A dual-label time-resolved fluorescence immunoassay (TRFIA) for screening of Coronary atherosclerosis based on simultaneous detection of Lp-PLA$_2$ and HsCRP

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ABSTRACT

Atherosclerosis is the underlying cause of most coronary events. The conventional method for coronary atherosclerosis detection is morphological examination or coronary arterionyraphy. These methods are complex and time-consuming. In this study a two-step dual-label TRFIA was developed for the simultaneous detection of Lp-PLA$_2$ and hsCRP in a single run. The performance of this assay was first evaluated using clinical serum samples, and then compared with commercialized kits. The sensitivity of this assay for Lp-PLA2 detection was 1 ng/mL (dynamic range, 0–1000 U/L), and the sensitivity for hsCRP detection was 1 mg/L (dynamic range, 1–1000 mg/L). High correlation coefficients (R) were obtained between the present dual-label TRFIA and commercially available kits(R = 0.99 for LP-PLA2 and hsCRP). The present dual-label TRFIA has high sensitivity, specificity, and accuracy in clinical sample analysis. It is a good alternative to the single-label diagnostic methods.

1. Introduction

Atherosclerosis is a systemic disease with focal rupture of vulnerable plaque and responsible for major clinical events, such as stroke and gangrene of the extremities [1,2]. Atherosclerosis accounts for more than 50% of all mortality in the Europe, USA and Japan [3,4]. The lesions result from an excessive, inflammatory-fibroproliferative response to various forms of insult to the endothelium and smooth muscle of the artery wall [5]. A large number of growth factors, cytokines and vasoregulatory molecules participate in this process [6]. Therefore, these specific molecules provide opportunities to develop diagnostic agents to detect lesions and coronary atherosclerosis.

Lipoprotein-associated phospholipase A2 (Lp-PLA2) is a calcium-independent serine and produced by inflammatory cells [7,8]. Lp-PLA2 is highly expressed in vulnerable plaques and associated with atherogenic lipoproteins [9]. Many clinical studies demonstrated that Lp-PLA2 was relevant to oxidized low density lipoprotein cholesterol and multifocal plaque vulnerability [10]. Importantly, systemic Lp-PLA2 has emerged as an independent marker for cardiovascular disease and events in several large population-based studies [11–13]. These data suggest Lp-PLA2 to be a better candidate for screening of coronary atherosclerosis using many kinds of technologies.

High-sensitivity C-reactive protein (hsCRP), produced in the liver, may provide an adjunctive method for global assessment of cardiovascular risk [14]. Several large-scale prospective epidemiological studies had shown that plasma levels of hsCRP were a strong independent predictor of risk of future myocardial infarction, stroke, peripheral arterial disease, and vascular death among individuals without known cardiovascular disease [15–20]. In addition, among patients with acute coronary ischemia [21–23], a stable angina pectoris [24], and a history of myocardial infarction [25], levels of hsCRP have been associated with increased vascular event rates.

The conventional method for coronary atherosclerosis detection is morphological examination or coronary arterionyraphy [26]. These methods are complex and time-consuming. To date, many commercialized kits were available to detect serum levels

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of Lp-PLA2 and hsCRP. However, they detect Lp-PLA2 and hsCRP separately. High cost, labor and time consuming are their major limitations. Time-resolved fluoroimmunoassays (TRFIA) is a sensitive, simple and inexpensive detection method that requires relatively small sample volume [27]. TRFIA is based on lanthanide chelates that have unique fluorescent properties, such as narrow emission peaks, high quantum yields, long Stokes shifts and long fluorescence lifetimes [28]. The long emission durations and narrow emission peaks allow the delayed measurement of different fluorescence, rendering this assay with the ability of multiplex detection.

In this study, a high sensitive and cost-effective dual-label TRFIA, using fluorescent lanthanide (Sm^{3+} and Eu^{3+}) chelates, was developed for the simultaneous detection of Lp-PLA2 and hsCRP in a single test run (Fig. 1). The present dual-label TRFIA has high sensitivity, specificity, and accuracy in clinical sample analysis. It is a good alternative to the single-label diagnostic methods.

2. Materials and methods

2.1. Materials

The LP-PLA2 and hsCRP standards were purchased from Abcam (Cambridge, MA). The monoclonal antibody (McAbs) for LP-PLA2 and hsCRP were purchased from Roche Diagnostics GmbH (Mannhein, Germany). Hormone-free bovine serum were obtained from Dingguo Biotech (Beijing, China). Eu^{3+}- and Sm^{3+}-labeling kits were obtained from PerkinElmer (Norwalk, CT, USA). BSA was obtained from Merck (Darmstadt, Germany). Centrifugal filters with molecular weight cut off 10 KDa and 50 KDa were purchased from Millipore (Bedford, MA). Sephadex G50 column was obtained from GE Healthcare (Uppsala, Sweden). Enzyme-linked immuno sorbent assay kits for LP-PLA2 and hsCRP were purchased from Roche Diagnostics GmbH (Mannhein, Germany). Other chemicals and reagent used were of analytical grade.

2.2. Coating of microplates

96-well microtiter plates were coated with 100 μL of coating buffer (50 mmol/L carbonate, pH 9.6) containing 8 μg/mL of antibodies (monoclonal antibody for LP-PLA2 and hsCRP) overnight at 4 °C. After coating, the plates were washed with PBS containing 0.05% Tween-20 (v/v) then blocked with blocking buffer (50 mmol/L Tris-HCl containing 3% BSA, m/v, pH 8.0) overnight at 4 °C. The blocking buffer was then removed and the coated plates were dried in a vacuum, and stored at −20 °C until use.

2.3. Labeling antibody and BSA with Sm^{3+} and Eu^{3+} chelates

The labeling procedure was performed using Sm^{3+} and Eu^{3+} labeling kits according to the manufacturer's instructions. Briefly, 1 mg of McAbs was washed 6 times with labeling buffer (50 mmol/L Na_2CO_3, pH 9.0) and collected using centrifugal filters. It was then suspended in 250 μL of labeling buffer, and mixed gently with 500 μg of Sm^{3+} chelates in 250 μL of the same buffer. The mixture was incubated overnight at room temperature, followed by collecting using Tris-HCl (50 mmol/L, pH 7.8) buffered Sephadex G50 column. The conjugated McAbs was preserved in Tris-HCl buffer (50 mmol/L, 0.1% BSA, m/v, pH 7.8) at 4 °C. hsCRP-bovine serum albumin conjugate was labeled with Eu^{3+} with the same procedure.

2.4. Assay procedures

Twenty five microliters of serum samples or standards were first added into the wells. After 4 times of washing, 200 μL of Sm^{3+} and Eu^{3+} labeled Lp-PLA2 and hsCRP antibody (1 μg/mL) were added, and the plate was incubated at room temperature for another 1 h. After 6 times of washing, 200 μL of enhancement solution (15 μmol/L β-NTA, 50 μmol/L TOPO and 0.1% Triton X-100, v/v) was added to each well and the plate was shaken gently for 5 min followed by the fluorescence readings (Auto DELFIA 1235, PerkinElmer).

2.5. Evaluation of the assay

Sensitivity of the assay was evaluated using a serial standard dilutions of Lp-PLA2 (0 ng/mL, 50 ng/mL, 100 ng/mL, 250 ng/mL, 500 ng/mL, 1000 ng/mL) and hsCRP (0 mg/L, 1 mg/L, 10 mg/L, 100 mg/L, 500 mg/L, 1000 mg/L). Averages (X) and standard deviations (SD) were calculated from twenty independent experiments (n = 20).

Accuracy of the assay was evaluated by analyzing the low, median and high concentrations of clinical samples. The intra- and inter- assay variations were obtained from ten independent experiments (n = 10).

Recovery of the assay was evaluated using standards spikes hormone-free serum. The recoveries (%) were established using the equation: Recovery = 100 × (measured concentration/spiked concentration).

For the specificity tests, different concentrations of follicle stimulating hormone (FSH), estradiol, testosterone luteinizing hormone (LH), cortisol and thyroid hormones (TH), were analyzed.

2.6. Comparison of TRFIA with commercialized kits

Parallel tests comparing the TRFIA method were carried out with Lp-PLA2 and CRP Enzyme-linked immuno sorbent assay kits. The appropriate controls were within the ranges provided by the manufacturer for all runs. Eighty-four clinical serum samples from Zhujiang Hospital (Guangzhou, China) and 316 samples from Nangfang Hospital (Guangzhou, China) were tested. The Enzyme-linked immuno sorbent assay kits was performed according to the manufacturer's instructions.

Receiver operator characteristic (ROC) curves were constructed, and the area under the curve (ROC–AUC) with a 95% CI (confidence interval) was calculated using 300 clinical samples from Nangfang Hospital (Guangzhou, China). Sensitivity and specificity were calculated using serum samples of patients confirmed by coronary atherosclerosis. Subgroups were analyzed according to the vulnerability of plaque.

2.7. Preparation of serum samples

Blood samples were immediately collected in 10 mL vacutainer serum Tube (Becton-Dickinson, Belgium) before surgery. Serum tubes were centrifuged at 1000 rpm for 8 min. Serum was collected, dispensed into multiple cryotubes and frozen at −75 °C within 4 h.

2.8. Statistical analysis

Quantitative data were presented as mean ± standard deviation (mean ± SD). Comparisons between the quantitative data were made using the paried-samples t test, whereas correlations between groups were calculated with Spearman's rank correlation. Spss 17.0 was used in the statistical analysis. $P < 0.05$ was considered statistically significant.