

Altered *in vitro* apoptosis of cultured mast cells prepared from an inbred strain of mice, NC/Kuj

T. HIRAGUN, E. MORITA, H. SHINDO, T. TANAKA, Y. KAMEYOSHI, T. OKABE, M. KANNO* and S. YAMAMOTO

Department of Dermatology and *Immunology and Parasitology, Hiroshima University School of Medicine, Hiroshima, Japan

Summary

Background An inbred strain of mice, NC, develops dermatitis associated with highly elevated serum IgE and dermal mast cell hyperplasia.

Objectives and methods To clarify the mechanisms for the dermal mast cell hyperplasia in NC, we prepared bone marrow-derived mast cells (BMMCs) from three strains of mice, NC/Kuj, C57BL/6 and BALB/c, and compare histamine contents, histamine release abilities, adhesive properties and apoptosis of the BMMCs.

Results Compared with BMMCs obtained from C57BL/6 and BALB/c, NC/Kuj BMMC possessed higher histamine content and higher adhesive ability to plastic plates, although histamine release from BMMCs was found to be similar in the three strains. The most intriguing finding is the lack of apoptosis in the BMMCs from NC/Kuj upon growth factor deprivation as determined by DNA ladder formation and loss of membrane phospholipid asymmetry.

Conclusion The altered *in vitro* properties of mast cells in NC/Kuj partially account for an increase of dermal mast cells, which might be involved in the development of skin lesions in NC.

Keywords: adhesion, apoptosis, histamine, mast cells, NC

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Introduction

An inbred strain of mice, NC, which was established from Japanese fancy mice by Kondo in 1955 [1], has been known to spontaneously develop skin lesions. Recently, Matsuda *et al.* [2] have found that this strain was able to maintain intact in their skin integrity when bred under specific pathogen-free conditions, whereas the mice develop dermatitis when they were bred in conventional circumstances. They also have reported that their serum immunoglobulin (Ig) E levels are elevated in association with the development of skin lesions, indicating that immediate-type hypersensitivity to some environmental factors underlies their manifestation of skin lesions. Among the environmental factors we have found that the fur mite-infection is a potential factor in the development of skin lesions [3].

Mast cell hyperplasia has been observed as a prominent finding in the skin [2–4], indicating the possibility that a dysregulated function of mast cells is behind the induction of skin lesions in NC. To test this hypothesis, we prepared mast cells from bone marrow in NC and investigated some of their functional and biochemical properties.

Materials and methods

Animals

NC/Kuj, which is a substrain of original NC, were kindly provided by Dr J. Hayakawa (Institute for Experimental Animals, Faculty of Medicine, Kanazawa University, Kanazawa, Japan) and bred in the Research Facilities for Laboratory Animal Science, Hiroshima University School of Medicine. Six-week-old male NC/Kuj were used for experiments. Male, 6-week-old C57BL/6 and BALB/c mice were purchased from Japan SLC (Hamamatsu,

Correspondence: T. Hiragun, Department of Dermatology, Hiroshima University School of Medicine, Kasumi 1–2-3, Minami-ku, Hiroshima 734–8551, Japan (e-mail: hiragun@ipc.hiroshima-u.ac.jp).

Japan). All strains of mice were bred under specific pathogen-free condition.

Mast cell count and histamine content in skin

Approximately 1 cm² skin were carefully excised from the back of NC/Kuj, C57BL/6 and BALB/c, under anaesthesia, and divided into two pieces. One piece was immediately placed in 8% neutral-buffered formaldehyde (pH 7.4), embedded in paraffin, and cut into sections of 5- μ m thickness. Sections were stained with toluidine blue, and mast cells in the dermis were counted under a microscope at a magnification of $\times 400$ and expressed as the mean number of the cells in five fields. The other pieces of skin was cut into small pieces, denatured with 0.25 mol/L HClO₄ and histamine content in samples was determined by using an automated histamine analyser (Tosoh corporation, Osaka, Japan).

Preparation of bone marrow-derived mast cells (BMMCs)

BMMCs were prepared from 6-week-old male mice according to the method described previously [5–7]. Briefly, bone marrow cells were suspended at a density of 1×10^6 cells/mL in α -MEM (ICN Biomedicals, Aurora, OH, USA) containing 10% fetal calf serum (FCS), 2 mmol/L of glutamine, 100 U/mL penicillin G and 100 μ g/mL streptomycin (complete α -MEM) supplemented with 50 μ mol/L 2-mercaptoethanol, 5 ng/mL of recombinant murine IL-3 (rmIL-3, R & D Systems, Minneapolis, MN, USA) and 2 ng/mL of rmIL-4 (R & D Systems) and cultured at 37 °C in a humidified atmosphere of 5% CO₂ in air. Nonadherent cells were collected by centrifugation and resuspended in fresh medium every 7 days. After 4 weeks, more than 95% of the nonadherent cells were stained positively with alcian blue. Histamine content of BMMCs were determined by using an automated histamine analyser as described previously [7,8].

Histamine release assay

Histamine release assay of BMMCs was performed according to the method previously described [9]. Suspensions of BMMCs were incubated with 0.5 μ g/mL rat antidinitrophenyl (DNP) IgE overnight. The BMMCs were washed twice with complete α -MEM, and then 2×10^5 cells were dispensed in 1.5 mL polypropylene test tubes containing a series of concentrations of human serum albumin (HSA)-DNP (1–1000 ng/mL). After incubation at 37 °C for 30 min, the samples were centrifuged (150 g for 5 min at 4 °C), and then supernatants were mixed with same volume of 0.5 mol/L HClO₄. Cell pellets in the test tubes were resolved in 1 mL of 0.25 mol/L HClO₄ to release histamine. Histamine content in samples was determined by using an automated

histamine analyser and histamine release was expressed as percentage of total histamine content in the cells.

Adhesion assay

BMMCs (1×10^5 cells/well) were plated in 96-well multiwell plates (Becton Dickinson Labware, Franklin Lakes, NJ, USA) and incubated in fresh complete α -MEM supplemented with 5 ng/mL of rmIL-3 and 2 ng/mL of rmIL-4 for 3 h at 37 °C. The sample wells were gently washed twice with PBS and finally replaced with complete α -MEM containing 5 ng/mL rmIL-3 and 2 ng/mL rmIL-4. The number of adherent cells was calculated by using Cell Counting Kit[®] (Dojindo Laboratories, Kumamoto, Japan), which is constructed by a colourimetric assay based on the cleavage of the tetrazolium salt WST-1 to a formazan dye by mitochondrial dehydrogenase of viable cells [10]. The number of the adherent cells was estimated from the absorbance values obtained from the serially diluted BMMC standard samples (1×10^5 – 6.25×10^3 cells/well).

In an experiment, adhesion assay of BMMCs was performed in the presence of various concentrations of RGDS or RGEs peptides (Bachem California, Torrance, CA, USA).

Detection of apoptosis

BMMCs were deprived of growth factor for the indicated intervals. The cultured cells excluding trypan blue dye were counted using an improved Neubauer haemocytometer. After two days of growth factor-deprivation, genomic DNA was extracted from 1×10^6 of BMMCs by using Sepa Gene[®] (Sanko Junyaku, Tokyo, Japan) and the DNA laddering was detected by agarose gel electrophoresis. To detect the loss of membrane phospholipid asymmetry, BMMCs were double-stained with FITC-conjugated annexin 5 and propidium iodide in Apoptosis detection kit[®] (R & D Systems), and analysed by flow cytometer (FACSCalibur, Becton Dickinson Immunocytometry Systems, San Jose, CA, USA) according to the manufacturer's instructions [11–13].

Statistical analysis

Data were analysed by Mann–Whitney's *U*-test. Probability (*P*) values less than 0.05 were considered to be significant.

Results

Mast cell counts in skin

Mast cell counts in skin specimens of NC/Kuj were more than twice compared with those of C57BL/6 and BALB/c

Table 1. Mast cell counts and histamine contents of skin* of three strains of mice, NC/Kuj, C57BL/6 and BALB/c

Strains	<i>n</i>	Mast cell count (cells/HPF)	Histamine content ($\mu\text{g/g}$ wet tissue)
NC/Kuj	4	$50 \pm 10^{**}$	$66.4 \pm 3.5^{**}$
C57BL/6	4	23 ± 3	14.8 ± 0.7
BALB/c	4	19 ± 7	23.8 ± 0.6

*Approximately 1 cm^2 skin was obtained from the back of each mouse and divided into two pieces. One portion was prepared for microscopic examination and the other prepared for the assay of histamine content. $^{**}P < 0.01$ vs C57BL/6 and BALB/c.

(Table 1). Histamine contents were paralleled for the mast cell counts in the three strains of mice, NC/Kuj, C57BL/6 and BALB/c (Table 1).

Histamine release and adhesive properties of BMMCs

After 4 weeks of culturing bone marrow cells with IL-3 and IL-4, almost pure mast cell preparations were obtained from the three strains of mice, NC/Kuj, C57BL/6 and BALB/c. Histamine content of BMMCs obtained from NC/Kuj was significantly higher than those of C57BL/6 and BALB/c (Table 2). The BMMCs released histamine in a dose-dependent fashion for HSA-DNP after sensitization with anti-DNP IgE antibody, and the maximum release was achieved at a concentration of 100 ng/mL HSA-DNP. These cells released up to 70% of total histamine when stimulated with 100 ng/mL HSA-DNP. Percentage histamine release was found to be similar in these three strains of mice.

Table 2. Characteristics of BMMCs obtained from three strains of mice, NC/Kuj, C57BL/6 and BALB/c

Strains	Histamine contents* (pg/cell)	Histamine release** (%)		
		Medium	HSA-DNP (100 ng/mL)	Adhesion to plastic plates*** (%)
NC/Kuj	0.67 ± 0.09 ($n = 14$)****	5.2 ± 2.1	52.8 ± 0.9 ($n = 4$)	32.4 ± 1.0 ($n = 6$)****
C57BL/6	0.09 ± 0.04 ($n = 8$)	2.9 ± 1.4	65.2 ± 5.9 ($n = 4$)	9.3 ± 1.8 ($n = 6$)
BALB/c	0.04 ± 0.01 ($n = 6$)	2.5 ± 1.4	53.7 ± 9.4 ($n = 4$)	6.3 ± 1.4 ($n = 6$)

*Histamine contents of the BMMCs were determined with automated fluorometric-HPLC system after denaturation with 0.25 mol/L HClO_4 . **BMMCs were incubated with anti-DNP IgE overnight, then incubated with HSA-DNP to release histamine. Cells were centrifuged and histamine content of supernatants and cell pellets were determined. Data were expressed as percentages of total histamine content in the cells. ***BMMCs (1×10^5) were seeded in a 96-well multiwell plate and incubated for 3 h. Then the wells were washed with PBS and percentages of adherent cells were calculated by Cell Counting Kit[®]. Data are expressed as percentages of adherent cells from total cells. **** $P < 0.01$ vs C57BL/6 and BALB/c.

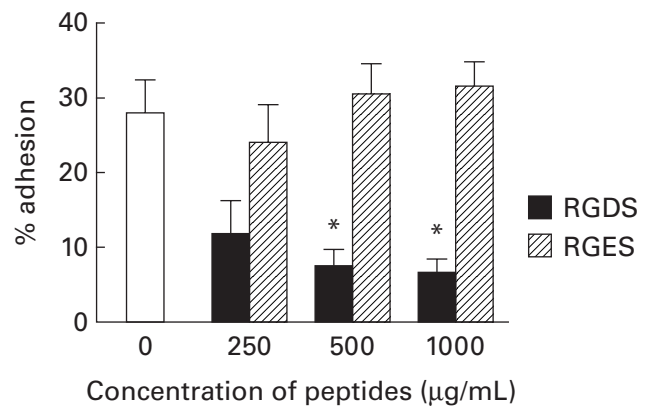


Fig. 1. Adhesion of BMMCs obtained from NC/Kuj was abolished by RGDS peptide. BMMCs (1×10^5) were plated and incubated in the presence or absence of RGD(E)S peptides for 3 h. Then the wells were washed twice with PBS and adherent cells were calculated by Cell Counting Kit[®]. Data are mean $\pm$ SEM of three independent experiments performed in duplicate and expressed as percentages of adherent cells from the total cells. $^{*}P < 0.01$ (vs control RGES peptide)

An adhesion of BMMCs obtained from NC/Kuj was significantly higher than those of C57BL/6 and BALB/c (Table 2). Since mast cells have been reported to express integrins on their cell surface that are involved in adhesion and activation of mast cells [14], next we examined the involvement of integrins on the enhanced adhesion of BMMCs from NC/Kuj. When the RGDS peptide, a specific inhibitor for the adhesion of cells to matrix components mediated by integrins, was added the peptide significantly reduced the adhesion of NC/Kuj BMMCs to the plastic plate in a dose-dependent manner, whereas a control RGES peptide had no effect (Fig. 1).

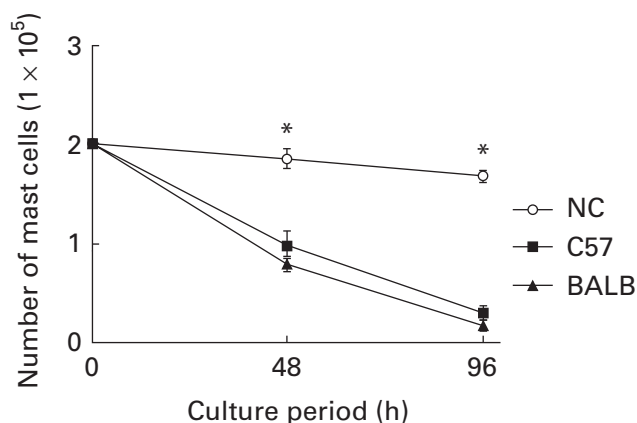


Fig. 2. BMMCs obtained from NC/Kuj survived upon IL-3 deprivation. BMMCs (2×10^5) obtained from NC/Kuj, C57BL/6 and BALB/c were cultured in the absence of IL-3 and IL-4 for 96 h. The numbers of BMMCs excluding trypan blue were counted. Data are mean $\pm$ SEM of three independent experiments. * $P < 0.01$ (vs C57BL/6 and BALB/c)

Apoptosis in BMMCs

Cell survival was compared among BMMCs from NC/Kuj, C57BL/6 and BALB/c in the absence of IL-3 and IL-4. Viability of BMMCs from C57BL/6 and BALB/c decreased rapidly and reached the minimum at 96 h after deprivation of the cytokines. In contrast with these BMMCs, the BMMCs obtained from NC/Kuj mostly survived even after 96 h (Fig. 2). Both BMMCs from C57BL/6 and BALB/c died by apoptosis on growth factor removal, as characterized by DNA fragmentation (Fig. 3), but a reduced extent of DNA laddering could be seen in NC/Kuj BMMCs.

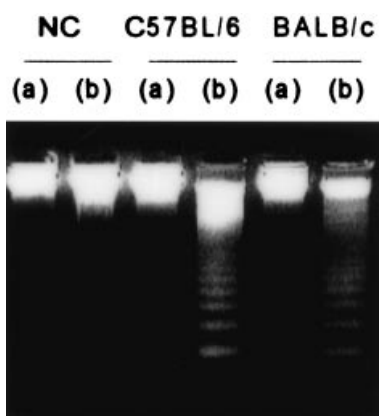


Fig. 3. DNA ladder formation in IL-3-deprived BMMCs. BMMCs obtained from NC/Kuj, C57BL/6 and BALB/c were cultured in the presence (a) or absence (b) of IL-3 and IL-4 for 48 h. Genomic DNAs were extracted and electrophoresed in 1% agarose gel

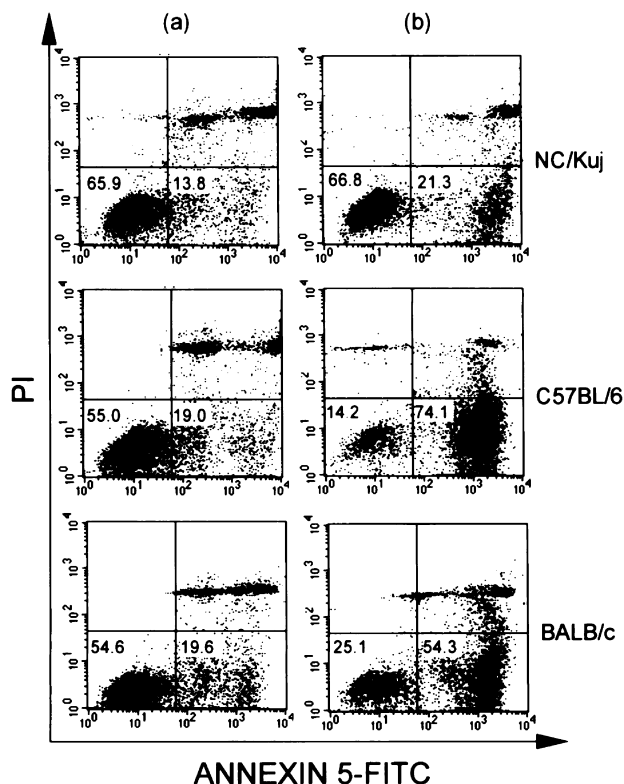


Fig. 4. Flow cytometric analysis of apoptosis in BMMCs. BMMCs obtained from NC/Kuj, C57BL/6 and BALB/c were cultured in the presence (a) or absence (b) of IL-3 and IL-4 for 48 h, then cells were stained with propidium iodide (PI) and FITC-conjugated annexin 5. Numbers in the figure represent the percentages of cells in corresponding areas

In addition, the percentages of annexin 5-positive apoptotic cells were remarkably increased in BMMCs obtained from C57BL/6 and BALB/c upon growth factor deprivation, however, no marked change was seen in NC/Kuj BMMCs (Fig. 4).

Discussion

Our investigation provides evidence that NC strain has a genetic abnormality in the development of mast cells, which results in dermal mast cell hyperplasia, and possible hypersensitivity to environmental antigens. A remarkably higher mast cell count in the skin was seen in 6-week-old NC/Kuj mice (Table 1), even if the mice were bred under specific pathogen-free conditions and had no skin lesions. The dermal mast cell hyperplasia in NC/Kuj is likely to be caused by the impaired apoptosis of the mast cell lineage. Although the histamine contents of dermal mast cells were found to be similar among these three strains of mice (Table 1), histamine content of BMMCs from NC/Kuj

was seven to 10 times higher than those of C57BL/6 and BALB/c when compared in the freshly prepared BMMCs from three strains (Table 2). One possible explanation of this discrepancy is that BMMCs are still immature mast cells, and BMMCs obtained from NC/Kuj differentiated further than those of C57BL/6 and BALB/c *in vitro*. We have already reported that, when BMMCs were co-cultured with 3T3 fibroblasts, they differentiated into connective tissue-type mast cells and their histamine contents increased to the levels similar to those of dermal mast cells [9]. The difference in histamine contents of these differentiated mast cells among three strains was far less than those found in BMMCs [9]. The higher histamine content of BMMCs obtained from NC/Kuj was not due to differentiation speed of mast cells because the histamine content of BMMCs in these three strains remained almost constant during the culture with IL-3 and IL-4 for up to 3 months (data not shown). This may be caused by an enhanced responsiveness to IL-3 and/or IL-4 during the culture with these cytokines. In addition, adhesive property of NC/Kuj BMMCs was also significantly higher than that of C57BL/6 or BALB/c (Table 2), implying another disorder in the function of NC mast cells. This enhanced adhesion to plastic plate was markedly inhibited by adding RGDS peptide (Fig. 1), which inhibits the binding of very late antigen-5 (VLA-5) or vitronectin receptor (VNR) to extracellular matrices, such as fibronectin and vitronectin, indicating that this enhanced adhesion of NC/Kuj BMMCs was due to the activated VLA-5 or VNR. As mouse mast cells are known to express VLA-4, VLA-5, VLA-6 and VNR [14–17], the enhanced adhesion of NC mast cells suggests the constitutive activation of these adhesion molecules on their surface.

The most intriguing finding in BMMCs from NC/Kuj is the lack of apoptosis upon growth factor deprivation. IL-3-dependent BMMCs undergo apoptosis upon deprivation of IL-3 [18], however, most BMMCs from NC/Kuj survived 96 h after IL-3 deprivation. The lack of apoptotic cells in NC/Kuj was confirmed by the reduction of both DNA ladder formation (Fig. 3) and loss of membrane phospholipid asymmetry (Fig. 4). These results suggest that this strain possesses some fundamental disorder(s) in mast cell differentiation, which is determined by the genetic background. Tsudzuki *et al.* [19] have studied the genetic background regulating dermatitis and hyperproduction of IgE in NC/Nga, and concluded that the dermatitis is controlled by one autosomal recessive gene and the IgE hyperproduction by two autosomal recessive genes. In this study we have not determined the molecule(s) responsible for the disorders in the growth of BMMCs in NC, but the abnormalities found in cultured NC mast cells are possibly due to the genes described by Tsudzuki *et al.*

In summary, BMMCs obtained from NC exhibit

abnormalities in their histamine content, adhesive property and apoptosis. These abnormalities may contribute to dermal mast cell hyperplasia and development of skin lesions in NC.

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