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Research paper

Absence of PTHrP nuclear localization and C-terminus sequences leads to abnormal development of T cells

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ABSTRACT

Parathyroid hormone-related protein (PTHrP), a ubiquitously expressed protein, is composed of four functional domains including N-terminus, mid region, nuclear localization signal (NLS) and C-terminus. Under the direction of NLS, PTHrP can enter cell nucleus from cytoplasm and stimulate mitogenesis. Although PTHrP is considered to have important developmental roles, the role of PTHrP NLS and C-terminus in developmental process remains unknown, especially in T-cell development. Here, we used a knock-in mouse model, which expresses a truncated form of PTHrP missing the NLS (87–107) and C-terminus (108–139) of the protein, to examine the role of PTHrP NLS and C-terminus in T-cell development. Our results showed that the truncated PTHrP (1-84) led to abnormal subpopulations, impaired proliferation and increased apoptosis in the thymus, indicating that PTHrP is involved in the development of T cells, and the NLS and C-terminus part is necessary for the normal role of PTHrP in T-cell development.

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1. Introduction

Parathyroid hormone-related protein (PTHrP) was originally identified in the blood of patients with hypercalcemia of malignancy as a humoral factor [1]. Studies have shown that PTHrP as a ubiquitously expressed protein had a wide distribution and diverse functions regulating cell differentiation, development, survival, and other physiological process [2–4]. These effects are mostly accomplished by interaction of secreted PTHrP with cell surface PTH/PTHrP receptors through paracrine or autocrine actions. In addition to the conventional interaction, a novel mechanism of action for PTHrP is being recognized. Intracellular PTHrP can get access to the nuclear compartment under the direction of a classical bipartite nuclear localization signal (NLS), which is located at PTHrP (87–107), and influence cellular events [5–10]. Henderson et al. has reported PTHrP can delay apoptosis induced by serum deprivation in chondrocytes, and this effect depends on the presence of intact NLS [5]. Likewise, De et al. has found the C-terminus of PTHrP as well as NLS is essential for cell proliferation in vascular smooth muscle cells [9,10]. Gu et al. reported that absence of nuclear localization and C-terminus sequences led to abnormal brain development and function [11]. Boras-Granic et al. also found that deletion of the NLS and C-terminus of PTHrP was able to impair embryonic mammary development [12]. These studies demonstrate that intranuclear PTHrP plays a role in cell proliferation and apoptosis [5,9–12]. The nuclear functions of PTHrP depend on the presence of intact NLS and C-terminus of PTHrP. Therefore, absence of NLS and C-terminus is supposed to hinder PTHrP's potential to enter cell nucleus and result in a loss of the nuclear function of PTHrP.

Through genetic manipulation, we created a stop codon (TGA) after the 84th amino acid residue of PTHrP and made a knock-in

Abbreviation: PTHrP, Parathyroid hormone-related protein; NLS, nuclear localization signal; DN, double negative; DP, double positive; KI, knock in; WT, wild type; DCFH-DA, 2′,7′-Dichlorofluorescin diacetate; ROS, reactive oxygen species.

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mouse model (*Pthrp* KI) which expressed a PTHrP (1-84) mutant missing regions containing NLS and C-terminus [13]. This mutant form of PTHrP remains signaling properties by interaction with the cell surface receptor (PTHR) but loses its nuclear localization and corresponding function in cell nucleus. As a result, the *Pthrp* KI mouse was found growth retardation and abnormal brain development, showing the extensive role of intra-nuclear PTHrP in tissue development and growth [13]. During our study of *Pthrp* KI mice, data have suggested that PTHrP may play a role in immune system development. In the present study, we aim to examine the biological consequence of PTHrP missing NLS and C-terminus in developmental T cells, in which cell proliferation and apoptosis are both critical events, by use of the *Pthrp* KI mouse model. Here, we report that loss of PTHrP (87–139) leads to abnormal subpopulation distribution and impairment of T-cell proliferation, indicating the NLS and C-terminus of PTHrP plays a role in the development of T cells.

2. Materials and methods

2.1. Reagents and antibodies

RPMI1640, FCS (fetal Calf Serum), L-glutamine and non-essential amino acids were from Invitrogen. Antibiotics and β-mercaptoethanol were purchased from Shanghai Sheng Gong Company. Propidium iodide (PI), DMSO (Dimethyl sulfoxide), DCFH-DA (2′, 7′-Dichlorofluorescin diacetate) and CFSE (5, 6-carboxyfluorescein diacetate, succinimidyl ester) were from Sigma-Aldrich. Antibodies for CD25-PE, CD44-FITC, CD4-PE, CD8-FITC, CD4-TC, CD8-TC and CD3-FITC were purchased from BD Pharmingen. TUNEL kit (Cat. No. QIA33) was from Merck.

2.2. Animals and treatments

Preparation of *Pthrp* knock-in mouse was described before [13]. Animals were allowed water and standard chow *ad libitum*. The animal studies were approved by the Animal Care and Use Committee of Nanjing University and have been carried out in accordance with the NIH guidelines for the care and use of Laboratory animals.

2.3. Thymocytes culture

Freshly prepared thymocytes from 14-day-old *Pthrp* KI or WT littermate mice were cultured in RPMI1640 supplemented with 10% FCS, 2 mM L-glutamine, 1 mM non-essential amino acids, 50 μM β-mercaptoethanol, 100 U/ml penicillin, and 100 μg/ml streptomycin for indicated time.

2.4. Cell population analysis and flow cytometry

Cell suspensions were prepared from lymphoid organs of 7-day-old or 14-day-old littermates with the appropriate genotypes. After red blood cell lysis, cells were filtered, washed, and stained with indicated antibodies. Normal serum cocktail was added first to eliminate nonspecific staining. For CD25 and CD44 staining of DN thymocytes, staining with tricolor-conjugated CD4 and CD8 antibodies was done with PE-CD25- and FITC-CD44-specific antibodies. The CD25 versus CD44 profiles were analyzed on cells gated on $CD4^{-}CD8^{-}$ [14].

2.5. Apoptosis analysis and cell cycle analysis

Freshly prepared thymocytes from 14-day-old WT or *Pthrp* KI mice were cultured as above and treated with vehicle or apoptosis inducer (anti-CD3/CD28 or dexamethasone) for 12 h. The using concentration for plate-bound anti-CD3/CD28 antibodies is 0.5 μg/ml respectively, and the dexamethasone is used with 50 nM. Cell suspensions were then prepared and stained with FITC-conjugated Annexin V for flow cytometry. For cell cycle analysis, cell suspensions were prepared and fixed with 70% ethanol. Then they were stained with propidium iodide and analyzed by flow cytometry.

2.6. ROS measurements

Cells were incubated with DCFH-DA (Sigma-Aldrich) at 5 μM final concentration for 20 min at 37 °C in the dark before flow cytometric analysis.

2.7. Western blot analysis

Cells were collected and washed with cold PBS. Protein samples were prepared as described previously [15]. Equal amounts (50 μg–100 μg) of proteins were resolved by 12% SDS-PAGE gels, transferred to PVDF membrane, and probed with indicated antibodies. The ImageJ software was used to quantify the western blot results.

2.8. TUNEL

Thymuses were freshly collected from 2 week old *Pthrp* KI and WT mice. Then the thymuses were fixed in PBS-buffered 4% formaldehye, embedded in paraffin, and sectioned at 5 μm using standard techniques. TUNEL assay was performed according to standard protocol provided by Merck's TUNEL Kit (Cat. No. QIA33). According to the protocol, Dark brown cells were determined as apoptosis cells. Typical results were shown.

2.9. Cell proliferation analysis by CFSE

1×10^7 splenocytes/ml fresh from 14-day-old mice were labeled with 2.5 μM CFSE in 0.1% bovine serum albumin/phosphate buffer saline at 37 °C for 10 min, washed twice with 10% FCS/RPMI 1640, and then cultured in RPMI1640 containing 10% FCS, glutamine, non-essential amino acids, penicillin/streptomycin, β-mercaptoethanol plus plate-bound anti-CD3 (0.5 μg/ml) and anti-CD28 (0.5 μg/ml) for 72 h. Then the splenocytes were washed with cold PBS and stained with anti-CD4-PE antibody. The $CD4^{+}$ gated cells were analyzed for CFSE fluorescence by flow cytometry.

2.10. Statistical analysis

Data are presented as means ± S.D.. All statistics were performed using Student's *t*-test with SPSS 11.5 software. A $P < 0.05$ was considered statistically significant.

3. Results

3.1. PTHrP NLS and C terminus deficiency disrupts normal development of T cells

To evaluate the effects of PTHrP NLS and C terminus deficiency on T cell development, thymus weight and thymocyte number were compared between *Pthrp* KI mice and their wild-type littermates. The overall thymus weight and thymocyte number were reduced in *Pthrp* KI mice at both postnatal day 7 and day 14 (Fig. 1A and B), reflecting the abnormality of T-cell development in *Pthrp* KI mice.

To further examine the effects of PTHrP NLS and C terminus deficiency, we measured the proportion of four distinct thymocyte subpopulations (DN, DP, CD4 SP, CD8 SP), four different T-cell

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