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The effect of carbohydrate moieties on immunoregulatory activity of yolkin polypeptides naturally occurring in egg yolk

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A B S T R A C T

Substances isolated from natural sources showing a character of endogenous regulators may have a circular meaning. One example is yolkin, which can be useful as a natural therapeutic agent for treating immunological disorders. In the present work we studied the effect of degree of deglycosylation on the biological activity of yolkin polypeptides. For this purpose, yolkin from hen egg yolk was fractionated by gel permeation chromatography (Sephacryl S 100 - HR and Zorbax GF- 250 columns) and enzymatically deglycosylated. The immunoregulatory activity of yolkin fractions (ysec, y4 and y9) were estimated by their induction of proinflammatory factors. The induction of cytokines IL-6, IL-10, and TNF-α in human whole blood cell cultures and nitric oxide from bone-marrow mouse macrophages BMDM were measured. The obtained results showed that all of the yolkin polypeptides, irrespective of size, exerted a strong cytokine and nitric oxide induction, indicating that all of the yolkin constituents possess these activities. The results indicated that the presence of carbohydrate in yolkin polypeptides is unnecessary to preserve cytokine inducing activity and NO generation. Furthermore the removal of carbohydrates from the yolkin by PNGase F led to a significant increase in the level of secretion of nitric oxide from the macrophages.

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1. Introduction

Vitellogenin (Vtg) is a large homodimeric lipoprotein of molecular weight in the range from 250 to 600 kDa present in serum which displays a similar structural characteristic in vertebrates and invertebrates (Chen, Sappington, & Raikhel, 1997; Tufail & Takeda, 2008). Vitellogenins of different origin exert a variety of biological activities. It has been proved that Vtg isolated from crustaceans (*Branchiostoma blecheri*) possesses hemagglutinating and antimicrobial activity (Zhang, Sun, Pang, & Shi, 2005), while insect Vtg, besides the main nutritive function, appears to be an antioxidant (Nakamura et al., 1999; Seehuus, Norberg, Gimsa, Krekling, & Amdam, 2006). Corresponding proteins isolated from fish serum have the ability to neutralize the infectious pancreatic necrosis virus (IPNV) (Garcia et al., 2010). In oviparous animals, Vtg is the protein precursor of the main yolk proteins. During formation of the egg, Vtg is proteolytically cleaved into several fragments and transported from the plasma into the oocyte by receptor endocytosis. Subsequently, Vtg was mainly considered as a source of nutrients for the developing embryo. Because Vtg has been so long recognized as a female specific protein, it is now ignored as being present in males, which indicates that Vtg fulfills unknown and additional functions, making it a particularly interesting protein (Polanowski, Zabłocka, Sosnowska, Janusz, & Trziszka, 2013).

Chicken Vtg consist of three species designated vitellogenins; I, II and III, with apparent molecular masses of: 260, 246 and 210 kDa, (Yamamura et al., 1995). It has been proven that Vtg II is enzymatically hydrolyzed into the main proteins belonging to the granule fractions of egg yolk, namely lipovitelin I (fragment of N-terminal region of Vtg, MW 120 kDa), phosvitin (phosphoseryl rich domain of Vtg, MW about 44 kDa) and lipovitelin II, a protein fragment of MW 32 kDa. These protein fragments corresponding to

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the N- terminal domain of Vtg are well characterized, but there is a little information about the protein fragments located in the Vtg C-terminal region (Polanowski et al., 2013; Yamamura et al., 1995).

It has been reported that the main egg yolk plasma proteins are accompanied by protein fragments released from the C- terminal region of Vtg. At first, yolk plasma glycoprotein YPG 40 was identified, an asparagine-linked carbohydrate chain composed of 284 amino acid residues from Ala (1567) to Thr (1850), the cysteine rich domain of Vtg II homologous to the D2 region of von Willebrand factor. The second protein is yolk plasma glycoprotein YPG 42 which is likely a proteolytic product of Vtg I. Recent studies have shown the presence in egg yolk plasma of another C terminal Vtg fragments named yolkin, complexed with immunoglobulin Y (Polanowski, Zabłocka, Sosnowska, Janusz, & Trziszka, 2012, 2013). It is a mixture of several proteins and peptides of apparent molecular weights ranging from 1 to 35 kDa. Yolkin polypeptides are homologous to several fragments of the C-terminal domain of Vtg II.

The host response to infection or injury initiates a cascade of events involving recruit-ment of immune competent cells like macrophages or lymphocytes. Those cells play a crucial role in the immune system both as antimicrobial effector cells and also as immunoregulatory cells, which induce, modulate or suppress immune response. They release a wide spectrum of inflammatory mediators like nitric oxide, reactive oxygen species, chemokines and cytokines (Mills, 2012; Rath, Müller, Kropf, Closs, & Munder, 2014). In our previous work we showed that yolkin is extremely active inducer of important cytokines for the immune response and possessed the ability to induce human blood cells to secrete cytokines such as: IL-1β, IL-6, IL-10 and TNF-α (Polanowski et al., 2012, 2013). It was also found that yolkin modulates nitric oxide release from the macrophage cell line J774.2 (Polanowski et al., 2012; Zabłocka, Sosnowska, Urbaniak, Janusz, & Polanowski, 2014). It has been proved that yolkin may mitigate the behavioral symptoms of aging, and support cognitive learning and memory in rats (Lemieszewska et al., 2016). Last study demonstrated its ability to stimulate blood cells to the production of pro-survival brain derived neurotrophic factor (Zambrowicz et al., 2017).

The aim. In view of yolkin, a relatively newly discovered polypeptide complex from egg yolk with not well characterized biological properties, the role of molecule glycosylation on its biological activity seems to be very interesting. Besides improving descriptions, we may indicate another technique for obtaining yolkin e.g. by chemical synthesis or with expression of recombinant protein in the microbial cell, where the presence of carbohydrates in polypeptides is unnecessary to preserve biological activity. Therefore, the aim of the study was a comparison of the immunoregulatory activity of glycosylated and enzymatically deglycosylated yolkin by examining the impact on cytokine and nitric oxide production by human whole blood cells and murine macrophages BMDM, respectively.

2. Materials and methods

2.1. Materials

RPMI 1640 medium, Dulbecco's medium and PBS were obtained from the Laboratory of Biopreparations of the Institute of Immunology and Experimental Therapy PAS. Tris (hydroxymethyl)aminomethane, Sephacryl S 100 - HR resin, bacterial lipopolysaccharide (LPS) from *E. coli* (serotype 055:B5), leukoagglutinin (PHA-L) from *Phaseolus vulgaris*, bovine serum albumin, concanavalin A were purchased from Sigma-Aldrich (St. Louis, MO, USA). L-glutamine and antibiotics (penicillin/streptomycin mixture) were purchased from BioWest (Nuaillé, France). Reagents for SDS-PAGE and protein marker were purchased from Bio-Rad (California, USA). Human IL-6 and IL-10 ELISA Max™ Deluxe Kit were obtained from BioLegend (San Diego, CA). PNGase F of *Flavobacterium meningosepticum*, was a recombinant protein designed with fusion Maltose Binding Protein (MBP) tag at N-terminus. The enzyme was purified from periplasmic space of K12 TB-1 E. coli strain (New England Biolabs) after transformation with pMal-p2 plasmid containing PNGase-coding sequence N-terminal 120 nt signal peptide coding sequence removed (genetic construct was a generous gift from Dr. Patrick von Roey, Wadswarth Center, USA). Details of purification procedure are available on request. Amylose resin was purchased from BioLabs (New England). Basic reagents for immunoblotting were purchased from Vector Laboratories.

2.2. Cell cultures

2.2.1. Whole blood samples

Whole blood samples from healthy donors were kindly provided by the Station of Blood Donation, 4th Military Hospital, Wrocław, Poland. Samples were collected into syringes containing 10 U/ml of heparin. Within 1 h after the collection, the blood was diluted 10-fold with RPMI-1640 medium supplemented with 100 units/ml penicillin, 100 mg/ml streptomycin, and 0.5 mg/ml L-glutamine. Whole blood samples were used for the determination of cytokines.

2.2.2. The murine bone - marrow –derived macrophages BMDM

The murine bone - marrow –derived macrophages BMDM was purchased from American Type Culture Collection (ATCC, USA). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM), containing 10% fetal bovine serum, antibiotics (penicillin, streptomycin and gentamycin) and 2 mM L-glutamine. Cells were grown under standard conditions in a humidified incubator at 37 °C and 5% CO_2.

2.3. Biological materials

Hen eggs were bought in local market.

2.4. Methods

2.4.1. Isolation of immunoglobulin Y (IgY) complexed with yolkin from egg yolk

The IgY containing yolkin was isolated from egg yolks according to the procedure described in details by Polanowski et al. (2013). Yolk plasma was fractionated with the use ammonium sulphate (40% saturation), dialyzed against water and then against 100 mM potassium phosphate buffer, pH 7.2 and clarified by centrifugation. It was a starting material for yolkin isolation.

2.4.2. Separation of yolkin polypeptides using gel permeation chromatography

2.4.2.1. Size - exclusion chromatography of saturated yolk plasma on sephacryl S 100 -HR resin. Starting material was chromatographed in an LC system (BioRad) on a Sephacryl S 100 - HR resin (K50/100 Pharmacia Ltd, Kent, UK). The column was calibrated using bovine serum albumin (66.0 kDa), ovalbumin (45.0 kDa) and lysozyme from hen egg white (11.4 kDa). Samples were loaded onto the chromatographic column (5.0 cm × 80.0 cm) and equilibrated with 100 mM potassium phosphate buffer, pH 7.2. The chromatography was conducted at 4 °C and flow rate of 1.1 ml/min. Fractions were collected in an intervals 4.0 min. Protein fractions from number 57 to number 68 were collected, dialyzed, lyophilized and identified as yolkin.

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