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Autoimmunity and cystatin SA1 deficiency behind chronic mucocutaneous candidiasis in autoimmune polyendocrine syndrome type 1

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ABSTRACT

Patients with the monogenic disease autoimmune polyendocrine syndrome type I (APSI) develop autoimmunity against multiple endocrine organs and suffer from chronic mucocutaneous candidiasis (CMC), a paradoxical complication with an unknown mechanism. We report here that saliva from APSI patients with CMC is defective in inhibiting growth of *Candida albicans in vitro* and show reduced levels of a salivary protein identified as cystatin SA1. In contrast, APSI patients without CMC express salivary cystatin SA1 and can inhibit *C. albicans* to the same extent as healthy controls. We evaluated the antifungal activity of cystatin SA1 and found that synthesized full length cystatin SA1 efficiently inhibits growth of *C. albicans in vitro*. Moreover, APSI patients exhibit salivary IgA autoantibodies recognizing myosin-9, a protein expressed in the salivary glands, thus linking autoimmunity to cystatin SA1 deficiency and CMC. This data suggests an autoimmune mechanism behind CMC in APSI and provides rationale for evaluating cystatin SA1 in antifungal therapy.

1. Introduction

Patients with autoimmune polyendocrine syndrome type I (APSI, APECED, OMIM 240300), suffer from recurring or persistent infections of mucosal surfaces with the yeast *Candida albicans (C. albicans)* known as chronic mucocutaneous candidiasis (CMC) [1]. In addition to CMC, APSI is characterized by autoimmune endocrinopathies, typically Addison's disease and hypoparathyroidism [2]. Organ-specific autoantibodies are a hallmark of the disease; most often they are specific for intracellular enzymes involved in the synthesis of hormones and neurotransmitters [3]. The AIRE gene, mutations of which cause APSI, is mainly expressed in thymic medullary epithelial cells and in peripheral antigen presenting cells, and is reported to function as a transcriptional regulator. The suggested role for AIRE in maintaining tolerance is to induce expression of peripheral antigens in the thymus, thereby mediating negative selection of autoreactive T cells [4,5]. AIRE has also been shown to be involved in peripheral tolerance mechanisms by regulating DC-mediated activation of T and B cells [6,7]. According to its monogenic origin, APSI has a higher prevalence in certain genetically isolated populations, *i.e.* Iranian Jewish, Sardinian and Finnish [8,9].

The events leading to CMC in APSI remain poorly understood. CMC affects nearly all APSI patients with the exception of those carrying the Y85C mutation which is the most common mutation in the Iranian Jewish population [8]. This selective susceptibility to mucosal *Candida* infections contrasts with the otherwise hyperactivated immune system of the APSI patients that cause the autoimmune destruction of endocrine organs. In line with a hyperactivated immune system, APSI patients do not suffer from increased susceptibility to other types of infections or to systemic *Candida* infections which occur in primary and secondary immune deficiencies. Furthermore, APSI patients appear to have functional intact immune responses to *C. albicans* antigens *in vitro* [10]. Two recent studies showed that APSI patients exhibit systemic neutralizing IgG antibodies against Th17 related cytokines, implicating that these have a role in the pathogenesis of CMC [11,12]. However, the notion that APSI patients are not susceptible to deep or systemic *Candida* infections suggests additional mechanisms. The aim of the present study was to investigate local oral defects in the anti-fungal immunity of APSI patients.

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2. Material and methods

2.1. Patients

Saliva was collected with informed consent from 3 APSI patients from Sweden, 10 APSI patients from Italy, 8 of whom had CMC as a component of their disease, and 6 APSI patients from Israel, of whom only one had developed CMC. Of the 5 patients from Israel without CMC, three were diagnosed with APSI and two had not yet been screened for AIRE mutations but were diagnosed with hypoparathyroidism. Saliva from 16 healthy individuals from Sweden in the age of 20–40 years were used as control samples. The saliva samples were collected using sterilized cotton cylinders according to manufacturer's instruction (Amersham Biosciences). The cylinders were centrifuged and the supernatant was carefully removed to avoid any pelleted residue. The protein concentration of the collected saliva was measured with BCA assay (Pierce). Saliva samples were stored at −80 °C until use. The study was approved by the local ethics committee.

2.2. Immunofluorescence staining

Five μm cryosections from oral pig mucosa or normal human submandibular gland (Biochain) were incubated with saliva (1:10 dilution) from 3 APSI patients or 3 healthy controls overnight at 4 °C. Immunoreactivity was detected with a rabbit α-human-IgA-FITC antibody (Dako) at 1:50 or 1:200 dilutions.

2.3. Western blot

Protein extract from oral pig mucosa, normal human submandibular gland (Biochain) or full length synthesized cystatin SA1 (Almac Science) was run on SDS-PAGE and transferred onto PVDF membrane (Bio-Rad). The membranes were incubated with saliva from APSI patients or healthy controls in 1:10 dilution overnight at 4 °C. Bound IgA was captured by rabbit α-human-IgA-AP in 1:500 dilution (Bio-Rad) and developed by BCIP/NBT color development solution (Bio-Rad). α-cystatin SA1-biotin antibody (RnD systems) and Streptavidin-AP (Dako) were used as controls. For identification of the reactive protein band in the human submandibular gland, a rabbit α-nonmuscular myosin IIA antibody (Sigma–Aldrich), diluted 1:1000, and an α-rabbit Ig-AP conjugated antibody (Dako), diluted 1:2000, were used.

2.4. 2D electrophoresis

The salivary protein profile of APSI patients and healthy controls ($n = 2$) was analyzed with 2-D electrophoresis using 3-10NL, IPG strips and pre-cast 4–12% Bis–Tris gels according to manufacturer's instructions (Invitrogen). The proteins were detected with coomassie brilliant blue dye.

2.5. In gel digestion (trypsin) and matrix-associated-desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry

Proteins from 2D gel electrophoresis were digested with trypsin and analyzed with matrix-associated-desorption/ionization mass spectrometry by the proteomics core facility at the Karolinska Institutet.

2.6. MTT assay

Inhibition of *Candida* growth was measured with a 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) reduction assay according to manufacturer's instructions (Sigma–Aldrich). *C. albicans* (ATCC 90028) was cultured in Brain Heart Infusion (BHI) medium for 24 h at 37 °C. The *Candida* yeast cells were diluted to 10^6 cells/ml in RPMI medium (Sigma–Aldrich) for transformation into hyphal form. *Candida* cells were incubated with cystatin SA1 (Almac Science), Nystatin (Sigma–Aldrich), Amphotericin B (Sigma) or Fluconazole (Diflucan, Pfizer), for 24 h at room temperature. OD values were measured at 550 nm with background reading at 690 nm. The OD values obtained from *Candida* cells cultured in medium were considered as 100% growth or 0% inhibition. All samples were tested in triplicates.

2.7. Flow cytometry

Saliva samples from APSI patients or healthy controls were incubated with *C. albicans* for 24 h in room temperature on a shaking board. As a negative control, a buffer was used with a similar composition as human saliva (2 mM KH_2PO_4, 1 mM $CaCl_2$, 50 mM KCl, 0.1 mM $MgCl_2$, pH 7.2). As a positive control, *Candida* cells were boiled at 99 °C for 20 min. The cells were stained with propidium iodide (PI) (BD Pharmingen) according to manufacturer's instructions and PI positive cells were considered dead. The cells were analyzed on a FACSCalibur with the CellQuestPro software.

3. Results

3.1. APSI patients have impaired anti-fungal activity of saliva

Healthy individuals have innate antifungal mechanisms that prevent oral colonization of fungal pathogens, such as the expression of anti-microbial peptides in the saliva [13]. To investigate whether this function was impaired in APSI patients we cultured *C. albicans* with saliva from APSI patients with or without CMC as a component of their disease, and measured the frequency of dead *Candida* cells. *Candida* cells incubated with saliva from APSI patients with CMC showed increased viability compared to saliva from healthy controls. In contrast, saliva from APSI patients without CMC could inhibit *Candida* viability to the same extent as saliva from healthy controls (Fig. 1). This shows that the oral antifungal immunity is impaired in APSI patients with CMC.

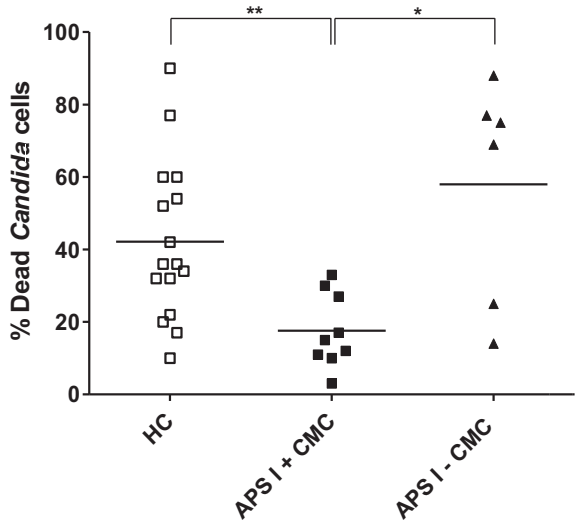

Fig. 1. APSI patients have impaired antifungal activity of saliva. *In vitro* antifungal activity of saliva from APSI patients with CMC (+CMC) or without CMC (−CMC) as a component of their disease, and healthy controls. *C. albicans* was incubated with saliva samples for 24 h and viable cells were identified as negative for propidium iodide, analyzed with flow cytometry. Each dot represents one individual, vertical line represents mean values. The experiment was repeated twice, the graph shows one representative experiment. The data was analyzed with Wilcoxon's rank sum test.