FEBS Open Bio 4 (2014) 342–346

journal homepage: www.elsevier.com/locate/febsopenbio

Establishment of a mast cell line, NCL-2, without *Kit* mutation, derived from NC mouse bone marrow

Takaaki Hiragun, Yuhki Yanase, Tsutomu Okabe, Makiko Hiragun, Mikio Kawai, Michihiro Hide *

Department of Dermatology, Integrated Health Sciences, Institute of Biomedical and Health Sciences, Hiroshima University, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8551, Japan

ARTICLE INFO

Article history:
Received 26 February 2014
Accepted 26 March 2014

Keywords:
Mast cells
Kit
FcεRI
Stem cell factor
IL-3

ABSTRACT

Immortal mast cell lines, such as RBL-2H3 and HMC-1 cells, are commonly utilized to investigate the function of mast cells. However, they are tumor cells carrying a gain-of-function mutation of *Kit*. We established an immortal mast cell line without *Kit* mutation, NCL-2, derived from NC mouse bone marrow. NCL-2 cells could be maintained without additional growth factors and thus could respond to exogenous growth signals. Moreover, NCL-2 cells expressed FcεRI and KIT, and release histamine and LTB_4 in response to antigen stimulation. This cell line could be a useful tool to analyze proliferation, differentiation, and function of normal mast cells.

1. Introduction

Mast cells play a pivotal role in type I hypersensitivity reactions via the high affinity IgE receptors (FcεRI) and antigen-specific IgE. The crosslinking of FcεRI leads to the activation of numerous signaling molecules, SYK, LAT, PLCγ, PKC, and finally results in degranulation, and arachidonic acid and cytokine production [1,2]. These mediators are involved in the pathogenesis of allergic disorders, such as urticaria, atopic dermatitis (AD), allergic rhinitis, and allergic asthma [2].

Previous studies on the function of mast cells, especially on degranulation and on signaling pathways mediated by FcεRI crosslinking, have been conducted mostly by the use of RBL-2H3 cells [3]. RBL-2H3 cells carry a gain-of-function mutation of *Kit* [4] thus they do not need exogenous growth factors, such as stem cell factor (SCF) or interleukin (IL)-3. Investigators can maintain the cells without expensive recombinant cytokines but they cannot assess cellular responses against SCF. In addition, it is possible that active KIT protein could affect other signaling pathways such as FcεRI-mediated signaling events [5]. Moreover, Passante et al. reported that RBL-2H3 cells share similarities with basophils and thus they are an imprecise model for mast cell mediator release [6]. MC/9 cells [7], the rodent mast cells without *Kit* mutation, need IL-3 to grow. HMC-1 cells, a human mast cell line derived from mast cell leukemia, carry a gain-of-function mutation of *Kit* and lack surface expression of FcεRI [8]. Another human mast cell line, LAD2 cells, responds to SCF but grows slowly even in the presence of 100 ng/ml of recombinant human SCF [9].

Here we established NCL-2 mast cell line without a gain-of-function of *Kit* mutation, which respond to SCF and IL-3, and release various chemical mediators against several stimuli including crosslinking of FcεRI.

2. Materials and methods

2.1. Reagents

Recombinant mouse IL-3 (rmIL-3) and stem cell factor (SCF) were purchased from R&D Systems (Minneapolis, MN, USA). Human serum albumin (HSA)-conjugated dinitrophenyl (DNP), ionomycin, and adenosine triphosphate (ATP) were from Sigma (St. Louis, MO, USA). Rat DNP-specific IgE monoclonal antibody was from Biosource International (Camarillo, CA, USA).

2.2. Animals

Male NC/Kuj mice (6 weeks) were kindly donated by Dr. J. Hayakawa, Institute for Experimental Animals, School of Medicine, Kanazawa University, Japan and Balb/c nude mice were purchased from Charles River Japan (Yokohama, Japan). Mice were maintained in closed racks with free access to food and water in the Institute of Laboratory Animal Science, Hiroshima University. This study was carried out in accordance with the Guideline for Animal

* Corresponding author. Tel.: +81 82 257 5235; fax: +81 82 257 5239.
E-mail address: ed1h-w1de-road@hiroshima-u.ac.jp (M. Hide).

http://dx.doi.org/10.1016/j.fob.2014.03.011

Experiments for Laboratory Animal Science Natural Science Center for Basic Research and Development (N-BARD), Hiroshima University.

2.3. Cells

The bone marrow-derived mast cells (BMMC) from 6 weeks old male mice were generated as previously described [10]. Briefly, bone marrow cells were suspended at a density of 1×10^6 cells/ml in Minimum Essential Medium α (MEMα) (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal calf-serum (FCS), 50 μM 2-mercaptoethanol, 2 mM L-glutamine, antibiotics (complete MEMα), and 5 ng/ml IL-3. The cells were cultured for 4 weeks, replacing half of the existing medium with fresh medium once a week. After 4 weeks, more than 98% of non-adherent cells were stained positively by alcian blue. The BMMC were subcultured about 100 times by 50-fold dilutions in the presence of IL-3. Subsequently, the cells were subcultured another 80 times by 100-fold dilutions without IL-3, and then a single clone (NCL-2) that grew without IL-3 was obtained by limiting dilution.

P815 murine mastocytoma cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen) supplemented with 10% FCS and antibiotics.

2.4. Release of histamine and LTB_4

NCL-2 cells were incubated in complete MEMα supplemented with 0.5 μg/ml of DNP-specific IgE overnight. After washing twice with complete MEMα, the cells were stimulated with antigen, ionomycin or ATP at the indicated concentration for 20 min at 37 °C. The supernatants were transferred into test tubes and centrifuged at 1500×*g* for 5 min at 4 °C to eliminate the cell components.

2.5. Measurement of histamine

After centrifugation, 200 μl of the supernatants were mixed with equal volumes of 0.5 N perchloric acid to precipitate proteins. Both cell pellets in test tube and residual cell in culture dishes were resolved in 1 ml of 0.25 N perchloric acid to recover all residual histamine. Histamine contents in the samples were measured fluorometrically by using an automated histamine analyzing system (Tosoh Corporation, Osaka, Japan) as described previously [11]. The histamine release was expressed as percentage of total cellular histamine.

2.6. Measurement of LTB_4

The amount of leukotriene B_4 (LTB_4) in the samples was measured using enzyme-immunoassay kits (Amersham, Buckinghamshire, UK). The measurements were performed according to the manufacture's instruction. The minimum detectable concentration of LTB_4 was 6.0 pg/ml and cross-reactivity for other related substances was less than 0.03%. In order to verify the measurement of LTB_4 by enzyme immunoassay, the supernatant of reaction mixture in one experiment was fractionated by reverse phase high performance liquid chromatography (HPLC) as described before [11].

2.7. Western blot analysis

For detection of IL-3-induced tyrosine phosphorylation of Jak2, 2.5×10^6 NCL-2 cells were stimulated with 100 ng/ml of rmIL-3 for 5 min at 37 °C, then quickly centrifuged at 10,000×*g* and lysed in the sample buffer (62.5 mM Tris–HCl, pH 6.8, 2% sodium dodecyl sulfate, 10% glycerol, 50 mM DTT, 0.1% bromophenol blue). The proteins were boiled at 95 °C for 5 min, centrifuged at 10,000×*g* for 10 min, electrophoresed with 5–20% polyacrylamide gel (Atto, Tokyo, Japan), and transferred to a PVDF membrane (Immobilon-P, Merck Millipore, Billerica, MA, USA). The membrane was incubated with rabbit anti-phospho-JAK2 polyclonal antibody (Biosource International, 1:1000 dilution) or anti-JAK2 antibody (Cell Signaling Technology, Beverly, MA, USA, 1:1000 dilution). After washed with Tris-buffered saline with Tween 20, the membrane was incubated with HRP-conjugated anti-rabbit IgG antibody (Cell Signaling Technology, 1:2000 dilution). The reacted antibodies were visualized by using ECL (GE Healthcare, Buckinghamshire, UK) under a luminescent image analyzer (LAS-1000 plus, Fuji Film, Tokyo, Japan).

For detection of SCF-induced tyrosine phosphorylation of KIT, NCL-2 cells (2.5×10^6) were stimulated with 100 ng/ml of rmSCF for 5 min at 37 °C and lysed in 100 μl of the lysis buffer (20 mM Tris–HCl, pH7.4, 150 mM NaCl, 10% glycerol, 1% NP-40, 1 mM phenylmethylsulphonyl fluoride, 0.15 U/ml aprotinin, 10 mM EDTA, 10 μg/ml leupeptin, 100 mM sodium fluoride, 2 mM sodium orthovanadate) at 4 °C for 30 min. Cell lysates were clarified by centrifuging for 10 min at 10,000×*g* at 4 °C and incubated with 30 μl of Protein G-Sepharose 4FF (GE Healthcare) to eliminate non-specific binding to Protein G. Then the samples were incubated with rat anti-mouse KIT monoclonal antibody (ACK2, GIBCO-BRL, 1:100 dilution) for 1 h at 4 °C. Protein G-Sepharose 4FF was used to collect the antigen-antibody complexes. These immunoprecipitatants were analyzed by immunoblotting using HRP-conjugated anti-phosphotyrosine antibody (4G10) (Merck Millipore, 1:2000 dilution) or anti-KIT antibody (ACK2, 1:1000 dilution).

2.8. Flow cytometric analysis of FcεRI and KIT

NCL-2 cells (2×10^5) were suspended in 100 μl of PBS(−) containing 0.125 μg of FITC-conjugated anti-mouse FcεRI antibody, APC-conjugated anti-mouse KIT antibody (eBioscience, San Diego, CA, USA), or their isotype controls for 0.5 h on ice. Cells were washed and analyzed with Attune® Acoustic focusing cytometer (Applied Biosystems, Foster City, CA, USA).

2.9. Tumor growth in mice

NCL-2 and P815 cells (both 1×10^6) were suspended in 500 μl of PBS(−) and were injected subcutaneously into the left hind flank of Balb/c nude mice. Each tumor size was expressed as a product of a longest diameter and the shortest diameter. Three mice per group were used for the experiment.

2.10. DNA sequencing analysis of Kit

A DNA fragment including aspartic acid at codon 814 of *Kit* was amplified by PCR with sense primer: 5′-CCGGAATTCGAGACG TGACTCCTGCCATC-3′ and antisense primer: 5′-CCGCTCGAGCCCAT AGGACCAGACATCAC-3′. *EcoR I* site was adapted to the sense primer and *Xho I* site was adapted to the antisense primer. *EcoR I* and *Xho I*-digested PCR products were ligated into pME18S cut with the same enzymes. DNA sequencing of the plasmids was performed with ABI PRISMTM 310 genetic analyzer (Applied Biosystems). There was no substitution of Asp814 in 26 clones.

2.11. RT-PCR analysis of mouse mast cell proteases (mMCPs)

NCL-2 cells (3×10^3) were incubated with 100 ng/ml of IL-3 or SCF for 7 days and harvested for the extraction of total RNA with RNeasy Mini Kit (Qiagen, Hilden, Germany). The reverse transcription reaction of RNA was performed with QuantiTect™ reverse transcription kit (Qiagen). PCR analysis of the expression of mMCPs in NCL-2 cells were performed with Tks Gflex™ DNA polymerase

(Takara, Shiga, Japan) and the specific primers reported previously [12].

3. Results

3.1. NCL-2 cells grew without IL-3 or SCF, and expressed FcεRI and KIT on their surface

NCL-2 cells grew independently from exogenous growth factors except FCS (Fig. 1A). The doubling time of NCL-2 cells calculated by exponential growth equation was approximately 30.1 h. On the other hand, P815 cells showed more rapid growth than NCL-2 cells (Fig. 1B), and the calculated doubling time of P815 cells was approximately 12 h. The surface expression of FcεRI and KIT of NCL-2 cells were confirmed by flow cytometric analysis (Fig 1C and D).

3.2. Degranulation and LTB_4 production of NCL-2 in response to various stimuli

Next, we investigated whether NCL-2 could release chemical mediators in response to various external stimuli including antigens or not. NCL-2 cells were sensitized with anti-DNP IgE and then simulated with various concentrations of DNP-HSA. As shown in Fig. 2A and B, NCL-2 cells secreted histamine and produced LTB_4 in a dose-dependent manner. NCL-2 cells also showed degranulation in response to ionomycin and ATP as well (Fig. 2C and D).

3.3. Tumorigenic property of NCL-2 cells

We then estimated the tumorigenicity of NCL-2 cells in Balb/c nude mice. A mouse lymphoblast-like mastocytoma cell line, P815, showed rapid growth under the skin of nude mice and killed

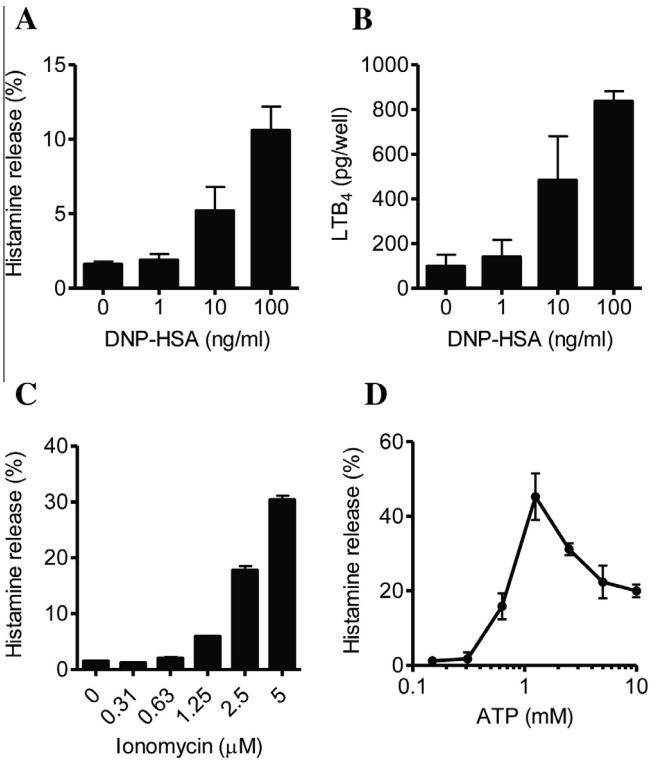

Fig. 2. NCL-2 cells release mediators in response to various stimuli. NCL-2 cells were sensitized with anti-DNP IgE, stimulated with DNP-HSA, and then the amount of histamine (A) and LTB_4 (B) released in culture supernatant were measured, respectively. NCL-2 cells were also stimulated with various concentrations of ionomycin (C) and ATP (D) and released histamine were measured. Data were expressed as mean ± SEM of three independent experiments done in duplicate.

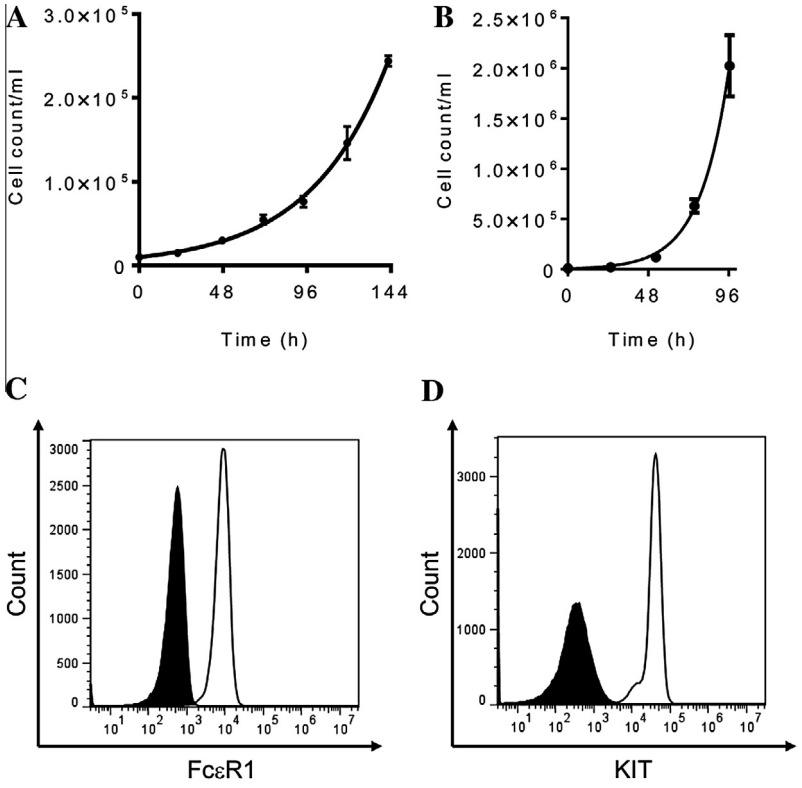

Fig. 1. NCL-2 mast cells are immortal and express FcεRI and KIT. 1×10^4/ml of NCL-2 cells (A) and P815 mastocytoma cells (B) were cultured without additional growth factors except for 10% FCS and the number of cells at each indicated time period was counted. Data were expressed as mean ± SD done in quadricate. NCL-2 cells were stained with FITC-conjugated anti-mouse FcεRI, APC-conjugated anti-mouse KIT, or their isotype controls. Surface expression of FcεRI (C) and KIT (D) of NCL-2 cells were shown as fluorescence intensity histograms (open area: specific antibodies, filled area: isotype controls).

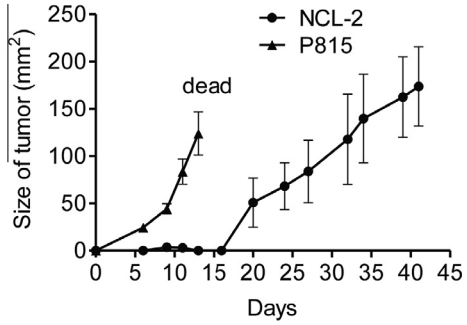

Fig. 3. Tumor formation of P815 and NCL-2 mast cell lines in nude mice. P815 and NCL-2 cells were injected subcutaneously into Balb/c nude mice. The size of each tumor at indicated time period was expressed as the product of a longest and a shortest diameter. Data were expressed as mean ± SEM of three mice of each group.

all of them within 2 weeks. Surprisingly, in contrast to P815, NCL-2 did not form tumors within 16 days. Even after inducing visible tumors, NCL-2 cells grew relatively slowly and did not kill mice at up to 40 days (Fig. 3).

3.4. Responses to exogenous growth factors, SCF and IL-3

Furthermore, we investigated whether or not NCL-2 cells could respond to exogenous growth stimuli. NCL-2 cells showed minimal tyrosine phosphorylation of KIT in quiescent state, and then showed robust tyrosine phosphorylation of KIT in response to SCF stimulation (Fig. 4A). NCL-2 cells also showed tyrosine phosphorylation of JAK2 in response to IL-3 stimulation (Fig. 4B).

3.5. The change in the expression profile of mMCPs by SCF and IL-3

NCL-2 cells were stimulated with 100 ng/ml of IL-3 and SCF for 7 days and the expression profile of mMCPs were studied. As shown in Fig. 5, NCL-2 cells, which were maintained without growth factors, already expressed mMCP-1, 4, 5, and CPA-3. Furthermore, the stimulation with IL-3 and SCF abolished the expression of mMCP-1, which connective tissue-type mast cells lack to express [13].

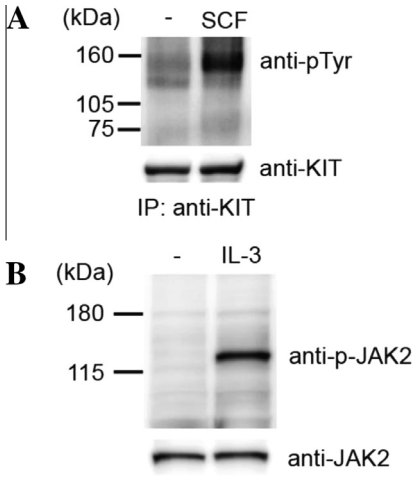

Fig. 4. NCL-2 cells respond to exogenous growth factors. (A) NCL-2 cells were stimulated with 100 ng/ml of SCF for 5 min and lysed. Cell lysates were immunoprecipitated with anti-KIT antibody. The immunocomplex was electrophoresed and blotted with anti-phosphotyrosine (anti-pTyr) or anti-KIT antibody. (B) NCL-2 cells were stimulated with 100 ng/ml of IL-3 for 5 min and lysed. Cell lysates were electrophoresed and blotted with anti-phosphorylated JAK2 or anti-total JAK2 antibody. A representative data of three independent experiments was shown for each panel.

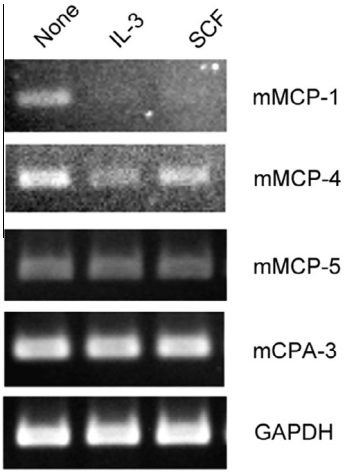

Fig. 5. The expression profiles of mMCPs in NCL-2 cells. NCL-2 cells (3×10^3) were incubated with 100 ng/ml of IL-3 or SCF for 7 days and harvested for RT-PCR analysis of the expression of mMCPs. A representative data of three independent experiments was shown.

4. Discussion

In this study, we established an immortal mast cell line, NCL-2, which can grow in the absence of SCF or IL-3. NCL-2 cells express KIT and functional FcεRI, and release histamine and LTB_4 in response to antigen stimulation. NCL-2 cells do not have a constitutively active mutation of *Kit*, thus show slow proliferation *in vitro* and substantially indolent tumor growth *in vivo* as compared with P815 mastocytoma cells. Moreover, stimulation with SCF and IL-3 induced tyrosine phosphorylation of KIT and JAK2, respectively.

NC mice were established as an inbred strain from Japanese fancy mice by Kondo in 1955. These mice spontaneously develop eczema and elevation of serum IgE similar to human AD in the conventional condition and thus are regarded as a mouse model of AD [14]. We previously reported that BMMC derived from NC/Kuj mice possess higher histamine content, higher adhesive ability, and especially, lack of apoptosis upon growth factor deprivation [15]. We, therefore, suspect that a certain population of BMMC from NC mice may survive without additional growth factors. In the process of establishing NCL-2 cells, we also obtained another immortal cell line, NCL-1, by the same long-term culture of BMMC of NC/Kuj mice. In contrast to NCL-2 cells, NCL-1 cells possessed an active mutation of *Kit* (D814F) on the single allele (data not shown). The mechanisms of immortality of NCL-2 remained unclear. However, we should keep in mind that BMMC of NC/Kuj has a potential to acquire a *de novo* active mutation of *Kit* in long-term cultures.

NCL-2 cells contained alcian-blue positive granules but not safranin-positive granules. Neither IL-3 nor SCF stimulation changed staining properties of granules even when NCL-2 cells were co-cultured with Swiss 3T3 cells for 2 weeks (data not shown). In general, BMMC express mMCP-5 and mCPA-3 without SCF, and express mMCP-4 with SCF [16]. On the other hands, NCL-2 cells express mMCP-4 without SCF stimulation, implying that NCL-2 cells might be partially differentiated mast cells. Furthermore, mucosal type mast cells (MMC) but not connective-tissue type mast cells (CTMC) express mMCP-1 [13]. IL-3 and SCF stimulation abolished the expression of mMCP-1, implying that these cytokines might shift NCL-2 cells towards CTMC-like cells.

In conclusion, NCL-2 cells do not need exogenous growth factors except for FCS to grow and are a useful tool to analyze both physiological and pathological functions of mast cells without the influence of constitutively active KIT.

Acknowledgments

We wish to thank Ms. Kazue Uchida for the expert technical assistances, and Dr. Faiz Kermani for his manuscript review. This work was carried out in part at the Analysis Center of Life Science, Natural Science Center for Basic Research and Development, Hiroshima University. The authors have nothing to disclose.

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