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Development of a fluorescent immnunochromatographic assay for the procalcitonin detection of clinical patients in China

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ABSTRACT

Background: Procalcitonin (PCT) has been recognized as a biomarker in severe inflammation, infection and sepsis. PCT detection in serum requires sensitive and specific antibodies. In this study, we generated monoclonal antibodies (mAbs) and developed fluorescent immunochromatographic assay for PCT detection.
Methods: Human recombinant PCT was used as immunogen. mAbs against PCT were developed and applied to fluorescent immunochromatographic assay for PCT detection in clinical samples.
Results: Out of 35 hybridoma cell lines secreting antibodies against the recombinant PCT, five sensitive and specific cell lines were selected and designated as F6, G2, C2, D2 and E5. All these antibodies have no cross reaction with calcitonin or calcitonin gene-related peptides (CGRP). After screening for pairing, mAb F6 was labeled with fluorescent microspheres and C2 was coated on a nitrocellulose membrane for immunochromatographic test. All 35 clinical samples were detected by the mAb F6–C2 test strips and the bioMérieux PCT assay. The test strips showed high specificity and sensitivity for PCT. Good correlation was observed between our immunochromatographic test strips and the bioMérieux PCT assay (R^2:0.986).
Conclusions: These newly developed anti-PCT mAbs and fluorescent immunochromatographic assay can serve as important diagnostic tools for a fast, reliable and point-of-care testing for easy determination of PCT in serum and diagnosis of bacterial infection, inflammation or sepsis.

1. Introduction

Procalcitonin (PCT), a peptide precursor of calcitonin (CT), is composed of 116 amino acids with a molecular weight of 13 kDa. PCT is normally produced by the C cells of the thyroid in healthy persons [1]. Normally, the physiological concentration of PCT in serum is below 0.1 μg/l. In the event of severe bacterial, parasitic or fungal infection, the concentration of PCT increases to several thousand-folds as compared to normal physiological concentrations [2,3].

PCT levels between 0.1 and 0.5 μg/l reflect local bacterial infections, whereas a systemic inflammatory response syndrome (SIRS) leads to serum PCT concentrations up to 2.0 μg/l. Serum PCT concentrations between 2 and 10 μg/l are considered to have been caused by sepsis, whereas concentrations ranging from 10 to 100 μg/l are indicative of the possibility of septic shock [4,5]. Therefore, PCT has been recognized as an important biomarker of the body' response to severe inflammation, infection, or sepsis. Compared with other inflammatory factors, such as C-reactive protein (CRP), white blood cells (WBC) count, tumor necrosis factor (TNF)-α, or interleukin-6 (IL-6), PCT is a more specific and sensitive biomarker for clinical diagnosis [6,7]. PCT assays can also be used to distinguish between viral infection and bacterial infection [8–10]. Bacteria co-infection has also led to unreasonable and excessive use of antibiotics leading to issues on antibiotics resistant species worldwide. Recent researches have shown that PCT can serve as a biomarker to guide the clinical application of antibiotics [8,10–12]. Thus, an effective and fast method to detect PCT concentration in the serum would be very helpful for early infection diagnosis and antibiotics progression monitoring.

Several methods have been developed to detect PCT levels in the blood, among which are chemiluminescence immunoassay, enzyme-linked immunosorbent assay and Thermo Fisher SPCT-Q-semi quantitative assay [13–16]. All these methods employ the use of antibodies with high sensitivity and specificity. Antibodies against PCT have been developed and applied to different platforms for PCT detection in many

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published papers [16–18], but developments in the point-of-care testing (POCT) of PCT are still in its early research stage.

2. Material and methods

2.1. Materials

Restriction endonucleases, *BamH I* and *Xho I*, and PrimeStar Hs mix, and PCR mix, DNA markers, protein and Western blot markers were from TaKaRa Biotechnology Co., Ltd. Plasmid and gel extraction kits were from the Tiangen Biotech. pET-28a plasmid, competent *Escherichia coli* cells (DH5α and BL21 (DE3) were preserved in our lab. Complete and incomplete Freund's adjuvant and ISO-2 kits were from Sigma-Aldrich. RPMI 1640 medium, fetal bovine serum (FBS), penicillin–streptomycin, Hypoxanthine and Thymidine (HT), hypoxanthine–aminopterin–thymidine medium (HAT) and Polyethylene glycol (PEG1500) were purchased from Gibco. Methyl cellulose was from Sigma-Aldrich. Myeloma cell line SP2/0-Ag14 was preserved in our lab. Goat anti mouse IgG conjugate was purchased from ZSGB-BIO. SuperSignal West Pico Chemiluminescent Substrate was from Thermo Fisher Scientific Inc. Six-to-eight-weeks-old Balb/c mice were purchased from Guangdong Animal Laboratory Center. F1 hybrid mice were purchased from the Animal Laboratory of Sun-Yat-Sen University.

Human recombinant procalcitonin with N-terminal thioredoxin tag (hrPCT-trx) was provided by Guangzhou Wondfo Biotech Co., Ltd. for use as standard hybridoma screening antigen. Human calcitonin (CT) and human calcitonin gene-related peptide (CGRP) were purchased from Sigma-Aldrich. N-Hydroxysuccinimide (Sulfo-NHS) and 1-ethyl-3-(3-dimethylaminepropyl) carbodiimide-HCl (EDC) were purchased from Pierce. Estapor Fluorescent Microspheres, sample pad, absorbent pad, and polyvinylchloride (PVC) pad were from Millipore. Nitrocellulose membrane (OEM Membranes) was from Sartorius. Blocking agent was from Fapon Biotech Inc. All chemicals used were of higher molecular grade.

2.2. Human samples and ethics approval

Serum and plasma samples were collected from the General Hospital of Guangzhou Military Command of PLA. Sera were stored at −80 °C until analysis. Ethical approval was obtained from the General Hospital of Guangzhou Military Command of PLA. All participants were provided written informed consent for the use of the clinical samples in this study.

2.3. Construction of the recombinant plasmid

The DNA sequence of human PCT was designed with codon optimization and synthesized by Sangon Biotech Co., Ltd. The primer sets were designed according to the gene sequence of PCT obtained from Genbank (Accession number: NM_001741.2). The forward primer and reverse primer were as follows: PCT-F, 5 -GATC**GGATCC**ATGGGCTTCCAAAAAT TCTCT-3 , PCT-R, 5 -GATA**CTCGAG**GTTAGCGTTCTGCGGCATGG-3 . The PCR product was digested with *BamH I* and *Xho I*and cloned into pET28a vector.

2.4. Protein expression and purification

The resultant construct carrying the PCT gene was then transformed into *E. coli* BL21 (DE3) cells and the recombinant PCT with his tag (hrPCT-his) protein was expressed and purified using the methods described previously [19,20]. After induction with isopropyl-β-D-thiogalactopyranoside (IPTG), the cultures were harvested by centrifugation. The cell pellet was lysed by sonication and the supernatant was loaded on a HiTrap Ni^{2+} column. The eluted protein was analyzed by SDS-PAGE.

2.5. Western blot analysis

The purified protein was transferred onto a polyvinylidene fluoride (PVDF). After blocking with 5% non-fat milk in 1X Tris-buffered saline (TBS) supplemented with 0.1% Tween-20 for 2 h at room temperature, the membrane was then incubated with anti-6X His tag antibody (1:2000) for 2 h at room temperature. PCT protein bands were visualized with Supersignal West Pico chemiluminescent substrate kit and scanned with MicroChemi Western Blot Analyser (DNR Bio-Imaging Systems Ltd., Israel).

2.6. Animal immunization

Five adult female Balb/c mice (6–8 weeks of age) were injected intraperitoneally (i.p.) and subcutaneously (s.c.) with 100 μg of hrPCT-his protein supplemented with Complete Freund's adjuvant. Booster immunizations were given with the antigen in Incomplete Freund's adjuvant (50 μg/mouse, i.p.) at 2-week intervals. A final boost was given (100 μg of the antigen in PBS, pH 7.4) three days before cell fusion. All the procedures do not violate any national guidelines and institutional policies for use of laboratory animals in research.

2.7. Hybridoma production and generation of mAbs

Mice were sacrificed and the spleen cells were fused with the myeloma cell line SP2/0-Ag14 using PEG 1500 as described previously [20]. Briefly, the fused cells were then mixed with methylcellulose-RPMI 1640 media supplemented with 15% (v/v) FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 1% (v/v) HEPES, 2% (w/v) methyl cellulose, 1% (v/v) HAT), and plated on petri dishes (35 mm). After 5–7 days of incubation, hybridoma clones were picked into 96-well plates containing complete RPMI 1640 medium supplemented with 1% HT. Cell culture supernatants were screened using indirect ELISA.

2.8. Enzyme-linked immunosorbent assays

The indirect ELISA assay was used to screen hybridomas which secrete antibodies against the PCT antigen. Microtitre plates were coated with 2 μg/ml of hrPCT-trx antigen in coating buffer (0.05 mol/l carbonate buffer, pH 9.6) and incubated over night at 4 °C. The plates were then washed with 0.015 M PBS buffer containing 0.1% Tween 20 and blocked with 3% BSA for 2 h at 37 °C before the addition of 100 μl of cell culture supernatant for 1 h at 37 °C. HRP conjugated goat-anti-mouse IgG (1:20000) was added to each well and incubated for 30 mins. The enzymatic reaction was visualized using substrate TMB with hydrogen peroxide and stopped by adding 50 μL 2 mol · l sulfuric acid to each well. The absorbance was measured at 450 nm using microplate reader (Thermo Fisher Scientific Inc.).

2.9. Ascite production and purification

F1 hybrid mice were injected intraperitoneally with mineral oil (0.5 ml/mouse) followed by injection with 5×10^6 of hybridoma cells after seventh day. Ten days later, ascites were collected and centrifuged at 6000 × *g* for 10 min. The IgG fractions were prepared by ammonium sulfate precipitation followed by purification on Protein A column. The titer values of both ascites and purified mAbs were determined by indirect ELISA.

2.10. Isotyping and affinity of mAbs

Commercial ISO-2 kits were used to determine the isotypes of the monoclonal antibodies. Antibody affinity was determined and calculated as described by David Beatty with little modifications [21]. 96-well plates were coated with hrPCT-trx and serial dilutions (2 μg/ml to 0) of mAbs were added and incubated for 1 h. After washing, goat