Cellular Immunology 266 (2010) 83–89

Contents lists available at ScienceDirect

Cellular Immunology

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

Ontogenesis of rat immune system: Proteasome expression in different cell populations of the developing thymus

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ARTICLE INFO

Article history:
Received 13 May 2010
Accepted 7 September 2010
Available online 15 September 2010

Keywords:
Immune proteasome
Thymus
Prenatal and postnatal ontogenesis
Rat
Antigen presenting cells
Thymocytes

ABSTRACT

Immune proteasomes in thymus are involved in processing of self-antigens, which are presented by MHC class I molecules for rejection of autoreactive thymocytes in adults and probably in perinatal rats. The distribution of immune proteasome subunits LMP7 and LMP2 in thymic cells have been investigated during rat perinatal ontogenesis. Double immunofluorescent labeling revealed LMP7 and LMP2 in thymic epithelial and dendritic cells, as well as in CD68 positive cells – macrophages, monocytes – at all developmental stages. LMP2 and LMP7 were also detected by flow cytometry in almost all thymic CD90 lymphocytes through pre- and postnatal ontogenesis. Our results demonstrate that the immune proteasomes are expressed in all types of thymic antigen presenting cells during perinatal ontogenesis, suggesting the establishment of the negative selection in the thymus at the end of fetal life. The observation of the immune proteasome expression in T lymphocytes suggests their role in thymocyte differentiation besides antigen processing in thymus.

1. Introduction

The ubiquitin–proteasome pathway, which degrades intracellular proteins, is involved in numerous cellular processes, including the supply of immunocompetent peptides to the antigen presenting machinery. Apart from the conserved "constitutive proteasome", a special type of proteasome called "immune proteasome" exists in vertebrates for better presentation of antigenic peptides on MHC class I molecules. The immune proteasomes contain interferon-γ (IFNγ) -inducible catalytic subunits LMP7 (β5i), LMP2 (β1i), and LMP10 (β2i), which replace X, Y and Z subunits constitutively expressed in most tissues [1,2]. The immune proteasomes have enhanced capability for generating MHC class I-binding peptides as compared to constitutive proteasomes, cleaving more efficiently after basic or hydrophobic residues [3,4]. Generated oligopeptides are transported into the endoplasmic reticulum, complexed to MHC class I molecules and transported to the cell surface via the secretory pathway to be recognized by CD8+ T lymphocytes [1,5,6]. The immune proteasomes are present both in lymphoid and non-lymphoid organs of adult animals, where they play different roles on creating antigenic epitopes or their precursors [7–9]. In the central lymphoid organ, the thymus, immune proteasomes are responsible for negative selection of thymocytes [7,10]. In antigen presenting epithelial and dendritic thymus cells, immune proteasomes generate self-peptides that are presented by MHC class I molecules for recognition by thymocytes and rejection of autoreactive thymocytes through apoptosis [11,12].

The processes described above play a particular role during immune system development. Nevertheless, the expression of immune proteasomes in developing lymphoid organs is not yet fully elucidated. According to our recent data immune proteasome subunits LMP2 and LMP7 were revealed in the rat thymus and spleen at the end of fetal life [9,13]. In this research, we investigated the ontogenetic pattern of the proteasome subunit expression: α1, 2, 3, 5, 6, 7 (20S and 26S proteasomes) and PA28α (20S proteasome activator) in the rat thymus. Besides, we defined the phenotype of thymic cells, expressing the immune proteasome subunits LMP7 and LMP2 during the perinatal ontogenesis.

2. Materials and methods

2.1. Animals

All the protocols of manipulations with animals have been approved by the animal care Committee of N.K. Koltsov Institute of Developmental Biology of the Russian Academy of Sciences. All efforts were made to minimize the number of used animals and their suffering. Wistar rats (nursery "Stolbovaya", Russian Academy of Sciences) of 200–250 g, embryos of days 18–21 (E18–E21, the day of conception was E1) and rats of days 1–30 of the postnatal development (P1–P30) were used in this study.

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doi:10.1016/j.cellimm.2010.09.001

2.2. Antibodies and reagents

Combined mouse monoclonal antibodies (mAbs) to the subunits α1, 2, 3, 5, 6, 7 and rabbit polyclonal antibodies (pAbs) to the immune subunits LMP7 and LMP2 of proteasomes were purchased from Affiniti (UK). Mouse mAbs to the OX-62 (Cederlaine, Canada), CD68 (Santa Cruz, USA) and cytokeratins 18 and 19 (Chemicon, USA) were used in the study. Mouse mAbs to β-actin were purchased from Santa Cruz (Germany). Peroxidase-conjugated anti-mouse IgG and anti-rabbit IgG Abs, and ECL reagents were purchased from Amersham Biosciences (UK). R-PE-conjugated mouse anti-rat CD90 (Thy-1) mAbs (BD Pharmingen, USA), Alexa-488- and PE-conjugted goat anti-rabbit IgG Abs (Invitrogen, USA), Alexa-546-conjugted anti-mouse IgG Abs (Invitrogen, USA) were used.

2.3. Protein content in the thymus

The protein concentration in the thymic tissue was measured by Lowry method [14]. Thymi from rats at the E18, E21, P1, P3, P5, P8, P19 and P29 were dissected, weighted, zonicated in 50 mM Tris–HCl buffer pH 7.5 (10:1 v/w). Homogenates were centrifuged at 10,000*g* for 20 min. Protein concentration was determined in supernatants.

2.4. Preparation of cleared homogenates from the thymus

All procedures were performed at 4 °C. The thymus was homogenized in a Braun Melsungen (Germany) homogenizer (glass–glass) in buffer containing 50 mM Tris–HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 10% glycerol, 10 mM $Na_2S_2O_5$, 1 mM phenylmethylsulfonyl fluoride, leupeptin (0.5 μg/ml), pepstatin (1 μg/ml), aprotinin (1 μg/ml) in the ratio 1:3 (w/v). The homogenates were centrifuged at 10,000*g* for 30 min. Supernatants (cleared homogenates) were used for further investigation.

2.5. Western blotting

After SDS–PAGE in 13% gel (5 μl of cleared homogenate per lane), the polypeptides were semi-dry transferred from the gel onto a nitrocellulose membrane. The membrane was incubated for 2 h at 20 °C in TNT buffer (10 mM Tris–HCl (pH 7.5), 150 mM NaCl, 0.1% Tween 20), then for 1 h in TNT buffer containing 5% defatted milk and mouse mAb to β-actin (1:200), the α1, 2, 3, 5, 6, 7 subunits or to PA28α (1:2500), washed several times in the TNT buffer and incubated for 1 h in the TNT buffer containing 5% defatted milk and peroxidase-conjugated secondary antibodies diluted 1:5000. After washing in TNT buffer, the membrane was treated routinely using the ECL system.

The image analysis was performed using "ImageJ" software. The relative quantities (the optical densities) of the immunoreactive bands on X-ray film were measured as the "gray level" (GL) which is related to the optical density (OD) of the specimen as follows [15]: ODSpecimen−ODBackground = log(GLBackground)−log(GLSpecimen). The dependence of the optical density on the amount of the protein subjected to Western blotting was evaluated preliminarily. For the further procedure, the range of the protein amount was chosen in which this dependence was linear.

2.6. Immunohistochemistry

The rat was perfused through the heart with PBS and 4% paraformaldehyde, thymus was dissected, postfixed for 1 h in the same fixative and frozen. Cryostat sections (10 μm) were subsequently incubated at room temperature with: (i) PBS containing 1% of BSA and 0.1% Triton X-100 for 1 h; (ii) PBS containing 1% of BSA, 0.1% Triton X-100, and mixture of primary rabbit pAb (to LMP7 or LMP2 (1:2500) and mouse mAb to OX62 (1:500) (or CD68 (1:500) or CK (1:100)) overnight; (iii) PBS containing mixture of secondary Abs (Alexa488-conjugated anti-rabbit IgG Abs (1:1000) and Alexa546-conjugated anti-mouse IgG (1:1000)) for 2 h. After being rinsed in PBS, sections were mounted in Mowiol and were analyzed under a fluorescent microscope Leica DM RXA 2 (Germany). Control experiments were performed by omitting primary or secondary Abs.

2.7. Flow cytometric analyses

Freshly prepared cell suspension from the thymus was incubated with specific Abs as indicated for each experiment. For two-color immunofluorescent analysis cells were exposed to mouse mAbs to CD90 or to OX62 for 1 h. Surface-stained cells were washed twice and fixed with 4% paraformaldehyde for 15 min. Then, for intracellular staining, thymic cells were treated with 0.1% saponin in PBS for 20 min and incubated with (i) unconjugated rabbit pAbs to LMP7 (or LMP2) over night at 4 °C and (ii) Alexa 488- or PE-conjugated anti-rabbit IgG Abs for 30 min. Double-stained cells were analyzed using Cell Lab Quanta SC (Beckman–Coulter). Background fluorescence was determined using PE-conjugated isotype-matched control mAbs and Alexa 488- or PE-conjugated anti-rabbit IgG Abs. The data were analyzed using "Quanta SC MPL" software (Beckman–Coulter).

2.8. Statistical analysis

The data are presented as means ± standard error. Statistical significance was determined by using non-parametric Mann–Whitney U test.

3. Results

3.1. The dynamics of the proteasome expression in the developing rat thymus

In this study we examined the expression of the total proteasome pool, which comprises the constitutive and immune forms, by Western blotting of cleared homogenates with using the combined mAbs to the subunits α1, 2, 3, 5, 6, 7. The subunits α1, 2, 3, 5, 6, 7 were detected in thymus at all studied periods of the development including the embryonic period (Fig. 1A). Their amounts did not differ reliably during development (Fig. 1A, B). The total protein concentration in thymus varied significantly during perinatal development (Fig. 1C). The protein concentration gradually increased from E18 to P1, then decreased 3-fold to P3–5 followed by the 4-fold increase to P8 and slight reduction to P19–29.

We have also studied the expression of PA28α – proteasome regulator – in the developing thymus. PA28α was detected at all studied developmental stages (Fig. 2A). Their expression level gradually increased from E18 to P19 (Fig. 2A,B).

3.2. The immune proteasome expression in the antigen presenting cells in rat thymus during the perinatal ontogenesis

Dendritic cells (DCs) in the thymus were identified using the OX62 mAbs that recognize an integrin expressed on rat DCs [16]. Although the OX-62 mAbs do not label all classical DCs and are not restricted to this cell type, in lymphoid tissues, the labeling correlates with DCs. Ox62 immunopositive cells were concentrated in the medulla and the cortico-medullary junction, scattered in the cortex. Immunohistochemistry for OX62 and LMP7 (LMP2)