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Production of monoclonal antibodies against *Chinemys reevesii* turtle vitellogenin and their usage for comparison of biochemical and immunological characters of vitellogenins and yolk proteins of freshwater turtles

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Abstract

Four hybridoma clones (ACV-1, -3, -4, and -5) were established for *Chinemys reevesii* (Reeves' turtle) vitellogenin (VTG) as a precursor protein of egg yolk and a biomarker of environmental pollution. Binding-inhibition experiments indicated that the epitopes of four mAbs were distinct. No binding of ACV-4 to *C. reevesii* VTG in the Western blot suggests that the epitope of ACV-4 would be dependent on the three-dimensional structure. ACV-1, -3, and -5 bound to *C. reevesii* VTG in the Western blot. The signal for ACV-1 and -5 disappeared by reduction of the VTG, suggesting that the construction of the epitopes for ACV-1 and -5 were dependent on the disulfide bridge in the VTG molecule. All four mAbs recognized *Trachemys scripta* and *Mauremys japonica* VTGs in the ELISA. The yolk proteins were tested for the binding of the mAbs in the Western blot. ACV-1 being capable to bind to the VTG in the reduced condition did not bind to any protein bands of the yolk. This indicates that ACV-1 recognizes a part of the VTG molecule that is not incorporated in the oocytes. Both ACV-3 and -5 bound to the 32- and 70-kDa yolk proteins. Since a mAb recognizes only one site (epitope) on a protein molecule, the 32-kDa protein originated from the 70-kDa one. An ELISA system using ACV-5 as the capture antibody and ACV-3 as the detecting antibody showed the lower detectable concentration (2 ng/mL) and a wide detectable range to 1000 ng/mL (R^2=0.999). The system was used to determine serum VTG levels of juvenile turtles treated with estradiol-17β or vehicle (corn oil). By the use of the mAbs described in this paper, basic and applied studies for turtle VTGs would be improved.

Keywords: Vitellogenin; Freshwater turtles; Monoclonal antibody; Yolk protein; Antibody binding; Cross reaction; Immunological distance

1. Introduction

Vitellogenin (VTG) is a maternal protein of yolk in oviparous vertebrates (Wallace, 1985). VTG is a lipophosphoglycoprotein with a high molecular weight (Ho et al., 1980; Kuchling, 1999). After being synthesized in the liver, VTG is released into the blood stream, from which it is taken up by the ovary and incorporated in oocytes (Wallace, 1985). Estrogen generally regulates the synthesis of VTG in female animals (Ho et al., 1981; Wallace, 1985) and evokes the synthesis and release of VTG in male animals (Ho et al., 1981; Mills et al., 2001). Bisphenol A, nonylphenol, and methoxychlor are recognized as xenobiotic estrogens due to their estrogen-like activities (Laws et al., 2000; Markey et al., 2001). When the xenobiotic estrogen(s) are spread into the environment, the VTG in blood from exposed animals is a good biomarker to estimate how the environment has been polluted.

The biochemical and immunological properties of VTG have been well characterized for birds and fish but only a few papers have been published on the VTGs of reptiles, especially freshwater turtles. A polyclonal antibody (pAb)

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was prepared against the plasma VTG of the painted turtle (*Chrysemys picta*) and its specificity among several species of turtles was examined (Gapp et al., 1979). The anti-*C. picta* pAb cross reacted with the plasma of *Chelydra serpintina*, but its cross-activity was weak. *C. picta* VTG has a molecular weight between 210 to 220 kDa based on sodium dodecyl sulfate-acrylamide gel electrophoresis (SDS-PAGE). The VTG of the red-eared turtle, *Trachemys scripta*, was purified (Palmer and Palmer, 1995). *T. scripta* VTG has a molecular weight of 214 kDa based on SDS-PAGE. Anti-*T. scripta* pAb was prepared and used to develop an enzyme-linked immunosorbent assay (ELISA). A pAb against the VTG of the painted turtle, *C. picta*, has risen (Irwin et al., 2001). The anti-*C. picta* pAb cross reacted with the *T. scripta* VTG. Recently, we developed a simple purification method of VTG of the Reeves' pond turtle, *Chinemys reevessi* (Tada et al., 2004). The *C. reevesii* VTG migrated as a single band with 200 kDa in SDS-PAGE. Also, we prepared a pAb against the *C. reevesii* VTG and used the pAb to develop an ELISA to quantify the serum VTG of the juvenile/adult or male/female of *C. reevesii*. We now describe the production and characterization of monoclonal antibodies (mAbs) against *C. reevesii* VTG and application of the mAbs to analyze yolk proteins and to quantify serum VTG of the juvenile male turtles experimentally exposed to estrogen.

2. Materials and methods

2.1. Turtle VTGs and preparation of mAbs

Adult female turtles (*C. reevesii*, *T. scripta*, and *Mauremys japonica*) were captured at a local river in Japan (Uji River in Kyoto City). The VTG of the individual species was purified from the serum of the estrogen-treated female turtle by EDTA–$MgCl_2$ precipitation followed by gel filtration (Tada et al., 2004).

C. reevesii VTG (10 μg in 50 μL) was emulsified with an equal volume of RIBI adjuvant system (Sigma-Aldrich, St. Louis, MO, USA). The emulsion (100 μL) was subcutaneously injected twice into a BALB/c male mouse at a three-week interval. The mouse was intravenously injected with the VTG without the adjuvant three days before cell fusion. Spleen cells of the immunized mouse were fused with mouse myeloma cells (X63Ag.653) grown in 15% fetal calf serum (Sigma) containing Dulbecco's modified MEM (Invitrogen, Carlsbad, CA, USA) with polyethylene glycol 4000 (Merck, Ltd., Japan, Tokyo, Japan). After HAT selection, ELISA was done for the growing hybridomas to check the antibody production in the culture medium. Antibody-producing hybridoma cells were cloned by the limiting dilution. The hybridoma cells were grown in the medium and each mAb was purified from the culture-supernatant by protein-G (Amersham Biosciences, Piscataway, NJ, USA) affinity chromatography. The protein concentration of the VTGs and mAbs was determined by Bradford's method (Bradford, 1976). The immunoglobulin class and subclass and the light chain type were determined by an immunoglobulin typing kit (Wako Pure Chemicals, Osaka, Japan).

2.2. ELISA, SDS-PAGE, and Western blot

Each well of a 96-well microplate (Iwaki, Funahashi, Japan) was coated with 100 μL of the VTG (10 μg/mL). Wells were washed with 0.05% Tween 20 in phosphate-buffered saline (PBS). After blocking of the unreacted sites of each well with 1% bovine serum albumin (BSA, Sigma), 100 μL of the hybridoma-culture supernatant was added to the wells. After 1 h incubation at 37 °C, the wells were washed as described. Peroxidase-labeled anti-mouse IgG (Bio-Rad Laboratories, Hercules, CA, USA) diluted 10,000-fold was added to the wells. After the incubation and washing, a substrate solution (0.4 mg/mL *o*-phenylenediamine and 0.006% H_2O_2) was added at 150 μL/well. The reaction was terminated after a 30-min incubation by addition of 1 N H_2SO_4 at 50 μL/well. The absorbance at 490 nm of the reaction products was then monitored.

C. reevesii VTG was applied to SDS-PAGE (Laemmli, 1970) for the Western blot (Towbin et al., 1979). The VTG was reduced in the SDS sample buffer containing 50 mM dithiothreitol. VTG was then transferred to a polyvinylidine difluoride (PVDF) membrane (Nihon Millipore K.K., Tokyo, Japan). The membrane was treated with 3% skim milk in PBS to minimize any non-specific reactions. The purified mAb (1 μg/mL), peroxidase-labeled anti-mouse IgG (1000-fold diluted, Bio-Rad), 3-3′ diaminobenzidine (0.5 mg/mL PBS, Sigma), and 0.003% H_2O_2 were used to visualize the reaction signal.

2.3. Biotinylation of mAbs and cross-inhibition analysis

The purified mAb was dialyzed against 0.02 M sodium carbonate buffer (pH 9.5) and then diluted to 1 mg/mL. Biotinamidocaproate *N*-hydroxy succinimide ester (NHS-biotin, Sigma) was dissolved in distilled water to 1 mg/mL. The NHS-biotin was added (0.1 mL per 1 mg of antibody) to the mAb solution and the mixture was gently stirred for 1 h at room temperature. The reaction products were dialyzed against PBS to remove any non-reacted NHS-biotin. BSA was added to the biotin-labeled antibody to 1% as a stabilizer. The wells of the ELISA plate were coated with the VTG, as previously described. After blocking and washing, the mixture of a biotin-labeled mAb appropriately diluted and a non-labeled mAb (10 μg/mL) were added to the well and the plate was incubated for 1 h at 37 °C. After washing, peroxidase-labeled streptoavidin (Sigma) diluted 1000-fold was added; then the above-mentioned ELISA procedures were carried. The binding–inhibition ratio was calculated as follows: [1 − (A_{490} in the presence of the