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Highly sensitive and quantitative human thrombospondin-1 detection by an M55 aptasensor and clinical validation in patients with atherosclerotic disease

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ABSTRACT

Aptamer-based biosensors (aptasensor) are powerful tools for rapid and sensitive biomarker detection. In this study, we report a DNA aptamer probe evolved from cell-SELEX that can recognize thrombospondin-1 protein in human plasma samples. The K_D value of the aptamer M55 binding to thrombospondin-1 was determined as 0.5 ± 0.2 μM with an R^2 of 0.9144. A horseradish peroxidase-linked short oligo was complementarily bound onto the 3′ end of the aptamer sequence to facilitate the 'smart' design of an M55-aptasensor for quantifying thrombospondin-1 protein in plasma samples. The limit of detection was 6.96 fM. Thrombospondin-1 is a glycoprotein with multiple biological functions, including inflammation, platelet aggregation and endothelial cell apoptosis, and is involved in the pathology of atherosclerosis. In total, 118 plasma subjects were analyzed by using the aptasensor measurement with 1 μL sample volume and 5 min incubation time. The thrombospondin-1 concentrations in ST-Elevation Myocardial Infarction patients with severe atherosclerotic plaque burden were statistically significantly higher than in the healthy volunteers without atherosclerosis conditions, suggesting that thromboposnidn-1 is a potential plasma biomarker for atherosclerosis progression.

1. Introduction

Apatmers are single-stranded oligonucleotides that are capable of binding numerous targets from small molecules to the whole living cells (Ohuchi, 2012). Aptamers are easily synthesized and chemically modified to provide various types of bioconjugations, which make them ideal materials for developing biosensors. An aptamer-based molecular probe usually consists of two parts: the sensing domain, which is an aptamer that can recognize the target, and a reporter, which can be measured by an analytical or imaging modality. The aptamer sensing domain selected by Systematic Evolution of Ligands by Exponential Enrichment (SELEX) is the core of the molecular probe. Upon the recognition event occurring, the signal then can be detected *in situ* through a reporter. These reporters may include fluorophores, radionuclides, enzyme catalysis, magnetic particles and other contrast reagents. Compared with antibody as the receptor for biosensors, the intra- and inter-hybridization of aptamer sequences enable the design of 'smart' biosensors to detect the target with high specificity and sensitivity (Holzhauser and Wagenknecht, 2012; Zhao et al., 2012; Shen et al., 2007).

Coronary atherosclerosis is the commonest manifestation of cardiac disease and the 2nd major cause of death in industrialized countries. It has an insidious systemic and progressive inflammatory disease occurring over multiple decades that often goes undetected until the onset of clinical symptoms (McCarthy, 2010). Vulnerable atherosclerotic plaques may be developed long before they can be detected using conventional diagnostic tools, since the early stage plaques do not protrude into the lumen. However, these 'silent' plaques are able to precipitate severe clinical events leading to myocardial infarction and cerebral stroke (Langer and Gawaz, 2006). Unfortunately, methods that are capable of screening and assessing "vulnerable" (prone-to-rupture) atherosclerotic plaques at risk of rupture, noninvasively and safely, are currently not available (Jacobin-Valat et al., 2011). Several recent studies on coronary arteries (Little et al., 1988; Libby, 1998) and carotid arteries (Lovett et al.,

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2004; Wasserman et al., 2005) have reported that additional factors, including plaque composition, presence and state of the fibrous cap, intra-plaque hemorrhage, plaque ulceration and plaque location, could be potentially important cellular and physiological markers for identifying future plaque rupture events. As a consequence, the current paradigm for the early detection, prognosis and risk prediction of atherosclerosis has shifted to the evaluation of biological processes at the molecular level. The discovery of biomarkers, which are able to report on the underlying atherosclerotic process, is therefore of great interest in biosensor development.

In this study, we utilized the cell based-Systematic Evolution of Ligands by Exponential Enrichment (cell-SELEX) method to select aptamer probes that can specifically recognize the atherosclerotic cell model, i.e. inflamed human aortic endothelial cells. The target protein of the aptamer probe was identified by MALDI-TOF MS and MS/MS as the thrombospondin-1 protein. Thrombospondin-1 is found in the platelet α-granules, and is also expressed by endothelial cells and macrophages (Lawler et al., 1998). In the early stage of atherosclerosis, the expression of thrombospondin-1up-regulates cell adhesion molecules expression and promotes monocyte binding to endothelium (Narizhneva et al., 2005). During the progression of atherosclerotic plaque, thrombosponidn-1 is up-regulated (Chavez et al., 2012) and involves in smooth muscle cell proliferation and migration (Patel et al., 1996). In the later stages of atherosclerosis, thrombospondin-1 plays a critical role in the development of the thrombus (Jurk et al., 2003). The infarcted heart directly expresses thrombospondin-1 locally after myocardial infarction (Sezaki et al., 2005; Chatila et al., 2007). To explore the role of plasma thrombospondin-1 level in the progression of atherosclerosis, we further developed an aptasensor based on the aptamer probe to analyze and compare thrombospondin-1 level among healthy volunteers, patients with angiographically normal coronary arteries, Non-ST-Elevation Myocardial Infarction (NSTEMI) subjects and ST-Elevation Myocardial Infarction (STEMI) subjects.

2. Experimental

2.1. Materials

Human aortic endothelial cells (CC-2535) and endothelial cell culture medium (CC-3162) were obtained from LONZA (Singapore). Chemicals and proteins were from Sigma-Aldrich (Singapore) if not otherwise specified. The iron oxide magnetic beads (65305, 65501) were obtained from Invitrogen (Singapore). Single-stranded DNA oligo was purchased from 1st Base, Singapore. SDS-PAGE reagents were from Bio-Rad (Singapore). The plasma samples and angiogram diagnosis information were provided by National University Hospital of Singapore. All statistical analysis results were generated by using SAS® 9 (Cary, NC).

2.2. Selection of aptamer for recognizing atherosclerosis cell model

The aptamers bound to the atherosclerosis cell model were obtained by the stimulus response cell-SELEX method as described previously (Ji et al., 2013). Basically, the human aortic endothelial cells were activated by 40 ng/mL tumor necrosis factor-α (Calbiochem, Billerica, MA) for 6 hours at 37 °C. 20 nmol of the ssDNA pool (5′-AGA TTG CAC TTA CTA TCT-50N-AAT TGA ATA AGC TGG TAT-3′) was then incubated with the activated cells for half an hour at room temperature. The sequences bound onto the activated cells were collected and amplified by asymmetric PCR. The amplified sequences pool was incubated with the activated cells again. After 3 rounds, the amplified sequences were first incubated with the untreated human aortic endothelial cells. The sequences that were unbound to the naïve HAE cells were collected and incubated with the activated cells. After 10 rounds, the ssDNA bound on the activated cells were amplified and cloned for identification.

2.3. Aptamer candidate N55 protein target identification by MALDI-TOF/TOF MS and MS/MS

Aptamer candidate N55 selected by SELEX was linked with biotin at the 5′ end. The biotin-N55 was coupled onto the Dynabeads® M-270 Streptavidin magnetic beads according to the manufacturer′s instruction. The beads that coupled with biotin-ssDNA library were used as control and uncoupled beads were used as negative control. 0.5 mg of biotin-N55 beads complex was incubated with 50 mL of old cell culture medium for 30 min at room temperature. The beads complex was then washed 3 times in 1 mL PBS and resuspended in 50 μL doubly distilled water (ddH_2O). The beads were then heated at 90 °C for 5 min to release the captured protein. The supernatant was collected by magnetic separation and resolved by SDS-PAGE on a 4–15% TGX precast gel. The coomassie blue stained band was excised and subjected to in-gel digestion.

Briefly, the excised gel pieces were washed with water and cut into 1 mm^3 for incubation in 200 μl of 50% acetonitrile/25 mM ammonium bicarbonate buffer, pH 7.8. After 3 times incubations for 5 min each the gels were dehydrated in 100% acetonitrile and dried using a SpeedVac. And then the gels were rehydrated with 10 ng/μl trypsin gold, (Mass Spectrometry grade, Promega, Madison, WI) in 25 mM ammonium bicarbonate buffer, pH 8.0, overlaid with 10 μl of 25 mM ammonium bicarbonate buffer, and digested for 16 h at 37 °C. The peptides were extracted sequentially with 20 mM ammonium bicarbonate buffer followed by 50% acetonitrile in 0.1% TFA. The pooled peptides were dried in SpeedVac and dissolved in 4 μl of 0.1% TFA.

Tryptic peptide of 0.6 μl was spotted onto Prespotted AnchorChip target plate (Bruker Daltonics, Fremont, CA) according to manufacturer′s protocol. The peptide mass fingerprint and MSMS spectra were acquired in UltraFlex III TOF-TOF (Bruker Daltonics, Fremont, CA) with the Compass 1.2 software package including FlexControl 3.0 and FlexAnalysis 3.0 with PACII peptide calibration standards. The peak list was submitted to in-house Mascot server (http://phenyx.bii.a-star.edu.sg/search_form_select.html) through BioTools 3.2 (Bruker Daltonics, Fremont, CA) with the database of SwissProt (517100 sequences) with peptide mass tolerance of 100 ppm and maximum 1 missed cleavage, considering global carbamidomethyl at cysteine (C) and variable modifications of oxidation at methionine (M).

2.4. Preparation of M55 aptasensor

Aptamer candidate N55 was first size minimized and base modified to enhance its stability (See Supplementary Data Fig. S1). The improved sequence was named M55. M55 was then linked with NH_2-group at the 5′ end followed by an 8-carbon PEG spacer. At the 3′ end, N55 was linked with a 3′-dT to prevent enzymatic degradation. 0.5 mL Dynabeads® MyOne™ Tosylactivated beads (Invitrogen, Singapore) were washed in 1 mL 0.1 M sodium borate buffer, pH 9.5. The beads were then resuspened in 835 μL of 0.1 M sodium borate containing 500 nM NH_2–M55. After adding 415 μL of 3 M ammonium sulfate in 0.1 M sodium borate buffer, pH 9.5, the mixture was allowed to react for 2 days at 37 °C under agitation.

The reaction mixture was washed three times in PBS and resuspended to 10 mg/mL in PBS. 100 μL of the M55-beads stock was incubated with 500 nM 5′-biotin-AGA TTG CAC TTA CTA TCT in 1 mL ddH_2O. After heating at 95 °C for 1 min, the mixture was cooled to room temperature and washed with PBS. The beads complex was resuspended in 1 mL PBS containing 50 ng/mL streptavidin-HRP (Abcam, Hong Kong) and incubated for 15 min