lines by using *Pst*I-digested and *Eco*RI-digested DNA and the *Bam*HI–*Stu*I internal probe to get a 6.5 kb and an 11 kb band as shown in Fig. 1A. Then we showed the loss of the wild type *Stu*I band in animals homozygous for the targeted allele (Fig. 1B).

2.2. Northern blot, HDC activity and histamine analysis

20 μg of the RNA extracted from the kidney was examined by Northern blot analysis using *HDC* cDNA as a probe. The low histamine (0.6 nmol/g diet) containing diets (L) were purchased from a commercial source (Nihon Nosan Kogyo K.K., Yokohama, Japan). The histamine content and HDC activity of the organs were measured essentially by the method as described in [10]. Briefly, for the histamine assay, organs were homogenized in 10 volumes of 3% perchloric

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Abbreviations: HDC, histidine decarboxylase; α-FMH, α-fluoromethyl histidine; BMMC, bone marrow-derived mast cell; MMCP, mouse mast cell protease; MC-CPA, mast cell carboxypeptidase A

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acid containing 5 mM sodium EDTA by a Polytron homogenizer (Kinematica, Lucern, Switzerland) and centrifuged at 10 000×*g* for 30 min at 4°C. An aliquot of 50 μl of the supernatant was injected into the high performance liquid chromatography system in which histamine was separated on a cation-exchanger TSK SP-2SW (6 mm inside diameter×15 cm, Toso, Tokyo, Japan) column and automatically mixed with *o*-phthalaldehyde in alkaline conditions. Fluorescence was measured with a fluoromonitor (L-7480, Hitachi, Tokyo, Japan) using excitation and emission wavelengths of 360 and 450 nm, respectively. The HDC activity was expressed as the amount of histamine synthesized from histidine per min per mg of protein. Protein was measured with the Protein Assay system (Bio-Rad, Tokyo, Japan).

2.3. Light and electron microscopy of mast cells

Bone marrow-derived mast cells (BMMCs) were prepared as described by Razin et al. [11] with minor modifications. The bone marrow cells were cultured at a concentration of $0.5–1\times10^6$ nucleated cells/ml in RPMI 1640 (Life Technologies, Tokyo, Japan) supplemented with 10% fetal calf serum (ICN Biomedicals, Aurora, OH. USA), 5 ng/ml interleukin-3 (R&D systems, Minneapolis, MN, USA), 100 IU/ml penicillin and 100 μg/ml streptomycin. Non-adherent cells were transferred to fresh BMMC medium at least once a week. After 10 weeks, BMMCs were stained with toluidine blue. For the peritoneal cell staining, 5 ml phosphate-buffered saline was injected into the peritoneal cavity and after slight massage it was collected and stained using the alcian–safranin method [12]. Tissues for electron microscopy were fixed in Karnovsky's and embedded in epoxy resin and analyzed using an electron microscope.

2.4. Reverse transcriptase (RT)-PCR

The cDNA prepared from peritoneal cell suspension via RNA was serially diluted in a 10-fold fashion and amplified with PCR. PCR was carried out with specific sets of primers. The primers used in PCR were as follows (5′ to 3′): mouse mast cell protease (MMCP)-4, GTG TTG AGT CTA GAC CAC ATT CTC G and ACA GGC CTC TTT ATC CAT GAT TCT C; MMCP-5, CCT GGG TTC CAG CAC CAA AG and TGG ACA ACC AAA TTC TCA TC; mast cell carboxypeptidase A (MC-CPA), TAT GAC TGT GGA TTT CCG AG and TAT AAT GGA TCT TCA AC; glycerol 3-phosphate dehydrogenase, GGT GCT GAG TAT GTC GTG GA and TGG TCC TCA GTG TAG CCC AA.

2.5. Western blot analysis

Peritoneal cells were solubilized in 50 μl of SDS–PAGE electrophoresis sample buffer/1×10^6 cells. These extracts were subjected to SDS–PAGE. The antiserum to MMCP-4 was produced in rabbits, whereas antisera to MMCP-5 and MC-CPA were produced in rats [13]. The second antibodies were either goat anti-rabbit Ig (Amersham) or goat anti-rat Ig (Sigma) conjugated to horseradish peroxidase. The filters were developed with the ECL system (Amersham) according to the protocol provided by the manufacturer.

3. Results

3.1. Creating a null allele of the HDC gene

The murine *HDC* gene contains 12 exons and spans 23 kb of genomic DNA [14]. Using gene targeting, we replaced intron 5 to exon 9 with an inverted PGK promoter-driven neomycin phosphotransferase (*neo*) gene (Fig. 1A). This replacement resulted in a null mutation since exon 8 contains the coding sequence for the putative binding site (TFNPSKW) to which pyridoxal 5′-phosphate, the coenzyme of HDC protein, is supposed to bind [15]. We analyzed the genotypes of 180 pups (Fig. 1B) from intercrosses between heterozygous mice, which showed the expected Mendelian heredity (28.9% wild type, 49.4% heterozygous, 21.7% homozygous), suggesting that HDC is not crucial for embryonic development. In addition to the homozygous viability, successive intercrosses suggested normal fertility. Using Northern blot analysis, we could not detect the expression of the *HDC* gene from the targeted allele (Fig. 1C). Therefore, we concluded that a null allele had been created.

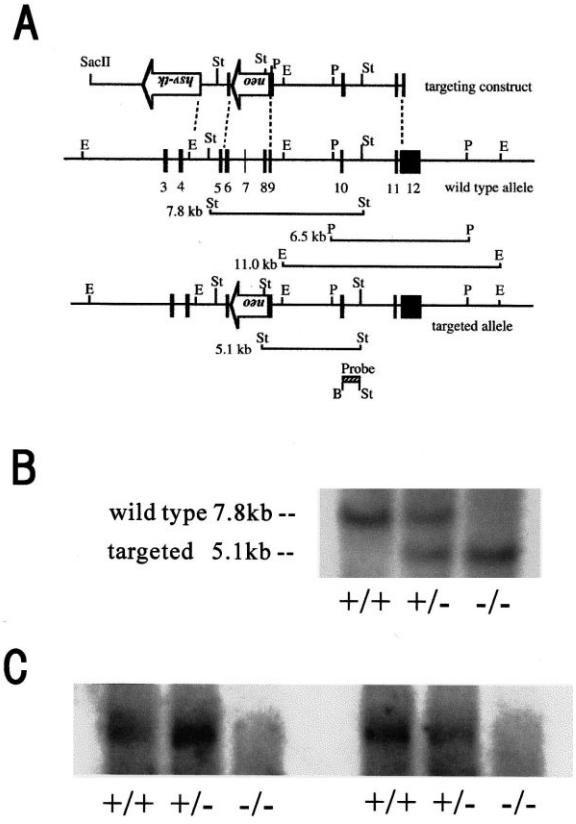

Fig. 1. Generation of a targeted mutation in *HDC* gene. A: Targeting strategy for removal of exons 6–8. The exons are indicated by black boxes. The probe used for Southern blotting analysis of the 3′ arm recombination is shown as a shaded bar and the diagnostic restriction fragments with their expected sizes as lines. B, *Bam*HI; E, *Eco*RI; P, *Pst*I; St, *Stu*I. B: The three genotypes are represented in the F2 generation. C: Northern blot analysis using the *HDC* probe, revealing a predominant 2.7 kb fragment in the kidney of $HDC^{+/+}$ and $HDC^{+/+}/HDC^{\Delta6-8}$ mice but not in $HDC^{\Delta6-8}/HDC^{\Delta6-8}$ mice.

3.2. Histamine levels and histamine synthesis in the HDC-deficient animals

The histamine levels and the histamine synthesis activity were measured in various organs of $HDC^{\Delta6-8}/HDC^{\Delta6-8}$ mice under low histamine diet (L) conditions (Table 1). The tissues of the $HDC^{\Delta6-8}/HDC^{\Delta6-8}$ animals lacked the histamine synthesis activity. The tissue histamine levels of various organs were practically zero except for that of brain.

3.3. Mast cell histology and biochemistry

The mast cell numbers in 13 randomly chosen microscope fields in mesenterial membrane preparations were evaluated. Wild type mice had 94 ± 11 cells/field, whereas $HDC^{\Delta6-8}/HDC^{\Delta6-8}$ mice had 32.9 ± 7.1 cells/field, representing a significant decrease of 65%. In the BMMC preparation, mast cells of $HDC^{\Delta6-8}/HDC^{\Delta6-8}$ mice showed pale and sparse granules compared to those of the wild type mice with toluidine blue staining (Fig. 2A,B). The number of the mast cells determined by safranin staining was reduced in the $HDC^{\Delta6-8}/HDC^{\Delta6-8}$ mice peritoneal cell suspension (Fig. 2C,D). Electron microscopy (Fig. 2E–H) revealed further details of the reduced gran-

Table 1
Histamine content (H) and HDC activity (HDC) in organs of animals segregating a HDC null allele

Organ	Genotype	HDC^{+}/HDC^{+}	$HDC^{+}/HDC^{\Delta 6-8}$	$HDC^{\Delta 6-8}/HDC^{\Delta 6-8}$
Plasma	H	0.32 ± 0.06	0.13 ± 0.01	0.04 ± 0.01
Skin	H	21.22 ± 4.25	19.21 ± 0.29	0.26 ± 0.06
	HDC	2.12 ± 0.56	0.60 ± 0.08	0.00 ± 0.05
Brain	*H	58.67 ± 9.83	31.61 ± 5.15	18.41 ± 2.74
	HDC	0.24 ± 0.04	0.12 ± 0.01	0.00 ± 0.00
Stomach	H	4.36 ± 0.50	2.76 ± 0.22	0.10 ± 0.05
	HDC	1.98 ± 0.22	1.23 ± 0.32	0.03 ± 0.02
Spleen	H	0.94 ± 0.52	0.19 ± 0.04	0.04 ± 0.01
	HDC	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00
Kidney	H	2.11 ± 1.28	1.07 ± 0.12	0.01 ± 0.00
	HDC	18.03 ± 0.01	13.13 ± 1.44	0.01 ± 0.00

Histamine levels are given in: *pmol/g tissue for brain, nmol/g tissue for skin, stomach, spleen and kidney and nmol/ml for plasma. HDC concentrations are given in pmol/mg protein/min (mean ± S.E.M., $n = 4$).

ular contents. The mast cells of $HDC^{\Delta 6-8}/HDC^{\Delta 6-8}$ mice were very heterogeneous in terms of staining strength of their granule. Some mast cells possessed fairly empty granules (Fig. 2F); on the other hand some other mast cells possessed granules with smaller granular contents inside them (Fig. 2H). The total number of secretory granules per mast cell of $HDC^{\Delta 6-8}/HDC^{\Delta 6-8}$ mice seemed to be similar to that of wild type mice.

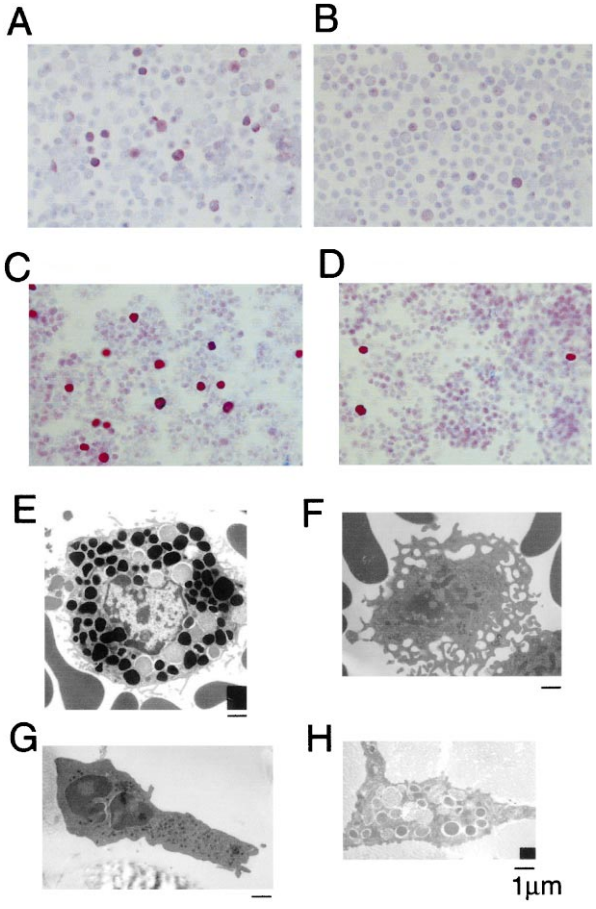

Fig. 2. A,B: BMMC preparation from wild type (A) or $HDC^{\Delta 6-8}/HDC^{\Delta 6-8}$ (B) mice stained with toluidine blue. C,D: Peritoneal cell suspension from wild type (C) or $HDC^{\Delta 6-8}/HDC^{\Delta 6-8}$ (D) mice stained with alcian blue and counter-stained with safranin O. Transmission electron microscopy of mast cells from the peritoneum (E,F), from the skin of the back (G,H) of wild type (E,G) and $HDC^{\Delta 6-8}/HDC^{\Delta 6-8}$ (F,H) mice.

The reduced granular content of the mast cells was very interesting since histamine is not the major component of the mast cell granules. Because the reduction in the contents of the granules may reflect reduced granular protease, we assessed the mast cell proteases in terms of their mRNA expression and protein level using RT-PCR analysis and Western blotting, respectively. The amount of MMCP-4, MMCP-5 and MC-CPA was decreased in $HDC^{\Delta 6-8}/HDC^{\Delta 6-8}$ mice in Western blotting (Fig. 3B), whereas the RNA expression level seemed to be not so affected (Fig. 3A). Histamine therefore might be one of the important mediators for maintaining proteases in the mast cell secretory granules or it might affect

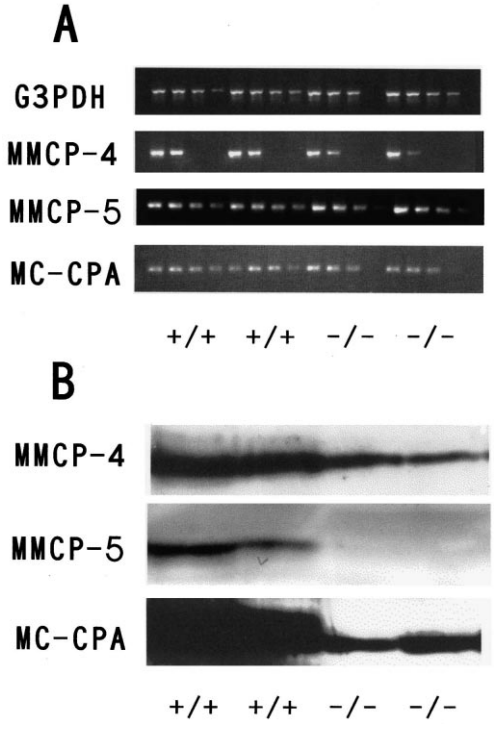

Fig. 3. The protease expression in peritoneal cells. A: RNA expression was analyzed using RT-PCR. Each PCR amplification set from one animal was comprised of four serially 10-fold dilutions of cDNA as the template and assessed on 2% agarose gel. B: Western blotting was carried out for the assessment of protease at the protein level. The extracts from 5×10^5 cells were subjected to SDS–PAGE.

the synthesis of these proteases. Further study is needed to explain the underlying mechanism.

4. Discussion

HDC is the unique enzyme that produces histamine. Gene targeting of the *HDC* gene was performed to create histamine-deficient mice. The targeting vector used in this study was designed to replace exons 6–8 and part of exon 9 of the *HDC* gene with an inverted *neo* selectable marker. We anticipated that, even if a truncated protein were produced from the targeted locus, this alteration would remove the putative binding site of pyridoxal 5′-phosphate, an essential coenzyme for HDC activity [15], and, therefore, would render the protein non-functional. Interestingly, however, the inverted *neo* selectable marker abolished the *HDC* gene expression at the transcriptional level. This finding further supports our previous observation that the inverted *neo* marker has an efficient transcriptional knockdown effect on a targeted allele [16].

$HDC^{\Delta 6-8}/HDC^{\Delta 6-8}$ mice lack histamine-synthesizing activity from histidine and basically lack histamine in several organs. Although the results of human genomic DNA blot hybridization analysis strongly suggested that HDC is specified by a single gene [17], because a certain amount of histamine was detected in the brain of $HDC^{\Delta 6-8}/HDC^{\Delta 6-8}$ mice, there is still a possibility that, in brain, there is another enzyme catalyzing histamine production. Blocking the endogenous production of histamine does not necessarily create a histamine deficiency, as dietary histamine intake can be an alternative source. Therefore, in the experiments described we decided to control for the dietary histamine uptake by supplementing the histamine-deficient diet with a defined amount of histamine.

Tissue histamine is localized in the specialized membrane-enclosed secretory granules of mast cells. The granules, which account for $\sim 50\%$ of the total mast cell volume, are comprised primarily of heparin and basic proteins. Although the nature of the mechanism by which histamine is stored in granules has not been established, there is evidence suggesting that heparin provides the histamine binding sites [18,19]. The reduced granulation phenotype of mast cells in the case of histamine deficiency is interesting since histamine is not the major component of the mast cell granules. Because the subcutaneous and peritoneal mast cells of $HDC^{\Delta 6-8}/HDC^{\Delta 6-8}$ mice stained weakly with safranin, they appeared to contain less heparin in the granules. Possibly, the lack of histamine could disturb the normal electrostatic balance between the positively charged histamine molecules and the negatively charged heparin proteoglycan in such a way that heparin storage is impaired. It is noteworthy that the lack of heparin by gene disruption results in a similar phenotype in the secretory granules, with a large reduction in the overall contents of secretory granule components, including histamine and proteases [13,20]. The present findings thus indicate that also the lack of histamine could result in a general reduction in the secretory granule contents. Further evidence for this was obtained when the contents of mast cell proteases were analyzed at the protein level by Western blotting. These experiments demonstrated a large reduction of the mast cell proteases MMCP-4, MMCP-5 and CPA in the $HDC^{\Delta 6-8}/HDC^{\Delta 6-8}$ mice. Since all of these proteases have high positive net charges, their reduction in the $HDC^{\Delta 6-8}/HDC^{\Delta 6-8}$ mice could be an effect of a possible overall disturbed electrostatic balance in the granules, e.g. a reduction in balancing negatively charged heparin proteoglycan to interact with.

For revealing the role of histamine, earlier attempts involved the injection of HDC blockers such as α-FMH into animals. Even though α-FMH significantly decreased the level of histamine in various organs, it was difficult to achieve complete and long lasting elimination of histamine in vivo. Here we demonstrated that the targeted deletion of the *HDC* gene provides a unique in vivo model of histamine deficiency. Studies using $HDC^{\Delta 6-8}/HDC^{\Delta 6-8}$ mice will enable a detailed understanding of the role of histamine in various physiological and pathological processes.

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