Biosensor-based Binding Assay for Platelet-Derived Growth Factor Receptor-α Autoantibodies in Human Serum

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Abstract

Biosensors are versatile tools for monitoring molecular interactions. Herein, we report a novel assay designed to detect anti-platelet-derived growth factor receptor α (PDGFRα) autoantibodies in the serum of patients affected by systemic sclerosis (SSc), an autoimmune disease of the connective tissue. The assay was based on resonant mirror sensing, and used recombinant PDGFRα as molecular "bait" for anti-PDGFRα autoantibodies (IgG class). The selection and optimization of the analytical procedure required a preliminary investigation on the preservation of the native-like conformation of the receptor following the immobilization procedure. The PDGFRα-based biosensor was used to test IgG purified from sera of SSc patients and healthy controls (HC). The specificity and the reversibility of the interaction permitted a rapid analysis, a single detection test requiring only a few minutes. Remarkably, this assay could discriminate between SSc patients and HC. This receptor-based biosensor, based on the reversible interaction between a blocked macromolecule and soluble ligands, and with major advantages such as the rapidity, the reusability of the capturing surface and the low cost per single test, could represent a promising approach for the diagnosis of SSc and other diseases characterized by anti-receptor autoantibodies or other bioactive ligands to cellular receptors.

Keywords: Systemic sclerosis; Scleroderma; PDGFR; Autoantibodies; Biosensor

Introduction

Systemic sclerosis, or scleroderma, (SSc) is a chronic autoimmune disease of the connective tissue characterized by clinical heterogeneity, unpredictable course, high morbidity/mortality and lack of adequate disease-modifying drugs [1]. The variety and the clinical relevance of autoantibodies in SSc patients have been extensively studied [2-5]. Agonistic autoantibodies (abs) targeting the PDGF receptor (PDGFR) have been detected by a cell-based bioassay based on the production of reactive oxygen species (ROS) with immunoglobulins (IgG) purified from serum of SSc patients [6] and proposed as novel biomarkers of SSc. However, subsequent reports questioned the presence of agonistic anti-PDGFR abs in SSc [7,8]. To address this issue, we sought to circumvent the pitfalls associated with cell-based assays and obtain a specific binding assay for detection of anti-PDGFRα IgG. Thus, we developed a solid-phase assay based on a recombinant human PDGFRα conformer spanning the extracellular, transmembrane and part of the intracellular regions fused to a poly-Histidine tail (His tag). PDGFRα-His to be used as a capturing molecule for autoantibodies. Besides providing adequate sensitivity/specificity, the resonant mirror sensing platform was chosen since it allows the conformational control of the immobilized recombinant PDGFRα, which we hypothesized to be critical for the specific detection of agonistic IgG likely binding to conformational epitopes of the receptor. On such background, herein we propose a rapid method for the detection of anti-PDGFRα IgG purified from human serum, based on the increase in the response signal upon their addition to a PDGFRα-derivatized biosensor, eventually enabling discrimination between SSc and healthy controls.

Materials and Methods

Reagents, chemicals, and devices

The carboxylate-functionalized cuvette and the immo-
staining. rhPDGFRα-His was purified from total protein extracts by metal-ion affinity chromatography (HiTrap chelating columns, Amersham) and dialyzed against PBS to remove imidazole traces. The quality of purified rhPDGFRα-His was assessed by immunoblotting with a rabbit anti-human PDGFα antibody (D01P, Abnova) followed by HRP-conjugated goat anti-rabbit IgG (Santa Cruz).

Patients

Patients (n=8) with a definite diagnosis of SSC [9] and sex- and age-matched healthy controls HC (n=8) were chosen upon informed consent as serum donors. The clinical features of SSc patients included in the study are described in table 1.

Purification of IgG from serum

IgG were purified by individual protein A/G resin columns (Pierce) from sera of 8 SSc patients and 8 healthy controls (HC). After elution with glycine at pH 2.2 and neutralization with Tris buffer, the fractions containing IgG were subjected to size exclusion chromatography (5000 MWCO, Thermo Scientific) to remove trace amounts of contaminating cytokines. The absence of PDGF contaminations in IgG preparations was verified by immunoblotting with a primary polyclonal rabbit anti-human PDGF-BB antibody (Abcam) with detection limit of 0.1 ng cytokine/200 µg IgG.

Immobilization of rhPDGFRα-His

The sensing chamber was thermostatted at 25°C. The carboxylate surface was washed and equilibrated with PBS buffer (10 mM Na2HPO4, 2.7 mM KCl, 138 mM NaCl, pH=7.4), then activated by addition of an equimolar mixture of N-hydroxysuccinimide and N-ethyl-N-(dimethylaminopropyl) carbodiimide hydrochloride [10]. rhPDGFRα-His was dialed against 10 mM CH3COONa buffer pH 5, then anchored to the carboxylic surface via the N-terminus of histidine tail. Free carboxylic sites on the sensor surface were deactivated by injection of 1 M ethanolamine, pH 8.5. The surface was finally re-equilibrated with PBS. Conformational control ligands (namely, PDGF-BB and mab322) were added, and binding kinetics were monitored up to equilibrium. Dissociation steps were performed by a single 1 min wash (80 µL) with fresh PBS buffer, whereas surface regeneration was achieved by serial PBS washes (the number of washing cycles depending on the levels of captured ligand) to rhPDGFRα baseline recovery prior to any further addition of ligands. Raw data were globally fitted to both monophasic and biphasic models. The validity of each model to fit a particular time course was assessed by a standard F-test procedure [11].

Binding assays

IgG purified from serum of patients and HC were identified with a rabbit anti-human PDGFα antibody (D01P, Abnova) followed by HRP-conjugated goat anti-rabbit IgG (Santa Cruz). The absence of PDGF contaminations in IgG preparations was verified by immunoblotting with a primary polyclonal rabbit anti-human PDGF-BB antibody (Abcam) with detection limit of 0.1 ng cytokine/200 µg IgG.

Optimization of the binding assay

To develop an efficient biosensor-based assay for the detection of ligand binding to PDGFRα, the best experimental conditions were achieved taking into account all the parameters such as PDGFRα and ligand concentrations, immobilization pH, temperature, buffering. The binding surface containing immobilized receptor was obtained as described in the Experimental Section (Figure 1). A shift in sensor response (ΔR ≈ 1000 arcsec) upon rhPDGFRα-His immobilization was reported: these conditions resulted in the coupling of a partial ‘Langmuir’ monolayer (70% surface occupied) corresponding to a final surface concentration of 1.7 ng/mm² (approximately equivalent to 7 mg/mL). These data were adequate, since immobilization levels should be moderately high for screening applications, in order to maximize the number of available binding sites, and to limit steric hindrance/shielding effects due to overcrowding, which could affect the analysis. The immobilization protocol was optimized after several experiments based on the variation of receptor concentration and immobilization pH value. The immobilization pH value of 5 (chosen upon the PDGFRα isoelectric point=5.5) allowed an optimal immobilization of rhPDGFRα-His with preservation of its native-like conformation as assessed by conformational control ligands. A receptor concentration of 300 µg/mL was chosen to obtain the optimal surface density of PDGFα molecules: in fact, the assay sensitivity kinetic was monitored up to equilibrium. The dissociation of the complexes and the regeneration of the PDGFRα monolayer were carried out by serial PBS washes. Each IgG sample was analyzed in triplicate. Values falling outside the 95% confidence interval were considered significantly different from controls. Detection procedures were replicated on different days both on the same and on different PDGFRα-functionalized surfaces. Additionally, the number of regeneration cycles that a sensor surface can withstand without significant loss of sensitivity and accuracy of the assay, and the stability of the sensing surface throughout multiple measurements were evaluated and assessed.

Table 1: Characteristics of SSc patients.

may be impaired not only by an insufficient surface density, but also by an excessive surface density potentially hindering the accessibility of soluble ligands to the binding domains of the receptor or favoring dimerization between blocked PDGFRα macromolecules in absence of ligands, consequently reducing the number of available binding sites on the sensing surface. Preservation of the native-like conformation of immobilized rhPDGFRα-His was tested each time using PDGF-BB and mab 322, ligands binding only to conformational epitopes of the extracellular PDGFRα domain. As highlighted in figure 2, disruption of the native folding caused loss of rhPDGFRα-His binding to these ligands. Local and global fit analysis of the interaction data generally revealed monophasic kinetics. Specifically, monoeponential analysis of association curves residuals was not affected by measurable systematic errors (a bieponential model did not considerably improve the quality of the fit as judged by an F-test, 95% confidence).

Application of the binding assay

Biosensor-based assay was applied to human IgG purified from serum. The test allowed the identification of anti-PDGFRα IgG fractions in total IgG pools. The final in-assay concentration of total IgG granting an unambiguous response within 1 min was 120 µg/mL (0.8 µM). Importantly, the analysis of IgG binding to rhPDGFRα-His generally discriminated between SSC and HC group, given the different extent of their responses (Figure 3). In fact, SSC signals were always higher than 2 SD above the mean value of the HC population (AR= 8.35 ± 1.77 arcsec): specifically, all SSC IgG samples generated responses in the range of 12-18 arcsec, whereas responses obtained with HC samples were lower than 8.2 arcsec, except for one HC sample (12 arcsec).

Reusability and efficiency of the biosensor

The effect of different regeneration agents on the biosensor reusability was studied. The complete dissociation of the IgG-receptor complexes was performed by exposure to both acidic and neutral buffer solutions (PBS binding buffer). After performing two rapid 15 sec pulses of 10 mM HCl (80 µL each), the biosensor surface could be used without loss of activity for 10 measurement cycles before any significant loss of binding capacity was observed. On the other hand, the sensing surface resisted to a higher number of experimental cycles if it was washed repeatedly with 80 µL of PBS buffer (biosensor response did not change more than 5% after 50 regeneration cycles). In this case, the number of washes varied with the levels of the captured ligand and with the affinity of the ligand-receptor complex. Consequently, the use of PBS provided an efficient (although slower) desorption of the ligand without degrading the immobilized receptor.

Conclusions

Biosensors are versatile tools with a wide range of applications [12-14]. From the immunological perspective, biosensors allow the real-time and label-free monitoring of the recognition events associated with the formation of antigen-antibody complexes. To our knowledge, the use of biosensors for autoantibody detection in serum of patients affected by autoimmune diseases has never been reported.

Here in we describe the development of a human PDGFRα biosensor for the detection of anti-PDGFRα autoantibodies in the serum of patients affected by systemic sclerosis, whose biological implications have been discussed in the introduction. The PDGFRα biosensor assay is based on the ability of anti-PDGFRα IgG purified from serum to specifically recognize a natively folded, surface-bound human PDGFRα, and makes use of a biosensor-based method to measure the response upon binding of the analytes, i.e. IgG purified from serum of different SSC patients and healthy controls. The PDGFRα biosensor displayed some general advantages such as the rapidity of the test and the minimal consumption of reagents. In fact, it was generally possible to obtain unequivocal results suitable for rapid testing of the biological samples within 1 minute, using only few microliters of IgG preparations, and the biosensor could be stored at 4°C for weeks after the first experimental session and reused for subsequent analytical sessions afterwards. Specific advantage of this assay was the excellent sensitivity/specifcity ratio, enabling detection of the very small anti-PDGFRα autoantibody fractions within the total IgG pools in all cases analyzed, but with a clear cutoff value discriminating between the IgG
samples obtained from SSc patients and those obtained from HC. Critical, from this perspective, was the preservation of the PDGFRα native-like conformation in the biosensor, that can certainly account both for the sensitive detection of anti-PDGFRα autoantibodies in the serum of SSc patients and for the identification of a difference between SSc and HC IgG. The only HC subject characterized by a biosensor response over the cutoff value will undergo clinical follow-up in order to monitor the possible onset of SSc.

In conclusion, this receptor-based biosensor could represent a promising tool for the diagnosis and clinical monitoring of SSc. The same methodological approach may be rewarding in other diseases characterized by anti-receptor autoantibodies or by bioactive pathological ligands to cellular receptors.

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