Journal of Autoimmunity 41 (2013) 175–181

Contents lists available at SciVerse ScienceDirect

Journal of Autoimmunity

journal homepage: www.elsevier.com/locate/jautimm

Epigenetic dysregulation in salivary glands from patients with primary Sjögren's syndrome may be ascribed to infiltrating B cells

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ARTICLE INFO

Article history:
Received 19 February 2013
Accepted 19 February 2013

Keywords:
Sjögren's syndrome
DNA methylation
B cells
PKC delta
Rituximab

ABSTRACT

Sjögren's syndrome (SS) is an autoimmune exocrinopathy characterized by an epithelium injury with dense lymphocytic infiltrates, mainly composed of activated T and B cells. Present at the interface of genetic and environmental risk factors, DNA methylation is suspected to play a key role in SS. To clarify this point, global DNA methylation was tested within salivary gland epithelial cells (SGEC), peripheral T cells and B cells from SS patients. Global DNA methylation was reduced in SGEC from SS patients, while no difference was observed in T and B cells. SGEC demethylation in SS patients was associated with a 7-fold decrease in DNA methyl transferase (DNMT) 1 and a 2-fold increase in Gadd45-alpha expression. The other DNA methylation/demethylation partners, tested by real time PCR (DNMT3a/b, PCNA, UHRF1, MBD2, and MBD4), were not different. Interestingly, SGEC demethylation may be attributed in part to the infiltrating B cells as suspected in patients treated with anti-CD20 antibodies to deplete B cells. Such hypothesis was confirmed using co-culture experiments with human salivary gland cells and B cells. Furthermore, B cell-mediated DNA demethylation could be ascribed to an alteration of the PKC delta/ERK/DNMT1 pathway. As a consequence, part of the SGEC dysfunction in SS may be linked to epigenetic modifications, thus opening new therapeutic perspectives in SS.

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1. Introduction

Sjögren's syndrome (SS) is a chronic autoimmune disease affecting exocrine glands, mainly the salivary and lacrimal glands, leading to its designation as an autoimmune epithelitis [1]. Systemic features may be present [2], autoantibodies are detected, and patients with SS have a 20–40 fold increased risk of developing B cell lymphoma [3]. Activated mononuclear cells infiltrate exocrine glands, mainly T and B cells, and such infiltration evolves with the lesion severity and disease manifestations. According to Christodoulou et al., B cells predominate in severe inflammatory lesions, and a B/T cell positive ratio correlated with the infiltration grade and the biopsy focus score [4].

The etiology of SS is multifactorial and arises from an interplay of genetic predispositions, immunological dysregulation, environmental factors and epigenetic modifications [5]. Experimental models support a role for DNA methylation in SS and lupus. Indeed, hydralazine and procainamide, two DNA methylation inhibitors, induce SS with immunological features of a systemic lupus erythematosus (SLE)-like disease [6,7]. DNA methylation at the 5-carbon position of cytosines (5MeCyt) in CpG dinucleotides is tightly regulated during cell development and the cell cycle. The DNA methyl transferases DNMT3a/b and DNMT1 are responsible for the establishment and maintenance of CpG methylation patterns, respectively. To be effective, DNMTs need to be associated with DNA binding proteins such as the proliferation cell nuclear antigen (PCNA) and the DNA repair/transcription factor ubiquitin-like PHD and RING finger domains 1 (UHRF1) [8]. In contrast, DNA demethylation is related to the methyl-CpG-binding domain (MBD) protein-2 and -4 when associated with the stress protein Gadd-45α that can initiate DNA demethylation by replacing a 5MeCyt with an unmethylated cytosine [9].

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http://dx.doi.org/10.1016/j.jaut.2013.02.002

Most of our knowledge regarding DNA demethylation in autoimmune diseases came from the analysis of $CD4^+$ T cells and B cells in patients with SLE [10–12]. Several mechanisms have been described to explain DNA demethylation in SLE patients. The first one, described by Gorelik et al., is related to a defective PKC delta (PKCδ)/ERK pathway observed both in idiopathic and drug-induced SLE patients [13]. Interestingly, the $PKC\delta^{-/-}$ mice reproduce the SS disease with reduced salivary gland function, B cell infiltration and autoantibody production [14]. The second mechanism is related to the expression of miRNAs that down regulate DNMT1 [15,16], and the third to the overexpression of DNA demethylating factors such as Gadd-45α [17], and MBD2/4 [18].

Because DNA demethylation is commonly observed in autoimmune diseases [19], and suspected to be associated with autoantibody production [20], we decided to characterize DNA methylation in SS. The results indicate that salivary gland epithelial cells (SGEC) are demethylated in SS and that this defect may be attributed in part to the presence of infiltrating B cells.

2. Material and methods

2.1. Patients and controls

Eight patients fulfilling the American–European consensus group criteria for primary SS that displayed salivary gland (SG) focus score of at least 3 were enrolled in the study [21]. Peripheral blood was collected by phlebotomy using lithium heparin tubes, and minor labial SGs were obtained by biopsy. SGs obtained before and after two infusions of anti-CD20 at 4 months (Rituximab, Roche) were available for two patients [22,23]. Peripheral blood controls were obtained from 8 healthy subjects and SG control samples consisted of SG specimens collected from patients who did not meet the criteria for SS, although they had described sicca symptoms and, as such, had undergone an SG biopsy.

2.2. Mononuclear cell isolation

Peripheral blood mononuclear cells (PBMC) were isolated by centrifugation on ficoll-hypaque (PAA Laboratories, Linz, Austria). Next, T and B cells were enriched by rosetting with sheep erythrocytes. After another ficoll-hypaque centrifugation, T cells were purified from the sheep erythrocyte bound fraction, and B cells from the unbound fraction by negative selection using specific antibodies (Ab) coated magnetic microbeads (EasySep, Stem Cell technologies, Vancouver, Canada). Purity of T and B cells was tested by flow cytometry and was over 95%.

2.3. Epithelial cell cultures and co-cultures

In order to obtain long term SG epithelial cells (SGEC) [24], SG from biopsy were cut in small fragments and incubated with supplemented basal epithelial medium (SBEM). SBEM is composed of three volumes of Ham's F12 medium (Gibco/Invitrogen, Carlsbad, CA) and one volume of Dulbecco's modified Eagle's medium (DMEM; Lonza Inc., Allendale, NJ) supplemented with decomplemented fetal calf sera (FCS) 2.5% (Eurobio, Les Ulis, France), L-glutamine 2 mM, epidermal growth factor (EGF) 10 ng/mL (Promega, Mannheim, Germany), and insulin 0.5 μg/mL (Novo-Nordisk, Badsvaerd, DK). Cells were incubated at 37 °C and 5% CO_2.

The human salivary gland (HSG) cell line established from irradiated neoplastic epithelial duct cells [25] was cultivated in DMEM supplemental with decomplemented FCS 10%, L-glutamine 2 mM, non-essential amino acids 1% (Sigma–Aldrich, St Louis, MO), and antibiotics (Panpharma SA, Fougères, France). Repression of DNMTs was achieved by incubating the cells for 48 h with 5-azacytidine 0–50 μM, PD98059 0–50 μM, and rottlerin 0–50 μM (all from Sigma–Aldrich). In the co-culture experiments, HSG cells were seeded at 5×10^5 cells into 6-well flat-bottomed culture plates (Nunc, Kamstrup, DK). After 4 h allowing cells to attach, the Ramos human B cell line (ATCC, Rockville, MD) was co-cultured for 48 h at different ratios. There were one, five and ten Ramos B cells, respectively, for one HSG cell. To address the implication of the PKCδ, rottlerin at 25 μM was added or not in the co-culture.

2.4. DNA global methylation

DNA was extracted from cells using the QIAmp DNA blood kit (Qiagen, Inc., Valencia, CA). The DNA concentration and the 260:280 nm absorbance ratios were calculated using a Nanodrop 2000c spectrophotometer (Thermo Scientific Nanodrop technologies, Wilmington, DE).

The global 5methyl cytosine (5MeCyt) DNA content was measured by ELISA according to the protocol described by Balada et al. with modifications [18]. Briefly, high-affinity microplates (Nunc) were pre-coated 90 min at 37 °C with 100 μL poly-L-lysine 0.01% (Sigma–Aldrich) to attach DNA. DNA samples adjusted at 1 μg in carbonate/bicarbonate buffer 0.1 M pH 9.6, were denatured at 95 °C for 5 min, kept on ice more than 5 min, and then incubated overnight at 4 °C. After four washes with PBS-Tween 0.01%, 200 μL of PBS with bovine serum albumin (BSA) 1%, used as blocking solution were added and incubated 1 h at room temperature (RT). After washing, 100 μL of mouse IgG anti-5MeCyt (Calbiochem, La Jolla, CA) diluted at 1:5.000 in PBS-BSA 1% were added per well and plates were incubated 2 h at RT. After six washes, 100 μL of alkaline phosphatase-labeled goat anti-mouse IgG diluted at 1:5000 in PBS-BSA 1% were added and incubation was carried out for 1 h at RT. After extensive washing, color was developed with 100 μL p-nitrophenyl-phosphate (Sigma–Aldrich) in carbonate/bicarbonate buffer 0.1 M pH 9.6. Plates were kept for 3 h 30 at 37 °C, and optical density (OD) was read at 405 nm using Titertek Multiscan microplates (Flow laboratories, Rockville, MD). Each sample was tested in triplicate and non-specific background OD (one well without DNA) was subtracted from the corresponding tested sample. To minimize experimental variability, a reference sample (GC content 41.2%, Sigma–Aldrich) was included in each run.

2.5. RNA isolation and real time quantitative PCR

Two micrograms of mRNA extracted from the cells using the RNAble extraction kit (Eurobio) were reverse transcribed in cDNA using reverse transcriptase and random primers (Gibco Invitrogen).

The RT-qPCR were performed in 20 μL mixtures in wells containing 300 ng cDNA, 500 nM specific primer for either DNMT1, DNMT3a, DNMT3b, UHRF1, PCNA, Gadd-45α, MBD2, MBD4, and HDAC1 (Table 1), plus 1X SYBR green PCR master mix (Applied Biosystems, Foster City, CA). Each assay included the reaction mixture with no template as a negative control. The level of cDNA was normalized to GAPDH cDNA. Cycle threshold values corresponding to PCR cycle numbers above baseline emission were determined and the relative cDNA expression levels calculated using the $2^{-\Delta\Delta ct}$ method.

2.6. Immunofluorescence staining of tissue sections

Biopsy specimens were embedded in optimal cutting temperature (OCT)-compound snap frozen in isopentane, cut into thick tissue sections, and mounted onto poly-L-lysine coated slides. In the first experiments, serial sections were incubated with a rabbit anti-CD20 polyclonal Ab (pAb, Beckman Coulter, Brea; CA) diluted at 1:50 in PBS

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