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Determination of calprotectin in gingival crevicular fluid by immunoassay on a microchip

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ABSTRACT

Objectives: Gingival crevicular fluid (GCF) contains calprotectin, which appears to be a useful biomarker for periodontal diseases because of its high level in GCF from periodontally diseased pockets. To determine calprotectin in GCF that has a very small volume, sandwich enzyme-linked immunosorbent assay (ELISA) on a microchip was performed and its utility was estimated.

Design and methods: Anti-calprotectin primary antibody was discharged on a microchip using a piezoelectric inkjet printing system. Calprotectin standard and calprotectin in GCF samples from eleven subjects were determined by the ELISA method with the prepared microchip and their values were compared with those obtained by conventional ELISA.

Results: Using the ELISA on a microchip, a reasonable standard curve of calprotectin protein (1.56–100 ng/mL) was obtained. Calprotectin in GCF samples was quantified and showed reasonable values in accordance with the condition of periodontal diseases. The values determined by the microchip method and conventional ELISA showed a significant linear relationship ($R^2 = 0.981$).

Conclusions: Calprotectin in GCF was determined using the ELISA on a microchip with high efficiency and this ELISA method for calprotectin determination may become a useful method for diagnosing periodontal diseases.

Introduction

Proteins in body fluids are measured by multiple methods such as immunoassays, enzyme–substrate and color reactions, and used as biomarkers for the diagnosis of various diseases. Enzyme-linked immunosorbent assay (ELISA) is a major method to determine protein biomarkers and has a high specificity, but needs a certain sample volume and a long time for measurement. Recently, biomarker analysis using a microchip has been studied for point-of-care testing (POCT) of disease diagnosis and enables rapid, accurate diagnosis [1–3]. Interferon-γ was determined by a sandwich immunoassay on a quartz glass microchip [4] and immunoglobulin E was rapidly, automatically measured using a micro-ELISA system [5]. Microchip electrophoresis enabled the accurate quantitation of glucose and amylase activity in human plasma in several minutes [6,7]. Recently, we quantified carboxyterminal propeptide of type I procollagen (PICP) in 0.4 μL of serum using a sandwich ELISA on a microchip at high accuracy and sensitivity [8]. These studies suggest that an ELISA on a microchip is suitable to determine a very small amount of protein in a sample.

Periodontal diseases have been diagnosed using clinical markers including probing depth (PD), gingival index (GI), bleeding on probing (BOP) and tooth mobility. However, these markers are not necessarily accurate indicators and do not indicate the change of periodontal disease activity [9,10]. Gingival crevicular fluid (GCF) contains many components that are derived from blood, periodontal tissues and bacteria [11]. Inflammation-related proteins, cytokines, proteolytic enzymes and the degraded products of periodontal tissues in GCF have been studied as biomarkers of periodontal diseases [12,13]. GCF samples can be used for biomarker determination without pretreatment, such as preparation of serum or plasma in the case of blood. However, GCF in one gingival crevice or periodontal pocket has a very small volume (0.05–1.50 μL) [14], and it is difficult to quantify multiple proteins in one GCF sample using conventional ELISA.

Calprotectin, a complex of S100A8 and S100A9 proteins, is expressed in neutrophils, monocytes/macrophages and epithelial cells, and increases in body fluids in some inflammatory diseases [15–17]. Calprotectin was also found in GCF and showed a high level in GCF from periodontal pockets with inflammation; its level positively correlated with the levels of clinical markers and other GCF biomarkers of

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periodontal diseases [17–20]. The determination of GCF calprotectin appears to be useful for accurate diagnosis of periodontal diseases. However, at present, since the determination of calprotectin is performed using a conventional ELISA kit, it requires a constant sample volume and over 4 h for the assay. Some GCF samples cannot be analyzed because of small sample volume. These problems make it difficult to use calprotectin as a biomarker of periodontal diseases. To determine calprotectin accurately in a small volume of GCF for a short time, we developed a sandwich ELISA system using a microchip and compared its efficiency with that of the conventional ELISA method.

Materials and methods

Reagents and materials for microchip determination

A microchip made of cyclic olefin copolymer (COC), 70 mm in length and 30 mm in width (Fig. 1), has four microchannels of 60 mm in length, 0.3 mm in width and 0.1 mm in depth and hole reservoirs at both ends of one microchannel (BS-X2321; SUMITOMO BAKELITE Co., Ltd., Tokyo, Japan). A microchip enables the assay of four samples. The surface of the microchip was treated with a polymer solution containing p-nitrophenyl ester to bind amino groups of antibody to the microchannel surface. Blocking and washing solutions for a microchip assay were purchased from SUMITOMO BAKELITE. Anti-calprotectin primary antibody was anti-human MRP8/14 (calprotectin) monoclonal antibody (clone IDCP2; Immundiagnostik AG, Bensheim, Germany) and the secondary antibody was anti-human S100A8/A9 (calprotectin) monoclonal antibody (clone 27E10; Hycult Biotechnology, Uden, The Netherlands). The secondary antibody was labeled with peroxidase using Peroxidase Labeling Kit-NH_2 (Dojindo Laboratories, Kumamoto, Japan) and purified using IgG Purification Kit-G (Dojindo Laboratories). The used calprotectin antigen was the standard calprotectin protein in HUMAN CALPROTECTIN ELISA KIT (Hycult Biotechnology). Microchannels on a microchip were sealed with a polymethylmethacrylate-based adhesive layer (47 μm thickness; TOYO INK MFG. Co., Ltd., Tokyo, Japan). The peroxidase-labeled immune product was detected with SuperSignal® West Femto (Thermo Scientific, Rockford, IL, USA).

Coating primary antibody on microchip

Anti-calprotectin primary antibody (50 μg/mL) mixed with a fixing buffer (SUMITOMO BAKELITE) was immobilized on the surface of the microchannel according to a modified version of a procedure described previously [8]. Briefly, antibody solution was ejected onto the bottom of the microchannel using Pulseinjector® (CLUSTER TECHNOLOGY Co., Ltd., Osaka, Japan) at a frequency of 20 Hz and a driving voltage of 16 V. One hundred droplets (5.3 nL) were ejected onto the bottom of the microchannel (Fig. 1) and incubated for 4 h at room temperature on a microchannel, which was sealed with polymethylmethacrylate film.

GCF preparation

GCF samples were obtained from eleven subjects who gave their written informed consent to participate in this study. The study was approved by the Ethics Institutional Review Board for Clinical Research of Tokushima University Hospital and performed in accordance with the Declaration of Helsinki. The eleven subjects included three who were periodontally healthy (53-, 64- and 65-year-old females) and eight periodontitis patients (36-, 36-, 46- and 71-year-old males and 54-, 59-, 64- and 65-year-old females) who had not

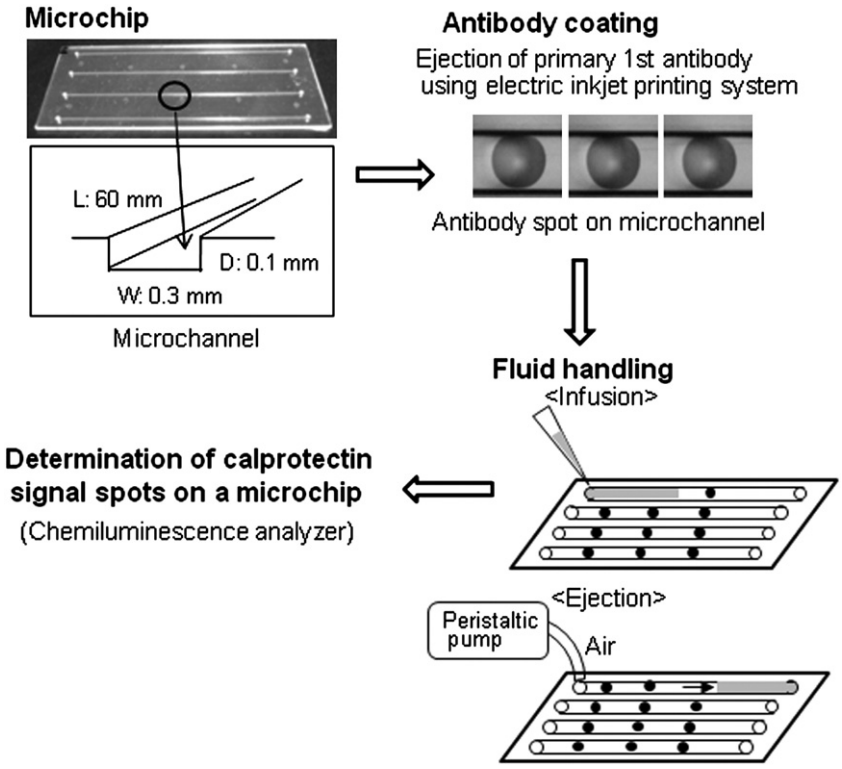

Fig. 1. Procedure of determination by ELISA using a microchip. COC-microchip is 70 × 30 mm (length and width) in size and has four microchannels with a size of 60 × 0.3 × 0.1 mm (length, width, depth). A primary antibody was ejected onto a microchannel using a piezoelectric inkjet printing system. The spots on a microchannel show the discharged droplets of anti-calprotectin primary antibody. Fluids were infused into a microchannel by a pipette and ejected by air of a peristaltic pump. Calprotectin signal spots were assayed by a chemiluminescence analyzer.