Large defects of type I allergic response in telomerase reverse transcriptase knockout mice

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Abstract: Telomerase is critically important for the maintenance of a constant telomere length, which in turn, is related to the concepts of longevity and oncogenesis. In addition, it has been well documented that telomerase activity is expressed in immune cells in a highly regulated manner. We have studied systemic anaphylaxis in mouse telomerase reverse transcriptase knockout (mTERT<sup>−/−</sup>) mice to understand the significance of telomerase activity and telomere stability in mast cells, which induce a type I allergic response. Compared with wild-type mice, mTERT<sup>−/−</sup> mice displayed largely attenuated, IgE-mediated, passive anaphylactic responses, which were observed even in the early generations of mTERT<sup>−/−</sup> mice, and had decreased numbers of mast cells in vivo and impaired development of bone marrow-derived mast cells (BMMCs) induced by IL-3 or stem cell factor in vitro. Moreover, in mTERT<sup>−/−</sup> mice, BMMCs exhibited a large morphology and low proliferation rate, while they possessed a comparable degranulation capacity and cell surface expression level of c-kit and FcεRI. These findings imply that telomerase activity has a definitive impact on the type I allergic response by altering the character of effector mast cells. J. Leukoc. Biol. 82: 429–435; 2007.

Key Words: anaphylaxis · mast cell · TERT

INTRODUCTION

Telomerase is a ribonucleoprotein enzyme, which adds telomeric repeats to chromosome ends to extend or maintain telomere length, which consequently avoids replicative senescence [1]. Telomerase consists of two essential subunits, the template RNA [telomerase RNA (TR)] and the catalytic subunit [telomerase reverse transcriptase (TERT)]. A defect of TR or TERT results in a lack of telomerase activity.

In knockout (KO) mice with TR deletion mutants (mTR<sup>−/−</sup>) on a C57BL/6 background, sterility, splenic atrophy, reduced proliferative capacity of B and T cells, abnormal hematological parameters, and progressive loss of organismal viability were observed coincident with telomere shortening in the late generations [Generations 4–6 (G4–G6) [2]]. In addition, the survival rate of the late generations decreased dramatically with age in comparison with wild-type (WT) mice. Splenocytes in the late generations of mTR<sup>−/−</sup> mice displayed telomere shortening, genomic instability, and a decreased number of germinal centers after immunization [3]. Recently, a number of studies have investigated the telomere length and telomerase expression in leukocytes during the activation, differentiation, and maturation process in combination with aging [4, 5]. As the function of the immune system is highly dependent on the capacity for extensive cell division and expansion of immune cells, these studies may shed new light on the regulation of the immune system. Although deep involvement of the telomere structure and telomerase activity in immune reactions is easily assumed, their precise role and physiological function remain unclear.

Previously, mice with a TERT deletion (mTERT<sup>−/−</sup>) have been reported as a similar phenotype to mTR<sup>−/−</sup> mice, which showed apparently normal macroscopic or microscopic findings in the early generations but developed multiple abnormalities in the late generations [6]. Here, we report that mTERT<sup>−/−</sup> mice display large anaphylactic response defects even in the early generations, thereby indicating a pivotal role for this enzyme in allergic reactions.

MATERIALS AND METHODS

Antibodies

Rat anti-mouse FcyRIIB/III (2.4G2) and mouse anti-TNP IgE (IGELa2) were purified from the hybridoma soup by ion exchange chromatography on DEAE cellulose (Merck, Whitehouse Station, NJ, USA) and by affinity isolation with a protein G column.

Animals

C57BL/6 mice were purchased from Charles River Japan (Yokohama), and 6- to 24-week-old mice were used as WT mice. Male and female mTERT<sup>−/−</sup> mice were used.

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with a C57BL/6 genetic background were described previously [6]. G1 TERT\(^{−/−}\) mice were obtained by crossing mTERT\(^{+/-}\) and mTERT\(^{+/-}\) mice. G1 TERT\(^{−/−}\) mice were mated to generate G2 mice, which were bred further to obtain successive generations. Mice were housed in the Animal Unit of the National Institute for Longevity Sciences (Aichi, Japan), an environmentally controlled and specific, pathogen-free facility, and experiments were performed according to guidelines for experimental animals defined by the facility.

### Induction of passive systemic anaphylaxis (PSA) and monitoring of rectal temperature

Mouse IgE anti-TNP mAb (1 mg/kg) were administered i.v. through the tail vein, and after 24 h, mice were injected with 50 mg/kg TNP-OVA. Changes in body temperature associated with systemic anaphylaxis were monitored by measuring changes in rectal temperature using a rectal probe coupled to a digital thermometer (Natsume Seisakusyo Co., Tokyo, Japan) as described elsewhere [7–9].

### Serum histamine level

Mouse serum was obtained from the subocular plexus 5 min after antigen challenge. Histamine levels in the plasma samples from 10 mice were quantified using ELISA and were performed by SRL, Inc. (Tokyo, Japan).

### Bone marrow-derived mast cell (BMMC) culture

c-kit-positive cells in BM cells were positively selected by MACS sorting using c-kit microbeads (Miltenyi Biotech, Gladbach, Germany). To keep mast cells in culture, BM c-kit\(^{+}\) cells were cultured at 3–5 \times 10^5 cells/ml in a medium containing 66 ng/ml recombinant mouse stem cell factor (rmSCF; Peprotech, Rocky Hill, NJ, USA) and/or 5 mg/ml rmIL-3 (R&D Systems Ltd., Abingdon, UK) with 10% FCS (Sigma, Poole, UK). Medium was changed every 3 or 4 days. When more than 90% of cells were differentiated into mast cells \(\sim 3\) weeks later, they were used for the experiments.

### Measurement of telomerase activity

Telomerase activity was measured with the telomerase PCR-ELISA kit (Roche Diagnostics, Mannheim, Germany), according to the manufacturer’s instructions as described previously [10]. In brief, the cell extract (representing \(10^5\) cells) was incubated for 30 min at 25°C in a reaction mixture of telomerase and then subjected to 40 cycles of PCR amplification (94°C for 30 s, 58°C for 30 s, and 72°C for 90 s). PCR products were detected by ELISA, and the absorbance of samples was measured at 450 nm with a Microtiter plate reader (Bio-Rad, Hercules, CA, USA). All assays were performed in triplicate. The level of telomerase activity in the positive cell extract supplied in the kit was set to 100%, and the relative specific telomerase activity (RTA) of each extract was expressed as a percentage of the positive control standard.

![Fig. 1. IgE-mediated systemic anaphylaxis in mTERT\(^{−/−}\) mice. Changes in rectal temperature (RT) during IgE-mediated systemic anaphylaxis were measured. IgE-mediated systemic anaphylaxis was induced in WT (G1) or early generations of mTERT\(^{−/−}\) mice (G2, G3). After mice received 1 mg/kg anti-TNP IgE i.v., 50 mg/kg TNP-OVA was injected 24 h later to induce systemic anaphylaxis. Data are shown as mean \(\pm\) s.d. \(*\), \(P < 0.05\).](http://www.jleukbio.org)

Mast cell degranulation

BMMCs were adjusted to \(2 \times 10^5\) cells/ml in culture medium and labeled with 1 \(\mu\)Ci/ml \([3H]\)-serotonin (Amersham Biosciences, Uppsala, Sweden) overnight at 37°C. Then, cells were washed three times and resuspended at \(2 \times 10^5\) cells/ml in the medium. Degranulation was carried out in 96-well plates after incubation with 5 \(\mu\)g/ml anti-TNP IgE, followed by cross-linking with 10 \(\mu\)g/ml TNP-OVA for 1 h at 37°C. Cell-free supernatants were collected and counted in a scintillation counter (experimental release). Total counts per 50 \(\mu\)l-labeled cells (total release) and spontaneously released counts (spontaneous release) were also determined to calculate the percentage of serotonin release as follows: [(experimental release–spontaneous release)/total release–spontaneous release] \(\times 100\%\).

Flow cytometry

For flow cytometric analysis, we used the following mAbs: FITC-, PE-, or biotin-conjugated, anti-mouse IgE (R35-72), anti-c-kit (2B8), and anti-TNP IgE. All antibodies were purchased from BD PharMingen (San Diego, CA, USA). Cell surface staining was carried out using standard techniques. Two million cells were incubated for 30 min at 4°C with antibodies, and flow cytometric analysis was performed with FACSCalibur using CellQuest software (BD PharMingen). Dead cells were eliminated from the analysis on the basis of propidium iodide (PI) staining.

Apoptosis assay

BMMCs were deprived of IL-3 for 24 h and cultured in the presence of IL-3 for an additional 48 h. Also, 0.01 mM dexamethasone (DXM) was used to induce apoptosis. After incubation, cells were stained with PI and Annexin V using Annexin V-FITC apoptosis detection kit I (BD PharMingen) and subjected to FACS analysis.

Proliferative response of mast cells

BMMCs were activated with rmIL-3, rmSCF, or rmIL-3 plus rmSCF. After 24 h of stimulation, proliferation was determined by [3H]-thymidine uptake as described previously [11]. In brief, culture wells were pulsed with [3H]-thymidine (1 \(\mu\)Ci/ml) for 12 h before being collected on glass fiber filters and counted in a scintillation counter. Experiments were performed in quadruplicate.

Senescence-associated \(\beta\)-galactosidase activity (SA-\(\beta\)-Gal)

To investigate expression of SA-\(\beta\)-Gal in BMMCs, the cultured cells were stained histochemically with \(\beta\)-Gal (pH 6.0) using the Senescence \(\beta\)-Galactosidase staining kit (Cell Signaling Technology, Danvers, MA, USA). After 2 h incubation, samples were developed and observed under the microscope.
Histological study

Cultured cells (2 x 10^5) were placed on slides and fixed in 4% paraformaldehyde. Tissues from mouse ear were fixed in 10% (vol/vol) neutral-buffered formalin and embedded in paraffin. The specimens were sectioned at 5 μm. Then, prepared slides were stained with 0.5% toluidine blue (pH 4.0). Mast cells were identified by the presence of abundant numbers of metachromatically stained granules. The number of mast cells was counted under a light microscope (/mm²).

RESULTS

Attenuated IgE-mediated systemic anaphylaxis in mTERT⁻/⁻ mice

We investigated IgE-mediated PSA using the early generation (G1, G2, G3) of mTERT⁻/⁻ mice. To elicit the anaphylactic response, mice were sensitized with IgE specific for TNP, followed by i.v. administration of TNP-OVA. In all generations tested, mTERT⁻/⁻ mice showed a largely attenuated anaphylactic response as compared with WT mice, and with successive generations, the attenuation became greater (Fig. 1).

Thus, a marked diminution of the IgE-dependent PSA reaction was observed, even in the early generations of mTERT⁻/⁻ mice. We also examined serum histamine levels during anaphylaxis by ELISA, but no significant difference was observed between WT and mTERT⁻/⁻ mice (WT: 13,267 ± 3786 nM; mTERT⁻/⁻: 15,333 ± 3164 nM, mean ± SD; P=0.51).

High levels of telomerase activity in mouse mast cell

Mast cell is the effector cell of systemic anaphylaxis through the binding of IgE to its surface receptor FcεRI. First, we measured the telomerase activity of mast cells during differentiation from WT BM cells maintained under standard culture conditions. BMMC, determined by c-kit⁺ cells, showed a rapid increase of telomerase activity immediately after starting culture in the presence of IL-3 (Fig. 2A). They reached a maximum after 3 days of culture and sustained high levels of activity thereafter. It has been reported that differentiation and maturation of mast cells are influenced by various microenvironments including numerous growth factors such as IL-3, IL-4, IL-9, IL-10, nerve growth factor, and the fibroblast-derived SCF [12]. In addition, monomeric IgE leads to a potent production of cytokines from BMMCs and enhances BMMC survival by preventing the apoptosis of BMMCs [13]. To investigate the effects of these cytokines and monomeric IgE on telomerase activity, BMMCs were restimulated with IL-3, SCF, or monomeric IgE in various combinations. However, no significant alteration of telomerase activity was observed in BMMC with these stimulations (Fig. 2B).

Characteristics of mast cells in mTERT⁻/⁻ mice

Mast cells degranulate and release chemical mediators when FcεRI on the cell surface is cross-linked by IgE and antigens. Furthermore, monomeric IgE stimulation enhances cell surface expression of FcεRI on mast cells. To clarify whether a loss of telomerase activity affects these functions, we first measured granular release from mast cells. As shown in Figure 3A, after 10 min incubation with IgE followed by 1 h stimulation with antigen, the degree of serotonin release from BMMCs of mTERT⁻/⁻ G2 mice was the same as that of WT mice. After a longer incubation period of 24 h, the serotonin release from BMMCs increased in WT and mTERT⁻/⁻ mice but exhibited no significant difference between them. These data indicated that the disruption of the TERT gene did not alter the mast cell function of FcεRI-mediated degranulation.

Next, we performed flow cytometric analysis to examine whether deletion of the TERT gene influences FcεRI and c-kit
expression on mast cells. As shown in Figure 3B, the expression levels of FcεRI and c-kit on BMMCs from mTERT−/− G2 mice were comparable with those from WT mice. Similarly, peritoneal mast cells isolated from mTERT−/− G2 mice and WT mice, 24 h after i.v. administration of IgE, displayed equivalent levels of FcεRI and c-kit expression on the cell surface. Altogether, flow cytometric analysis revealed no considerable difference in the cell surface markers on mast cells between mTERT−/− and WT mice.

Impaired proliferation and development of mast cells in mTERT−/− mice in vitro and in vivo

IL-3 and SCF are essential factors in mast cell differentiation in vitro as well as in vivo. First, we examined the induction of BMMCs from mTERT−/− G2 mice and WT mice in the presence of IL-3 or SCF using a standard technique. Judging from the number of mast cells expanded, BM cells from mTERT−/− G2 mice showed a limited capacity for development into BMMCs (Fig. 4A). Especially, mTERT−/− G2 BM cells died in 3 weeks under culture with SCF. As impaired development of BMMCs was observed in mTERT−/− mice, we investigated the apoptotic tendency of BMMCs. Apoptosis was induced by cytokine (IL-3) depletion or DXM stimulation, the standard technique to trigger cell death of leukocytes. In all conditions tested, BMMCs from mTERT−/− mice exhibited slightly higher rates of cell death in the early phase of apoptosis (Annexin-V+/PI−) and the late phase of apoptosis or necrosis (Annexin-V+/PI+), although the differences were not significant (Fig. 4B). Next, we examined the proliferative responses of BMMCs. In Figure 4C, BMMCs from mTERT−/− G2 mice displayed a largely reduced proliferation rate as compared with those from WT mice in response to IL-3, SCF, or both.

Morphological features of mast cells

By microscopic analysis, the development of mast cells was judged by toluidine blue staining. The presence of acid toluidine blue-positive, granule-containing cells in the culture of BM-derived cells was observed in WT and mTERT−/− mice. However, most BMMCs from mTERT−/− mice displayed an enlarged morphology with big nucleus and vacuoles, as often seen in senescent cells (Fig. 5A, left). These large cells were also stained deeply with β-Gal under a pH 6.0 condition, which indicates SA-β-Gal activity (Fig. 5A, right). To investigate the development of mast cells in vivo, we performed a histopathological analysis. Each ear of WT and mTERT−/− mice was fixed and stained with acid toluidine blue. As shown...
in Figure 5, B and C, the number of mast cells in ear dermis was significantly lower in mTERT<sup>−/−</sup> mice. These results indicated that the lack of mTERT expression largely inhibits the proliferation and development of mast cells in vitro and also in vivo.

**DISCUSSION**

Leukocytes derive from BM hematopoietic stem cells and circulate in blood and tissues. Their distinctive process of differentiation and rapid proliferation in immune responses require involvement of numerous biological factors, including telomerase activity and telomere maintenance, which are implicated in the regulation of the replicative lifespan of dividing cells. In addition, in aging, the attrition of telomere length in leukocytes and decline of immune functions are observed [2, 3]. Thus, the function of the immune system seems deeply associated with telomere length and telomerase activity in leukocytes during differentiation, activation, and also aging. Accumulating data derived from analysis of telomere length and telomerase expression in hematopoietic stem cells and lymphoid lineage cells indicate that telomerase activity is highly regulated in hematopoietic stem cells and lymphoid cells and plays a pivotal role in the telomere maintenance and replicative lifespan of cells [4, 5], although its precise physiological function remains to be elucidated. Contrary to the considerable information available on lymphoid lineage cells such as T and B cells, few reports have addressed the issue of telomere length and telomerase activity in myeloid lineage cells, which are involved mainly in innate immunoresponses and tend to have a short lifespan and high turnover rate [10, 14].
In this report, we analyze the impact of telomerase activity on anaphylactic responses, which are mediated by mast cells. It has been reported that telomerase is expressed during human mast cell development from hematopoietic stem cells and mast cell progenitor cells. Although mast cell progenitors showed a rapid increase in telomerase activity preceding proliferation in response to SCF and IL-3 or IL-6, the induction was transient, and telomerase activity declined to basal levels. No telomerase activity was detected in human mature mast cells [14]. To clarify the physiological role of telomerase expression in mast cells, we examined anaphylactic responses and mast cell functions using TERT KO mice. We chose to evaluate a passive rather than an active model of anaphylactic response, as mTERT -/- mice might exhibit dampened, humoral immune responses as a result of impairment of IgE production from B cells. To elicit an anaphylactic response, mice were injected with IgE specific for TNP, followed by i.v. administration of TNP-OVA. In this study, significantly attenuated IgE-mediated PSA was observed even in the first generation (G1) of mTERT -/- mice (Fig. 1). The defects in the anaphylactic response became more apparent with successive generations in the mTERT -/- mice (Fig. 2). Conversely, in the absence of telomerase expression, the development of mast cells from BM cells and the proliferation in response to IL-3 and SCF were impaired severely (Fig. 4). In microscopic analysis, WT and mTERT -/- BMBCs possessed abundant acid toluidine blue-positive granules equally, but mTERT -/- BMBCs displayed morphological features of senescence such as enlargement of cells and expression of SA-β-Gal activity. Histologically, mTERT -/- mice carried a smaller number of mast cells in ear dermis, reflecting their lower proliferation, higher turnover rate, or possibly, reduced migration of mast cells (Fig. 5). Taken together, these
results suggest that a defect of telomerase expression interferes with the proper development and proliferation of mast cells, resulting in a reduced number of mast cells and largely attenuated anaphylactic responses \textit{in vivo}.

The treatment of allergic disease, caused by foods, drugs, injections, insect stings, or inhalations, has assumed increasing importance. The most dangerous allergic reaction is anaphylaxis, which can be a life-threatening emergency. A number of drugs such as antihistamines, adrenalin, and steroids have been developed to manage anaphylaxis, but it still is not easy to predict, prevent, and treat. In the present study, we demonstrate the importance of telomerase activity in anaphylaxis, which may help to understand the age-related alteration of allergic responses and also suggest new, therapeutic strategies to control allergic responses.

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