Journal of Autoimmunity 39 (2012) 64–68

Contents lists available at SciVerse ScienceDirect

Journal of Autoimmunity

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

The salivary gland epithelial cells of patients with primary Sjögren's syndrome manifest significantly reduced responsiveness to 17β-estradiol

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ARTICLE INFO

Article history:
Received 4 January 2012
Accepted 7 January 2012

Keywords:
Sjögren's syndrome
Salivary gland epithelial cells
Estrogens
Estrogen receptors

ABSTRACT

Several lines of evidence indicate that salivary gland epithelial cells (SGEC) play an important role in the pathogenesis of primary Sjogren's syndrome (SS). Normal SGEC have been shown to possess functional estrogen receptors, however, the estrogenic response of SGEC in patients with SS has not been previously assessed. To address this issue, we comparatively tested cultured non-neoplastic SGEC lines from SS patients (SS-SGEC, $n = 8$) and from disease controls (control-SGEC, $n = 12$) in a standard estrogenic inhibition assay of cytokine-induced adhesion molecule expression, where the modulation of the expression of constitutive and interferon-gamma (IFNγ)-induced CD54/ICAM.1 molecules following treatment with 17β-estradiol (E2) was evaluated by flow cytometry. Similarly high ICAM.1 expression was induced by IFNγ in control-SGEC and SS-SGEC lines. E2-treatment did not modify the constitutive ICAM.1 expression in either control-SGEC or SS-SGEC lines. In line with previous results, E2-pretreatment of control-SGEC was found to impede significantly the IFNγ-induced upregulation of ICAM.1 ($p = 0.003$). However, such inhibition was not observed in the SS-SGEC lines ($p = 0.55$). Such aberrant response of SS-SGEC to estrogens did not appear to associate with altered expression of estrogen receptor (ER) proteins, as no discernible differences could be revealed by immunoblotting and immunohistochemistry in the patterns or the intensity of ERα and ERβ (ERβ1- and ERβ2-isoforms) protein expression in SGEC lines or minor salivary gland tissues between SS patients and disease controls. The deficient estrogenic responsiveness of SS-SGEC likely represents a manifestation of the intrinsic epithelial activation that characterizes SS and possibly indicates the perturbation of the immunoregulatory potential of estrogens in SS-epithelia.

1. Introduction

Sjögren's Syndrome (SS) or autoimmune epithelitis is a chronic autoimmune exocrinopathy that is mainly characterized by dysfunction and destruction of salivary and lacrimal glands associated with periductal mononuclear cell infiltrates and immune hyperactivity [1,2]. On the basis of the strong female preponderance of the disease, as well its usual occurrence around menopause, the role of estrogens have long been thought important for the development of SS [3–5]. In fact, although human studies have been so far inconclusive, evidence from animal models has strongly implied estrogen deficiency in the pathogenesis of the disorder [6–10]. Estrogens have been consistently shown to exert immunomodulatory effects in various types of cells [5], including SGEC [11], however, their role in the pathophysiology of human disorders appears quite complex [5].

On the other hand, glandular epithelia appear to have active participation in the induction and perpetuation of tissue inflammatory reactions of SS patients [1]. These patients manifest mononuclear infiltrations in various epithelial tissues (described as autoimmune epithelitis [2]) and increased epithelial expression of several inflammatory proteins in the lymphoepithelial lesion [1,12,13]. The inherent capacity of SGEC to participate to immune responses has been corroborated by phenotypic and functional analyses of long-term cultured non-neoplastic SGEC lines [14]. In addition, cultured SGEC lines derived from SS patients display increased constitutive expression of several types of molecules, a fact which apparently indicates the intrinsic activation status of the epithelial cells in these patients [1] and lends further support to the active involvement of these tissues in the pathogenesis of the disorder.

In a recent study, we have demonstrated the expression of ER proteins by epithelial cells in salivary gland tissues, as well as in

Abbreviations: SS, primary Sjögren's syndrome; SGEC, salivary gland epithelial cells; MSG, minor salivary gland; E2, 17β-estradiol; IFNγ, interferon-gamma.

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doi:10.1016/j.jaut.2012.01.005

cultured non-neoplastic SGEC lines [11]. Moreover, taking advantage of cultured SGEC lines, these studies have provided evidence for the functional responsiveness of SGEC to estrogens in a standard estrogenic inhibition assay. In a manner similar to other types of cells, the pretreatment of cultured SGEC with 17β-estradiol (E2) was found to impede the induction of the adhesion molecule CD54/ICAM.1 by interferon-gamma (IFNγ) [11]. Herein, the comparative assessment of cultured SGEC lines from SS patients and disease controls by the above functional assay had revealed significantly reduced responsiveness to E2 of SS-SGEC, a fact which may be pertinent to the pathogenesis of the syndrome.

2. Materials and methods

2.1. Tissue samples and cultured cell lines

Minor salivary gland (MSG) tissue samples and non-neoplastic cultured salivary gland epithelial cell (SGEC) lines were obtained with informed consent, from individuals who underwent MSG biopsy during their diagnostic evaluation for suspected Sjögren's syndrome. Individuals studied (all women) included 14 patients with primary SS (SS, median age: 53.5 years, range: 27–74 years, including 7 patients with serum antibodies to Ro[SSA] only and 4 with antibodies to both Ro[SSA] and La[SSB] antigens), diagnosed on the basis of the American European Consensus Group classification criteria for SS [15], and 12 disease controls (median age: 52.5 years, range: 30–80 years), who did not fulfill the SS classification criteria and did not present any histopathological or serological evidence for SS [15]. Long-termed cultured non-neoplastic SGEC lines were established from each MSG biopsy by the explant outgrowth technique, as previously [14]. SGEC lines obtained from SS patients (SS-SGEC) and controls (control-SGEC) were treated in an identical fashion, using serum-free, low calcium-containing culture medium. Under these culture conditions, the constitutive and the interferon-gamma-induced levels of ICAM.1 do not show appreciable differences between SS-SGEC and control-SGEC lines [14].

2.2. Assessment of estrogen responsiveness in unstimulated and interferon-gamma-treated SS-SGEC and control-SGEC lines

The estrogenic influence on constitutive and IFNγ-inducible expression of ICAM.1 were studied in SS-SGEC ($n = 8$) and control-SGEC ($n = 12$) lines, using E2 (Sigma–Aldrich, 10^{-7} M), IFNγ (Imukin; Boehringer Ingelheim, 500 IU/ml) or culture medium alone (sham treatment), as previously described [11]. The expression levels of surface ICAM.1 molecules (mean fluorescence intensity; MFI) were measured by flow cytometry [11]. The fold-induction of surface ICAM.1 expression by treatment with IFNγ alone or by IFNγ following E2-pretreatment, as well as the percent inhibition of IFNγ-induced ICAM.1 expression by E2-pretreatment were calculated as previously [11].

2.3. Detection of ERα and ERβ protein expression

The expression of ERα and ERβ isoform proteins was evaluated by immunoblotting and immunocytochemistry in SGEC lines (14 from SS patients and 9 from disease controls), as well as by immunohistochemistry in paraffin-embedded minor salivary gland (MSG) tissue specimens (for ERα; 12 from SS patients and 3 from controls, for ERβ1/ERβ2; 3 specimens each from SS patients and controls), as previously [11]. Antibodies applied in immunoblotting included MC-20 (for ERα, 4.0 μg/ml, Santa Cruz Biotechnology), H-150 (for ERβ1, 4.0 μg/ml, Santa Cruz), and pB2 (for ERβ2, 6.0 μg/ml; kindly provided by M. Alexis, National Hellenic Research Foundation, Athens, Greece), whereas antibodies used in immunocytochemistry and immunohistochemistry included 1D5 (for ERα, 10.0 μg/ml; Dako), mB1-C1 (for ERβ1, 0.5 μg/ml) and pB2 (for ERβ2, 6.0 μg/ml; both kindly provided by M. Alexis). In the immunoblotting experiments, densitometric quantification of the bands and normalization of the loaded proteins with the housekeeping protein β-actin was applied in order to compare the amounts of ERα and ERβ proteins detected in SS-SGEC and control-SGEC lines. The intensity of the bands detected was scored in a 4-grade scoring scale (from 0 to 3) according to the amount of protein detected.

2.4. Statistical analysis

Data are presented as median and range of values. Statistical analysis of results was performed by Mann–Whitney *U*-test and non-parametric Wilcoxon paired test, as well as by the Fischer's exact test, as appropriate.

3. Results

3.1. Non-neoplastic SGEC lines derived from patients with SS display reduced responsiveness to 17β-estradiol

The effect of E2 on constitutive and IFNγ-induced ICAM.1 expression was assessed in parallel, in control-SGEC and SS-SGEC lines. As previously described [14] both control-SGEC and SS-SGEC lines were found to express constitutively surface ICAM.1 molecules that were significantly upregulated following treatment with IFNγ. No statistically significant differences were observed between the control-SGEC and the SS-SGEC cell lines tested, in regard with either the constitutive ICAM.1 expression (control-SGEC; median MFI: 13.7, range: 5.5–28.7, SS-SGEC; median MFI: 12.5, range: 6.2–31.3) or the IFNγ-induced expression of ICAM.1 (control-SGEC; median MFI: 221.1, range: 107.5–362.5, SS-SGEC; median MFI: 183.1, range: 117.6–286.9).

Treatment of SGEC with E2 did not alter the constitutive ICAM.1 expression in either the control-SGEC or the SS-SGEC lines (data not shown). Pretreatment of control-SGEC lines with E2 was found to inhibit significantly the IFNγ-mediated ICAM.1 induction (sham-treated cell lines; median induction: 19.0-fold, range: 7.1–26.9, E2-pretreated cell lines; median induction: 8.7-fold, range: 5.7–23.2, $p = 0.003$). However, such inhibitory effect was not observed in the SS-SGEC lines (sham-treated; median induction: 14.4-fold, range: 4.7–20.7, E2-pretreated; median induction: 12.8-fold, range: 4.3–18.8, $p = 0.55$). In fact, significant (≥30.0%) estrogenic inhibition of the IFNγ-mediated ICAM.1 induction was observed in only 1 out of 8 SS-SGEC lines, compared to 9 out of 12 control-SGEC lines ($p = 0.02$) (Fig. 1A–C).

3.2. Detection of ERα and ERβ proteins in non-neoplastic SGEC lines and MSG tissue specimens derived from SS patients and disease controls

The ERα and ERβ protein analysis of SGEC by immunoblotting had revealed quantitative and qualitative variations among the individual cell lines studied that were derived from SS patients and controls (representative experiments shown in Fig. 2). The expression of both ERα protein species previously described in normal SGEC (i.e. the full-length 67 kDa and the 61 kDa ERα-Δ3 isoform) [16] could be identified in several of the control-SGEC and SS-SGEC lines tested. The full-length 67 kDa ERα protein was detected in 6/9 control-SGEC lines and in 12/14 SS-SGEC lines, whereas the 61 kDa ERα-Δ3 isoform was uniformly found along with the full-length ERα protein (in 4/6 control-SGEC and