Monoclonal Antibodies Directed Against Chicken β 2-Microglobulin Developed With a Synthesized Peptide

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We developed a panel of monoclonal antibodies (MAb) against chicken β 2-microglobulin (ch β 2M) by fusions between SP2/0 myeloma cells and spleen cells from mice immunized with a synthesized peptide corresponding to positions 91-119 of the COOH domain of ch β 2M. Two of them, 6E7 and 3D1, identified as IgG1/ κ , could react with ch β 2M protein from avian macrophage HD11 cells and human 293T cells transfected with pcDNA3.1-ch β 2M in immunofluorescence assays. Only a 12 kDa protein band of ch β 2M could be detected in the HD11 and 293T/ch β 2M cell lysates by Western blot analysis. Chicken β 2M in serum and plasma could be found in Western blot by MAb 3D1. Moreover, MAb 3D1 also recognized the ch β 2M antigen on the cell membranes in flow cytometry. Immunohistochemical staining with these MAbs revealed that ch β 2M was present in chicken thymus, spleen, and bursa. These MAbs will be good tools for analyzing the mechanism of the chicken immune system.

Introduction

β 2-MICROGLOBULIN (β 2M) IS A LOW MOLECULAR WEIGHT, non-glycosylated protein that is synthesized by all nucleated cells. It composes the small, invariable light chain subunit of the major histocompatibility complex (MHC) class I antigen through non-covalent linkage on the cell surface.⁽¹⁾ The function of β 2M is interacting with and stabilizing the tertiary structure of the MHC class I α -chain to present antigenic peptides to cytotoxic (CD8+) T lymphocytes.⁽²⁾ In addition, β 2M is extensively involved in the functional regulation of survival, proliferation, apoptosis, and even metastasis of cancer cells.^(3,4) Recent studies showed that antibodies against β 2M were highly cytotoxic against some solid⁽⁵⁾ or liquid tumors.^(3,6) However, there are only a few reports focusing on chicken β 2-microglobulin (ch β 2M).⁽⁷⁻¹⁰⁾ In this study, we developed a panel of monoclonal antibodies against ch β 2M with a synthesized peptide. These monoclonal antibodies could react with both linear ch β 2M and native ch β 2M.

Materials and Methods

Cell lines and cell culture

HD11 cells, a replication-defective avian leukemia virus MC29-transformed macrophage-like cell line, were kindly provided by Dr. XinAn Jiao (Yangzhou University, China). HD11 cells were maintained in Dulbecco's Modified Eagle's Media (DMEM, Life Technologies-Gibco, Bethesda, MD)

containing 10% fetal bovine serum (FBS) at 41°C and 5% CO₂. Human renal epithelial 293T cells were cultured in DMEM supplemented with 10% FBS at 37°C and 5% CO₂. The MDCC-MSB1 (MSB1) cells, a chicken T-lymphoblastoid cell line transformed with BC-1 strain of Marek's disease virus (MDV), were grown in RPMI 1640 medium (Life Technologies-Gibco) supplemented with 10% FBS and 10% tryptose phosphate broth (Sigma-Aldrich, St. Louis, MO) at 37°C and 5% CO₂. Chicken embryo fibroblasts (CEF) were made from 10-day embryos supplied by Merial-Vital Laboratory Animal Technology (Beijing, China) according to conventional procedures.

Cloning of the ch β 2M gene and sequences analysis

Based on the reported sequence of ch β 2M (GenBank: M84767.1, AY989898, and Z48921), a pair of specific primers was designed to amplify the full-length ch β 2M from the total RNA of the chicken peripheral blood mononuclear cells (PBMCs), as reported previously.⁽¹¹⁾ The primer sequences, which also contained the restriction site *Eco*R I in the forward primer (P1) and *Xho* I in the reverse primer (P2) (underlined), were as follows: forward primer (p1): 5'-TTGAATTCATGGGGAAGGCGGCGGC-3'; reverse primer (p2): 5'-TTCTCGAGTCAGAACTCGGGATCCCA-3'. PCR product of approximately 400 bp was inserted into a pGEMT-easy vector (Promega, Madison, WI), and positive clones (pGEM-T-ch β 2M) from an independent PCR reaction were sequenced by the

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Shanghai Sangon Company (China). The sequence was analyzed by DNASTar software (Madison, WI).

Synthesis of COOH-terminal ch β 2M fragments

The peptide was synthesized by GL Biochem (Shanghai, China) with a solid-phase method and purified by high performance liquid chromatography. The purity of the peptide was greater than 99%.

Preparation of monoclonal antibodies

BALB/c mice were immunized with 100 μ g synthetic peptide in complete Freund's adjuvant through a peritoneal injection, boosted with 150 μ g in incomplete Freund's adjuvant 2 weeks later and 200 μ g in 0.01 M/L PBS (pH 7.2) after another 3 weeks. Three days after the final injection, the spleen cells from the immunized mice were fused with SP2/0 myeloma cells according to standard procedures.⁽¹²⁾ The hybridoma cell supernatants were screened for specific antibodies. Positive hybridomas were subcloned twice by conventional limiting-dilution.

Construct of ch β 2M expression plasmids and transfection

The pcDNA3.1-ch β 2M plasmid was constructed by ch β 2M removed from PGEM-T-ch β 2M with *Eco*R I and *Xho* I inserted into a pcDNA3.1 plasmid vector (Life Technologies-Invitrogen, Carlsbad, CA). The recombinant expression plasmid pcDNA3.1-ch β 2M was transfected into 293T cells with Lipofectamine 2000 (Life Technologies-Invitrogen) according to the manufacturer's instruction. Then the cells were cultured for 48 h to express ch β 2M proteins. pcDNA3.1 vector DNA was as control.

Western blot analysis

Cell lysates were prepared from HD11 cells and 293T cells transfected with pcDNA3.1-ch β 2M or pcDNA3.1 by a lysis buffer (7 M urea, 2 M thiourea, 4% CHAPS, 65 mM DTT) in the presence of a protease inhibitor (Fermentas, Burlington, Canada) for 30 min at 4°C. The lysates were pelleted by centrifugation at 12,000 r/min for 10 min at 4°C and the supernatants were collected. The samples of the supernatants, chicken serum, and plasma were respectively diluted 1:5 in sample buffer (0.5 M Tris [pH 7.0], with 10% sodium dodecyl sulfate, 20% glycerol, and 5% β -mercaptoethanol) and heat denatured for 5 min at 100°C, then subjected to a 10% SDS-polyacrylamide gel and Western blot assay according to conventional procedures. Bovine serum and duck serum maintained in our laboratory were used as controls and treated in the same manner.

Immunofluorescence

HD11 cells, CEF cells, and 293T cells transfected with pcDNA3.1-ch β 2M or pcDNA3.1 were fixed with chilled acetone-alcohol (3:2) for 10 min and washed three times with PBS. The cells were then incubated with MAb diluted 1:200 in PBS at 37°C for 45 min and further incubated with goat anti-mouse IgG conjugated with FITC (Sigma-Aldrich) at 37°C for 45 min. For double staining, the specimens were counterstained with 4', 6'-diamidino-2-phenylindole (DAPI, Sigma-Aldrich) at 10 mg/L for 2 min at room temperature. After each incubation step, the cells on the coverslip were washed five times with PBS. Immunofluorescence was observed under confocal laser scanning microscope (Leica, Wetzlar, Germany). Normal mouse serum was used for negative control.

FIG. 1. Amino acid comparison between the chicken β 2M allele sequences with that of turkey (GenBank protein accession no. B53282), duck (BAE97673), human (M30683), and mouse (AAH85164). Nos. 1–119 above line, β 2M aa sequence numbers; nos. 1–20, site of signal peptide; nos. 91–119, site of synthetic peptide; dashes, similarity to chicken β 2M sequence; YVVVVY above the chicken β 2M sequences, immunoglobulin (Ig) motif; nos. 90–119, site of ch β 2M COOH-terminal 29 amino acids used as antigen.

FIG. 2. Western blot analysis of monoclonal antibodies against $\text{ch}\beta$ 2M. (A) Untransfected 293T cells lysate was not detectable with MAbs 6E7 and 3D1 (lanes a, d). 293T cells lysate transfected with $\text{ch}\beta$ 2M were detected (lanes b, e). 12 kDa protein in the HD11 cells lysates recognized by antibodies 6E7 and 3D1 (lanes c, f, respectively). (B) MAb 3D1 could react with the $\text{ch}\beta$ 2M in chicken serum (lane a) and plasma (lane b). No reactions with bovine serum (lane c) and duck serum (lane d) were observed.

Flow cytometry

To detect $\text{ch}\beta$ 2M antigen on the cell membrane, MSB1 cells cultured in logarithmic growth phase were collected by centrifugation at 800 rpm for 5 min. Then the cells were washed three times with cold PBS and incubated for 45 min at 4°C with 200 μL $\text{ch}\beta$ 2M MAb 3D1 at a 1:100 dilution in PBS with 1% BSA. The excess primary antibody was removed by three washes with cold PBS, and the cells were incubated for an additional 45 min with FITC-conjugated goat anti-mouse IgG (Sigma-Aldrich). After three washes, the cells were resuspended in PBS and 1.0×10^4 cells were analyzed by flow cytometry (FACScan Flow cytometer, BD Biosciences, San Jose, CA) using the CellQuest (BD Biosciences) software package. The cells incubated with equal concentrations of an irrelevant isotype MAb were used for control.

Immunohistochemical staining

Immunohistochemistry was performed using a Roly-One kit (XiYa JinQiao Co., Beijing, China) according to the man-

ufacturer's protocol. Briefly, frozen sections of thymus, spleen, and bursa from 28-day-old chickens (a White Leghorn specific-pathogen-free line) were fixed in chilled acetone for 30 min. The sections were then blocked with 5% horse serum in PBS and incubated with MAb diluted 1:500 with dilution buffer (provided in the Roly-One kit) in a moist chamber for 4 h at 37°C, followed by incubation with HRP-conjugated anti-mouse IgG (XiYa JinQiao Co.) for 2 h at 37°C. The sections were then developed with diaminobenzidine substrate and hydrogen peroxide, which produced a brown dark precipitate. The slides were counterstained with hematoxylin and observed under a microscope (Olympus, Tokyo, Japan).

Results

Chβ2M genes analysis and peptide selected

The $\text{ch}\beta$ 2M gene was amplified from chicken PBMCs by RT-PCR. Sequence analysis showed that the sequence of the cloned $\text{ch}\beta$ 2M was the same as that previously reported (M84767, AY989898, Z48921). It consists of 360 bp encoding 119 amino acids (aa) with a molecular weight of 12 kDa (Fig. 1). There are two conserved cysteines at positions 45 and 100. The immunoglobulin (Ig) motif YXCXVXH (X indicates any amino acid) were located at positions 98 to 104. $\text{Ch}\beta$ 2M showed 94.1% amino acid homology to the turkey β 2M gene (B53282) with only seven amino acid substitutions at positions 81, 86, 95, 96, 97, 99, and 103. There is a relatively low homology of 65.9% between duck β 2M and chicken β 2M. Multiple sequence alignment showed that $\text{ch}\beta$ 2M shared only 44.2% and 42.5% amino acid identity with human and mouse β 2M, respectively. Most of the amino acid substitutions were focused on the signal peptide domain and the C terminus, which suggest that the C-terminus' amino acids are specific to $\text{ch}\beta$ 2M. Antigenic analysis of $\text{ch}\beta$ 2M by DNASTAR software indicated that the 29 amino acids of the $\text{ch}\beta$ 2M COOH-terminus (NH₂-TPSSGSTYACKVEHETLKEPQVYKWDPEF-COOH, named TF29) is different from the mouse β 2M (Fig. 1). TF29 was selected.

MAb production

The mice were immunized with the synthesized peptide TF29. Fusions between SP2/0 cells and the mice spleen cells were made according to the conventional hybridoma procedure. A panel of monoclonal antibodies screened by ELISA, termed 3D1, 6E7, 1E2, 2E9, 7D5, was obtained. Immunoglobulin subtypes were identified as IgM for MAbs 1E2, 2E9, 7D5, and IgG1/ κ for MAbs 6E7 and 3D1.

MAbs recognized a 12 kDa of chβ2M

SDS-PAGE and Western blot results are shown in Figure 2. MAbs 6E7 and 3D1 specifically recognized a unique protein band, with a molecular weight of approximately 12 kDa, from the lysates of 293T/ $\text{ch}\beta$ 2M but did not react with the lysates of untransfected 293T cells. These MAbs also reacted with $\text{ch}\beta$ 2M antigen from HD11 cells. The same band was found in chicken sera and plasma in the Western blot analysis (Fig. 2B).

MAbs reacted chβ2M in immunofluorescence

MAb 6E7 demonstrated specific binding to 293T/ $\text{ch}\beta$ 2M and HD11 cells in immunofluorescence (Fig. 3A, c). There was

FIG. 3. Indirect immunofluorescence imaging of MAb 6E7 binding to different cells. (A) HD11 cells (a); CEF cells (b); 293T/ch β 2M (c); pcDNA3.1 transfected 293T cells (d). 200 $\times$ (B) ch β 2M on the cell membrane and cytoplasm of HD11 cells (green, a); nuclei stained with DAPI (blue, c); merged images (b). 650 $\times$

not any fluorescence signal observed in untransfected 293T cells (Fig. 3A, d). The staining pattern by confocal positioning indicated that the MAb 3D1 reacted with ch β 2M epitopes on both the cell membrane and in the cytoplasm of HD11 cells (Fig. 3B). HD11 cells (Fig. 3A, a) and CEF cells (Fig. 3A, b) demonstrated different expression levels of ch β 2M. Ch β 2M in HD11 cells produced a significantly stronger reaction than that in CEF cells. Flow cytometry analysis results showed that MAb 3D1 specifically could bind to the ch β 2M antigen on the membrane surface of MSB1 cells ($<1.0 \times 10^2$) (Fig. 4).

ch β 2M locations in tissues

Frozen tissue sections from chicken thymus, spleen, and bursa were immunostained with the MAbs. We found that MAb 6E7 reacted weakly with the bursal cortex and medulla and very weakly or not at all with bursal cortical cells. However the thymus sections showed much stronger staining

in medullary cells and mesenchyme, but weak staining in the cortical area. Spleen sections were strongly positive, but in a patchy fashion, with the most positive patches corresponding to lymphocyte-rich areas (Fig. 5).

Discussion

The current knowledge of chicken MHC class I structure and biological function has been advanced by the extensive development of MAb reagents.^(13–15) In previous studies, MAbs against chicken MHC I and II were generated using chicken peripheral blood lymphocytes.^(16,17) Herein, we analyzed β 2M sequences of different species and selected a polypeptide (TF29) as immunogen for developing monoclonal antibodies. There is relatively high homology between mammalian β 2M sequences but much lower homology between the mammalian and avian β 2M sequences. The 29 amino acid residues of the ch β 2M C-terminus have only 12

FIG. 4. Analysis of ch β 2M antigen on the cell membrane by flow cytometry with MAb 3D1. MSB1 cells incubated with the mouse isotype control antibody (A) and MAb 3D1 (B). Y-axis, cell counts; X-axis, relative fluorescence level.

FIG. 5. Immunohistochemical stain of frozen thymus (a), spleen (b), and bursa (c) sections with antibody 6E7. (d–f) Same tissue types as in a–c, negative controls stained with hematoxylin nuclear dye. Arrows show borders between cortex (top) and medulla (bottom). The tissues were from a 28-day-old SPF chicken. The bar represents 200 μ m.

and 13 aa homology with the mouse and human sequences, respectively. Among them, most amino acid substitutions are dispersed and discontinuous. It suggests TF29 is a better immunogen than chicken PBMC. The MAbs 6E7 and 3D1 are specific to the ch β 2M and no cross-reaction with human β 2M. Moreover, despite previous suggestions that β 2M was relatively conserved between the different poultry species, we found that antibody 6E7 and 3D1 could not react with duck serum β 2M.

During the last several years, the two-dimensional (2D) and three-dimensional (3D) structures of ch β 2M and MHC class I proteins have been frequently discussed. As originally noted,⁽⁸⁾ ch β 2M contains a typical β -sheet with no α -helix. Although the 20 amino acid residues of the C-terminus of ch β 2M consist of two discontinuous β -sheets, they may be freely exposed to the external environment.⁽¹⁸⁾ In our study, MAbs 6E7 and 3D1 could react both with the linear ch β 2M antigen in Western blot and the native ch β 2M antigen in flow cytometry. It suggests that antibody recognition of the ch β 2M antigen at C-terminal acid residues should be less influenced by MHC 1 conformation.

β 2M is particularly plentiful on the surface of white blood cells, and white blood cell membrane turnover is the principal source of serum β 2M. Serum β 2M is a significant diagnostic predictor, and increased levels of β 2M have been found to be of prognostic relevance in a wide variety of diseases.^(19–21) Serum Western blot immunoassays have been developed as a confirmatory test for many diseases, including *Mycoplasma hyopneumoniae*,⁽²²⁾ *Toxocara vitulorum*,⁽²³⁾ and severe acute respiratory syndrome (SARS).⁽²⁴⁾ Perhaps detection of chicken serum β 2M levels by serum blots or other immunological methods will prove highly useful for future study.

With our MAbs, the strongest staining was found in medullary cells and the mesenchyme in thymus. The staining pattern of lymphocytes in the bursa mesenchyme was much weaker than those in the thymus. We found that the ch β 2M antigen's tissue distribution, especially on sections of thymus and spleen, corresponded to that reported for Class I MHC antigens.⁽¹⁶⁾ The biological functions of ch β 2M still need to be

investigated, especially in immune regulation and cell-cell interaction. These MAbs will be good tools for analyzing the mechanism of the chicken immune system.

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Author Disclosure Statement

The authors have no financial interests to disclose.

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