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Short communication

Nanotechnologic biosensor ellipsometry and biomarker pattern analysis in the evaluation of atherosclerotic risk profile

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ABSTRACT

A proteoheparan sulfate coated, hydrophobic silica surface serves as lipoprotein receptor at which the Ca^{2+}-driven arteriosclerotic nanoplaque formation can be pursued by laser-based ellipsometry. Any lipoprotein from human blood can be very sensitively tested for its atherogenic properties. From the same blood sample, it is possible to determine the concentration and activity of a series of interacting biomarker molecules which, through a pattern analysis, allow to assess the state of health with respect to cardiovascular diseases. These two interlinked and complementary biosensors make a prospective cardio-cerebro-vascular risk stratification feasible, especially the sequelae of an underlying arteriosclerotic disease. Based on these diagnostic tools, an optimized therapy decision for the patient can be taken and the necessary preventive measures for the still healthy person.

1. Introduction

Within the general setting of the program of regenerative, diagnostic and therapeutic medicine, the salient focus and impact will be placed on the development of biofunctionalized materials for tissue engineering and regenerative medicine. These materials are mostly built up from degradable polymers or hybrid materials based on polymers, natural and/or tissue derived materials and inorganic materials. Biofunctionalization and development of nanostructured surfaces are tools to develop the necessary real-time exchange of information and matter between the biological system and the physical backbone material. One major area of research can be the development of biofunctionalized nanoparticles ("bionanosystems"), biomaterials for imaging and therapy – as new contrast agents or biomimetics for diagnostic purposes – and the use of nanotechnology fabrication tools in the synthesis process and the development of improved tissue-biomaterial interfaces. Another would be the construction of synthetic (bioactive) scaffolds for the regeneration of complex tissues, as cartilage, bone, blood vessels and nerves.

In westernized societies, atherosclerosis and its clinical sequelae heart disease and stroke are the underlying cause of about 50% of all deaths. Thus, the prevention or deceleration of atherogenesis is one of the most significant medical objectives since this is a matter of avoidance of myocardial and cerebral infarction. Epidemiological studies have revealed several important environmental and genetic risk factors associated with atherosclerosis (Paras et al., 2008; Libby, 2008). Although 'lifestyle risk factors' are taken into account in the guidelines, e.g., of the American Heart Association, unexpected deaths on grounds of heart or brain attack were again and again reported, even though the persons concerned were not rated as high-risk patients (Kavey et al., 2003; Sacco et al., 2006; Smith et al., 2006). Therefore, the guidelines were repeatedly actualized and extended, e.g., by Ca score determinations (Expert Panel/Writing Group, 2007; Kumanyika et al., 2008).

Through the complementary biosensor models presented here it is attempted to directly assess the cardio-cerebro-vascular risk. On the one hand, from the same patient's blood sample, the atherosclerotic nanoplaque formation can be measured by means of an ellipsometric approach and, on the other hand, additional information via determination and pattern analysis of a variety

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of biomarkers acquired on those risk factors which can lead to an increased nanoplaque formation. In the following, both biosensors as well as one measurement result each of a clinical trial are presented.

2. Subjects, materials and methods

2.1. Subjects and study design

This was part of a preventional, randomized, 12-week study comprising a 4-week dietary run-in phase followed by a study treatment period of 8 weeks, which was conducted in the Phase I–II study clinic of the UMHAPT "Zaritza Johanna" University Hospital, Sofia, Bulgaria. The project has been reviewed and approved by the local Ethics Committee and the Bulgarian Drug Agency. Eleven patients (2 male, 9 female) with early stage metabolic syndrome aged 26–48 years were recruited, provided that they fell within the additional inclusion criteria smoking (all patients) and blood lipoprotein(a) concentration >30 mg/dL (9 patients). After the first taking of a 45 mL blood sample, the standard therapy of the patients was 2×120 mg/d *Ginkgo biloba* special extract EGb 761 (Rökan® novo, Spitzner Arzneimittel, Ettlingen, Germany) over 2 months. No statins, no calcium antagonists and no nitrate compounds were given. No adverse events occurred and all the patients felt well during and after ginkgo intake. After 2-month medication, the second taking of a 45 mL blood sample was carried out. 1 mL of blood was required for the determinations in duplicate of all 13 biomarkers together, the remaining 44 mL for the preparation of the lipid fraction needed in the ellipsometry measurement.

2.2. Preparations and solutions

All experiments were carried out in a blood substitute solution. The normal blood substitute solution consisted of a Krebs solution simulating the extracellular ionic microenvironment of the proteoglycans and lipoproteins: Na^+ 151.16, K^+ 4.69, Ca^{2+} 2.52, Mg^{2+} 1.1, Cl^- 145.4, HCO_3^- 16.31 and $H_2PO_4^-$ 1.38 mmol/L (25 °C, pH 7.3). The Krebs solutions were gassed by 93% N_2/7% CO_2 to hinder VLDL/IDL/LDL oxidation (Siegel et al., 1999). The HS-PG preparation had a molecular weight of about 175 kDa as assessed by chromatography on Sephacryl S 400 columns calibrated with reference proteoglycans. Analytical data revealed an HS proportion of 82 g/100 g dry weight corresponding to four HS chains ($\sim 4 \times 36$ kDa) with an average sulfate content of 0.5 sulfate groups/disaccharide unit covalently linked to the protein core. The protein content (~35 kDa) was 18 g/100 g dry weight. Lipoproteins were isolated by sequential preparative ultracentrifugation essentially as described previously (Siegel et al., 2003).

2.3. Ellipsometry measurements in a bionanosystem

The adsorption of the lipoproteins and of HS-PG was monitored by *in situ* ellipsometry of a nanostructured surface, as detailed previously (Siegel et al., 1999, 2003). All measurements were carried out with an Optrel Multiskop (Optrel, Kleinmachnow, Germany) at 532 nm. Prior to adsorption, the ellipsometry measurements require a determination of the complex refractive index of the substrate (Malmsten, 1994). In the case of a layered substrate such as oxidized silicon a correct determination of the adsorbed layer thickness and mean refractive index requires a determination of the silicon bulk complex refractive index ($N_2 = n_2 - ik_2$) as well as of the thickness (d_1) and the refractive index (n_1) of the oxide layer. This is done by measuring the ellipsometric parameters Ψ and Δ in two different media, e.g., air and buffer. From the two sets of Ψ and Δ, n_2, k_2, d_1 and n_1 can be determined separately. The methyl layer is neglected for the methylated silica surfaces. Electrophoretic studies suggest such silanization only adds 0.5 nm or less to the double layer surface of shear (Burns et al., 1995). Similar results were obtained with ellipsometry. All measurements were performed by four-zone null ellipsometry in order to reduce effects of optical component imperfections. After optical analysis of the bare substrate surface, the proteoglycan solution was added to the cuvette, and the values of Ψ and Δ recorded. The adsorption was monitored in one zone, since the four-zone procedure is time-consuming and since corrections for component imperfections had already been performed. The maximal time-resolution between two measurements is 3–4 s. The adsorption temperature was 25 °C. Stirring was performed by a magnetic stirrer at about 300 rpm. A bulk concentration of 0.1 mg/mL proteoheparan sulfate (0.192 mmol/L in disaccharide units) and of VLDL/IDL/LDL at its native blood concentration in the patient were used throughout. Furthermore, the measurements were performed at different Ca^{2+} concentrations around those in the biological system. The normal blood substitute solution consisted of a Krebs solution.

From Ψ and Δ, the mean refractive index (n_f) and average thickness (δ_{el}) of the adsorbed layer were calculated numerically according to an optical four-layer model (Malmsten, 1994). It was previously shown that both, adsorbed amounts and adsorbed layer thicknesses, obtained with ellipsometry agree well with those obtained with other methods for proteins, polymers, and surfactants at model surfaces (Malmsten, 1994; Tiberg et al., 1994). The adsorbed amount (Γ) was obtained using values of the molar refractivity and the specific volume of 4.1 and 0.75, respectively. Throughout, the pH was kept around 7.38 by the bicarbonate/phosphate buffer, and by a continuous aeration of the cuvette solution (sample volume 5 mL) with a 93% N_2/7% CO_2 gas mixture (Aga, Sweden). The latter is included due to the necessity to keep the pH well regulated throughout these experiments in order to avoid both proteoglycan and lipoprotein degradation and calcium phosphate precipitation. As a physicochemical biosensor procedure, the ellipsometric technique is flawed by 5% at the most, so that reliable data could be expected for each patient. Further details can be found in earlier publications (Malmsten et al., 1993; Siegel et al., 1996).

2.4. MPO, oxLDL, 8-iso-$PGF_{2\alpha}$ and bilobalide determinations

The determination of myeloperoxidase (MPO) and oxidized low-density lipoprotein (oxLDL) were carried out by the MPO and the Oxidized LDL ELISAs (Mercodia, Uppsala, Sweden) using murine monoclonal anti-MPO antibodies and monoclonal antibodies 4E6 against oxidized apolipoprotein B molecules, respectively. 8-Isoprostane was determined after batch purification of the samples on 8-Isoprostane affinity sorbent (mouse anti-8-isoprostane covalently bound to Sepharose 4B, no. 416359, Cayman-Chemical, Ann Arbor, MI, USA) using a commercial EIA kit (Cayman no. 516351) according to the instructions of the manufacturer. The concentration of ginkgo-bilobalide in the VLDL/IDL/LDL particles after 2-month treatment of the patients was determined by a Varian 1200L GC-MS/MS Triple Quadrupole with GC CP 3800 (Varian, Darmstadt, Germany).

3. Results and discussion

3.1. Nanotechnologic biosensor ellipsometry

The bionanosystem presented can serve as a model for arteriosclerosis (Fig. 1). A hydrophobic methylated silica surface is coated by a monomolecular layer of isolated HS-PG depositing through its transmembrane hydrophobic core domain, thus rep-