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ORIGINAL ARTICLE

IL-6 Contributes to the TGF-β1-Mediated Epithelial to Mesenchymal Transition in Human Salivary Gland Epithelial Cells

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Abstract

To determine the role of IL-6 in bringing about the EMT, in SGEC obtained from healthy subjects. Human salivary gland (SGs) epithelial cells (SGEC) from primary Sjögren's syndrome (pSS) are able to synthesize interleukin (IL)-6, which is a critical mediator of the SGs modifications in response to chronic inflammation. Recently, a hypothetical link between epithelial-mesenchymal transition (EMT)-dependent salivary gland fibrosis and chronic inflammatory conditions has been suggested for pSS; the present study was conducted to evaluate this link. Primary cultures of human SGEC from salivary mucoceles were stimulated with increasing concentrations of IL-6 for 24–72 h. Microscopy, RT-PCR, Real-time PCR, immunoblotting and flow cytometry were used to detect morphological changes, mRNA and protein expression of the EMT markers E-Cadherin, Vimentin and Collagen type I following IL-6 stimulation. The data collected demonstrate that IL-6 can induce SGEC to undergo a morphological and phenotypical transition to a mesenchymal phenotype, in a dose-dependent manner. Decreased mRNA levels of E-Cadherin accompanied by higher mRNA levels of Vimentin and Collagen type I were observed in the IL-6-treated cells compared to control cells (all $p < 0.05$). This was confirmed at the protein level, demonstrating the decreased E-Cadherin expression, while Vimentin and Collagen type I expression was increased in IL-6-treated SGEC compared to controls (all $p < 0.05$). The results obtained corroborate the hypothesis that dysregulated cytokines IL-6 may contribute to the EMT-dependent fibrosis, offering a more complete understanding of the role of the EMT during SGs fibrosis in pSS.

Keywords Salivary gland · Sjögren's syndrome · IL-6 · EMT · TGF-β1

Introduction

The epithelial-mesenchymal transition (EMT) is a transdifferentiation process that modulates changes in cell states through a morphological and genetic transformation of epithelial cells to mesenchymal-like cells, that acquire an epithelial-mesenchymal plasticity (Lamouille et al. 2014; Nieto et al. 2016). This mechanism is characterized by a wide spectrum of inter- and intra-cellular morphological changes, most likely caused by the integration into cells of extracellular signals (Lamouille et al. 2014). Indeed, inflammatory molecular regulatory mechanisms affect the EMT program, and chronic inflammation is associated with several human diseases, including cancer and autoimmune disease (Radisky et al. 2007; López-Novoa and Nieto 2009). Therefore, the chronic activation of multiple pro-inflammatory signals characterizing various inflammatory diseases can trigger the EMT circuit, provoking the development of a pathological fibrotic state (Thiery et al. 2009; Wynn and Ramalingam 2012). Primary Sjögren's syndrome (pSS) is an autoimmune disease characterized by a severe, chronic inflammatory process with persistent dryness of the eyes and mouth, and lymphocytic foci infiltrates in lachrymal and salivary gland (SG) tissues (Humphreys-Beher and Peck 1999; Moutsopoulos et al. 1980; Ramos-Casals et al. 2005). Although fibrosis is not a main finding in the labial minor salivary glands (LMSG) of pSS patients and is more often shown in the LMSG of scleroderma patients (Drosos et al. 1988a) and in LMSG of elderly individuals (Drosos et al. 1988b), over the last few years, great attention has been focused on the chronic inflammatory state of pSS linked to

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SGs fibrosis. However, the mechanisms that regulate this disease and the role of the EMT in- linking inflammation and SGs fibrosis remain uncertain. Among numerous aberrant pro-inflammatory factors involved in the pathogenesis of pSS, interleukin (IL)-6 was identified as an important pleiotropic cytokine contributing to acute and/or chronic inflammatory conditions (Hirano 2010; Ogura et al. 2008). Previous molecular and pathological results showed, in fact, that human epithelial salivary gland cells (SGEC) are able to synthesize IL-6, which is a crucial mediator of the SGs response to a persistent inflammatory state (Lisi et al. 2010, 2012; Sisto et al. 2010). Recently, IL-6 has been demonstrated to be a potent inducer of the EMT in a number of tumour cell types (Masjedi et al. 2018; Suarez-Carmona et al. 2017) and therefore because the EMT is induced by a dramatic upregulation of cytokines and has an important role in triggering the progression of organ fibrosis, it would be interesting to evaluate whether IL-6-dependent EMT occurs in pSS SG tissue, an issue that has not yet been analysed. In the present study, we investigated whether IL-6 treatment can promote the EMT in healthy SGEC in vitro, analyzing morphological changes and epithelial and mesenchymal markers expression. The current research offers the prospect of identifying IL-6 as a new inducer of the EMT in SGs, and corroborates the hypothesis of a link between inflammation and fibrosis.

Materials and Methods

Reagents and Antibodies

Recombinant human IL-6 (Catalogue Number: I2786**,** Sigma-Aldrich, St. Louis, MO); mouse anti-human β-actin monoclonal antibody (mAb) clone AC-15 (1:100, Catalogue Number: A5441, Sigma-Aldrich, St. Louis, MO); mouse anti-human transforming growth factor (TGF)-β1 mAb (1:50, Catalogue Number: sc-130348, Santa Cruz Biotechnology, Santa Cruz, CA, USA); mouse anti-human E-Cadherin mAb (1:100, Catalogue Number: GA05961-2; Dako, Santa Clara, CA, USA), mouse anti-human Vimentin mAb (1:100, Catalogue Number: MA3-745; Thermo Fisher Scientific, Waltham, MA, USA); rabbit anti-human Collagen type I polyclonal antibody (pAb) (1:500, Catalogue Number: PA5-95,137; Thermo Fisher Scientific, Waltham, MA,USA).

Cell Culture and Cytokine Stimulation

The study included fifteen healthy selected volunteers awaiting removal of mucoceles. The healthy subjects had no complaints of oral dryness, and they had no present or past medical history of any autoimmune diseases, and normal salivary function. All subjects signed the informed consent form before participation. Healthy minor SGs, obtained by the explant outgrowth technique from the lower lip (Sens et al. 1985), were dissociated by enzymatic and mechanical means into suspensions of single cells and plated onto culture flasks. Healthy dispersal SGEC were re-suspended in McCoy's 5a modified medium (Catalogue number: M8403, Sigma-Aldrich, St. Louis, MO) supplemented with 1% heat-inactivated (56 °C for 30 min) fetal calf serum (to avoid excessive growth during treatment with interleukins), 1% antibiotic solution, 2 mM L-Glutamine, 50 ng/ml epidermal growth factor (Promega, Madison, WI, USA), 0.5 µg/ml insulin (Novo, Bagsvaerd, Denmark) and incubated at 37 °C, 5% CO_2 in the air. The contaminating fibroblasts were selectively removed with 0.02% EDTA treatment. Immunocytochemical confirmation of the epithelial origin of the cultured cells was routinely gained (Kapsogeorgou et al. 2001). To assess the activation of the EMT process, assays were carried out using healthy SGEC treated with different concentrations of IL-6 (10–20–50 ng/ml), at distinct incubation times (24–72 h). After 24 h, reverse transcriptase-polymerase chain reaction (RT-PCR) and quantitative Real-Time PCR (q-RT-PCR) for *TGFB1, CDH1, VIM* and *COL1A1* gene expression were performed. After 48 h of stimulation, E-Cadherin, Vimentin and Collagen proteins expression was detected by Western Blot and flow cytometry. After 72 h from treatment, SGEC morphological changes were evaluated. All experiments were performed in triplicate and repeated three times.

Assessment of Morphological EMT-Related Changes in SGEC

To confirm the activation of IL-6-EMT-dependent fibrosis, in vitro analysis of the morphological changes, as a hallmark of EMT, was performed in healthy SGEC after 24–72 h of treatment with IL-6 (10–20–50 ng/ml). Changes in cell morphology were assessed under phase-contrast light microscopy. Since the maximum morphological response was obtained at a dose of 50 ng/ml IL-6, this IL-6 concentration was used in all subsequent experimental procedures.

Gene Transcripts Amplification by RT-PCR and q-RT-PCR

Total RNA was extracted from untreated and IL-6-treated healthy SGEC following the manufacturer's protocol, and reverse-transcribed as previously reported (Lisi et al. 2010; Sisto et al. 2010). Total RNA was isolated using the TRIzol reagent (Catalogue number: 15596026, Thermo Fisher Scientific, Waltham, MA, USA) and first-strand cDNA was synthesized by M-MLV reverse transcriptase (Catalogue number: M1701, Promega, Madison, WI, USA) with 1 µg each of DNA-free total RNA sample and oligo-(dT)15 (Catalogue

number18080051, Thermo Fisher Scientific, Waltham, MA, USA). Subsequently, equal amounts of cDNA were amplified by PCR in a 20 µl reaction mixture containing 2 µM of each sense and antisense primer, PCR buffer, 2.4 mM MgCl2, 0.2 mM each dNTP, 10 µl of transcribed cDNA, and 0.04 U/µl Taq DNA polymerase. The primers used to amplify cDNA fragments were as follows: *TGFB1*: forward 5′-GGACACCAACTATTGCTTCAG-3′ and reverse 5′-TCC AGGCTCCAAATGTAGG-3′; *CDH1*, forward 5′-TTCCCT GCGTATACCCTGGT-3′ and reverse 5′-GCGAAGATA CCGGGGGACACTCATGAG-3′; *VIM*, forward 5′-AGG AAATGGCTCGTCACCTTCGTGAATA-3′ and reverse 5′- GGAGTGTCGGTTGTTAAGAACTAGAGCT-3′; and *COL1A1*, forward 5′- GAGCGGAGAGTACTGGATCG-3′, reverse 5′-TACTCGAACGGGAATCCATC-3′. Amplification products were electrophoresed on 1.5% agarose gel containing ethidium bromide and visualized under ultraviolet transillumination. For qRT-PCR, forward and reverse primers for all the genes tested and the internal control gene β-2 microglobulin (part n° 4326319E; β2M) were purchased from Applied Biosystems (Assays-On-Demand, Applied Biosystems). Each qPCR reaction was run in triplicate on an ABI PRISM 7700 sequence detector (Applied Biosystems). Relative mRNA expression ratios were calculated using the ΔΔCt formula, where Ct is the threshold cycle time value. The different expression of mRNA was deducted from $2^{-\Delta\Delta Ct}$.

Data Evaluation and Sequence Analysis

mRNA quantification data were based on the average of a set of three independent experiments, and densitometric analysis was performed by gel image software (Bio-Profil Bio-1D; ltfLabortechnik GmbH, Wasserburg, Germany). The intensity values for each band, relative to *GAPDH* (internal control for lane loading), were determined and expressed as arbitrary units. The identity of each PCR product was confirmed by the size, and the purified products were directly sequenced during the gene-specific forward or reverse primers.

Western Blot Analysis

TGF-β1, E-Cadherin, Vimentin and Collagen type I protein levels were determined in IL-6-treated healthy SGEC and analysed on sodium dodecyl sulfate–polyacrylamide gel by electrophoresis (SDS-PAGE) using untreated cells as control. The proteins (30 µg/lane) and prestained standards (BioRad Laboratories, Hercules, CA, USA) were loaded on SDS–polyacrylamide precast gels. After electrophoresis, the resolved proteins were transferred from the gel to nitrocellulose membranes. A blot buffer [20 mMTris/150 mM glycine, pH 8, 20% (v/v) methanol] was used for gel and membrane saturation and blotting. The blot conditions were the following: 200 mA (constant amperage), 200 V for 110 min. Subsequently, blots were blocked by phosphate-buffered saline (PBS) pH 7.2 with 0.1% (v/v) Tween 20, 5% w/v non-fat dried milk for 1 h and washed three times with 0.1% (v/v) Tween 20-PBS 1×. Membranes were incubated for 90 min with mouse anti-human TGF-β1 mAb, mouse anti-human E-Cadherin mAb, mouse anti-human_Vimentin mAb and rabbit anti-human Collagen type I pAb and for 30 min with the relative secondary conjugated Ab-HRP (Santa Cruz Biotechnology). Bands intensity was detected via chemoluminescence according to the protocol (Santa Cruz Biotechnology). Immunoblots were then incubated with stripping buffer (Thermo Scientific, Middletown, VA, USA) and probed with mouse anti-human β-actin mAb clone AC-15 as control.

Flow Cytometry

Flow cytometric analysis was performed to reveal TGF-β1, E-Cadherin, Vimentin and Collagen type I expression in IL-6-treated healthy SGEC. Cells were incubated with the anti-human TGF-β1, E-Cadherin, Vimentin and Collagen type I as primary antibodies and with secondary antibodies conjugated with Alexa fluor 488 (Invitrogen). The protein expression was analysed by a Becton–Dickinson (BD, Becton–Dickinson, Germany) FACS Canto tm II flow cytometer and BD FACS Diva software. Indirect staining using just the secondary antibody was performed, as negative standard, indicated in the figures as "negative". The events were acquired, and data were analysed as mean fluorescence intensity (MFI) using the FACS Diva software package (BD Biosciences). MFI values were compared with those observed in healthy untreated SGEC. This procedure was repeated for at least three passages.

Statistical Analysis

Normalised data of mRNA, protein and enzymatic activity were processed to calculate mean values ± SE. Differences between parameters were evaluated using Student's *t* test. Differences were considered statistically significant at $p < 0.05$.

Results

IL-6 Induces Morphological Changes in Human SGEC

To assess the effect of IL-6 treatment on EMT-induced fibrosis in human SGEC, in vitro analysis of the morphological changes, that are a feature of the EMT activation program, was performed in 24–72 h-treated SGEC with increasing

concentrations of IL-6 (10, 20, 50 ng/ml). As shown in Fig. 1, panels a–d, IL-6 induced EMT activation in healthy SGEC incubated with 20 and 50 ng/ml of IL-6. In the absence of treatment (panel a), SGEC had an epithelial-like morphology, but a more complete change to a mesenchymal phenotype was observed in cells treated with 50 ng /ml of IL-6. Indeed, phase contrast microscopy revealed that the epithelial cells underwent dramatic morphological changes, acquiring an elongated phenotypic morphology.

IL-6 Enhances TGF-β1 Expression in Primary Healthy SGEC

We analysed TGF-β1 expression to evaluate its key role in the EMT triggered by IL-6 in healthy SGEC (Fig. 2). To investigate whether or not IL-6 modulates TGF-β1 expression, healthy SGEC were cultured for 24 h in the presence of IL-6 (50 ng/ml). Total RNA was isolated, and gene expression was assessed by RT-PCR (panels A, B) and quantitative PCR (panel C). As seen, *TGFB1* mRNA expression increased significantly ($p < 0.01$), up to three-fold, with IL-6 treatment. To confirm whether the observed induction of *TGFB1* mRNA was correlated with the induction of TGF-β1 protein, healthy SGEC were cultured for 48 h in presence of IL-6 (50 ng/ml) and TGF-β1 protein expression was compared to that of untreated healthy SGEC. As shown in Fig. 3 (panels a, b) Western blot analysis confirmed the induction of TGF-β1 protein expression following IL-6 treatment. In addition, we validated our results by flow cytometry (Fig. 3, panel c), showing the MFI values, and observed a similarly increased TGF-β1 protein expression in healthy IL-6-treated SGEC compared to control ($p < 0.01$).

IL-6 Stimulation Induces a Reduction of E-Cadherin Protein Expression and Enhances the Expression of EMT-associated Mesenchymal Markers in Primary Healthy SGEC

To assess whether IL-6 was involved in the regulation of the expression of the epithelial and mesenchymal markers, cultured healthy SGEC were incubated with IL-6 (50 ng/ml) for 24 h, and RT-PCR analysis was performed for the epithelial gene *CDH1*, and for the mesenchymal genes *VIM* and *COL1A1* (Fig. 2). Experimental data showed that after treatment, a significant ($p < 0.01$), marked increase in the expression of *VIM* and *COL1A1* mRNAs was observed, while *CDH1* gene expression was profoundly affected by stimulation with IL-6 (Fig. 2, panels a, b) ($p < 0.01$). Quantitative Real-Time PCR (Fig. 2, panel c) confirmed these results, demonstrating that the majority of healthy SGEC was negative for *VIM* and *COL1A1* mRNAs, but, after IL-6 treatment, *COL1A1* and *VIM* mRNAs expression was induced (Fig. 2, panel c).

To validate these data, we assessed EMT markers by western blotting and flow cytometry analysis (Fig. 3 panels a, b, c). Proteins were collected from cultured cells after 2 days of treatment with IL-6 (50 ng/ml), and E-Cadherin, Vimentin and Collagen type I expression was evaluated by immunoblotting (Fig. 3, panels a, b). Western blot analysis showed a strong negative effect of IL-6 stimulation on E-Cadherin expression at the protein level (Fig. 3, panel a). Indeed, densitometric analysis of the results (panel b) showed significant inhibitory effects of IL-6 on the expression of the epithelial marker E-Cadherin ($p < 0.01$). The down-regulated E-Cadherin expression was accompanied by a simultaneous, significantly increased expression of

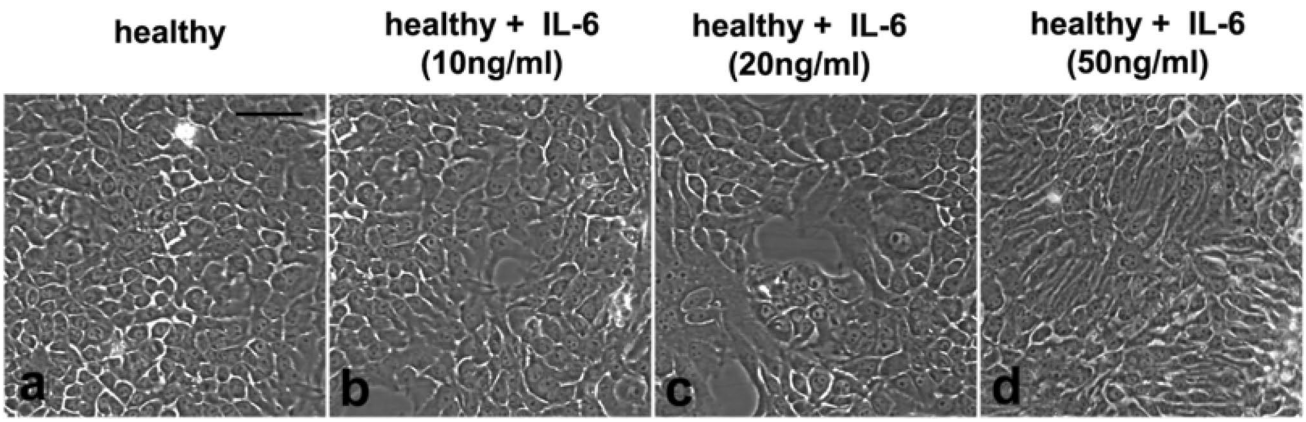

Fig. 1 Morphological changes in in vitro cultured SGEC treated with IL-6. SGEC derived from SGs biopsies of healthy donors were incubated with 10, 20, 50 ng/ml IL-6 for 72 h. (**a**) Untreated SGEC show a clear pebble-like shape and cell–cell adhesion. The cells treated with IL-6 at 10 and 20 ng/ml respectively, (**b**, **c**) show a decrease in cell–cell contacts and a more elongated morphological shape. The most evident morphological changes toward a mesenchymal phenotype were observed in SGEC subjected to 50 ng/ml of stimulation with IL-6 (**d**), that showed a high proportion of spindle-shaped cells. Changes in cell morphology were assessed under phase contrast light microscopy (original magnification, × 20). Bar = 20 µm

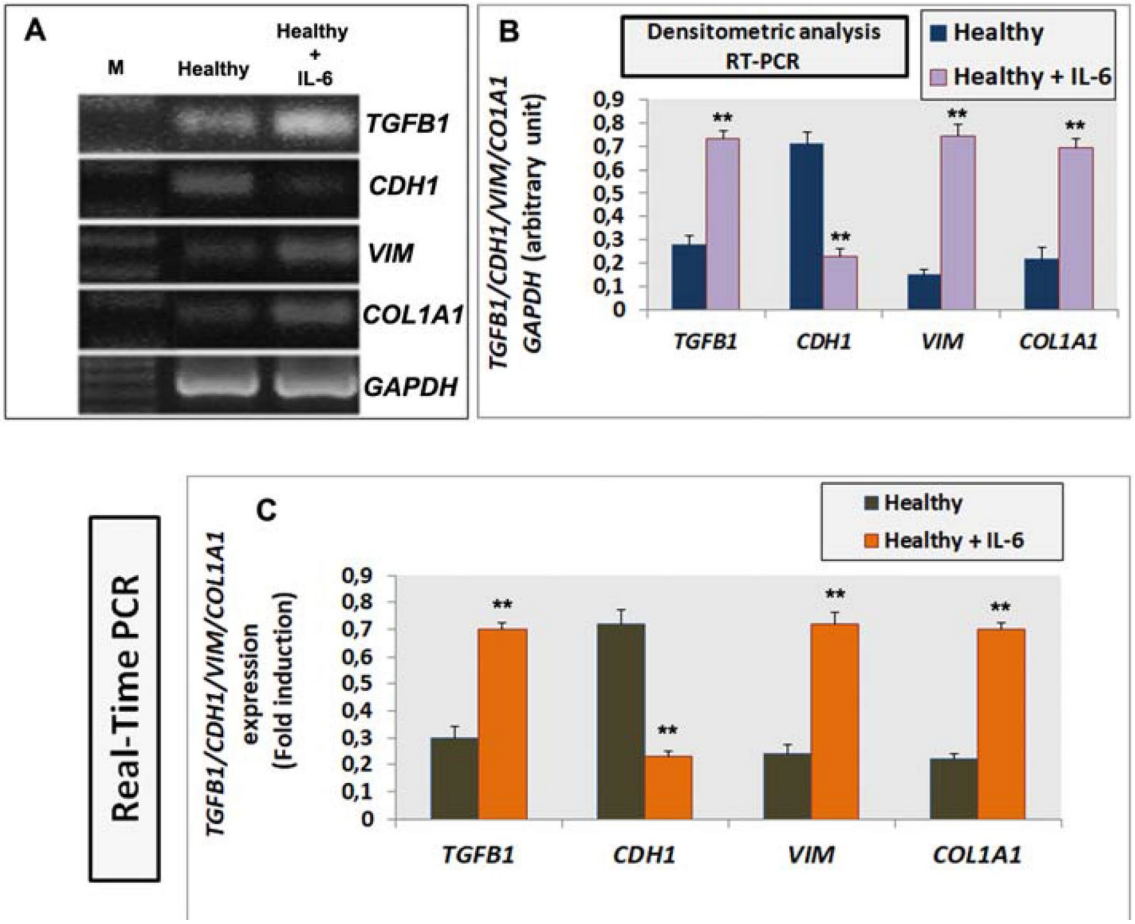

Fig. 2 *TGFB1*, *CDH1, VIM* and *COL1A1* gene expression was quantified by semiquantitative RT-PCR (panel **a**, **b**) and real-time PCR (panel **c**) in in vitro cultured healthy SGEC treated for 24 h with IL-6 (50 ng/ml) and in untreated control healthy SGEC. The images show that IL-6 treatment determined an increased gene expression of *TGFB1* and the mesenchymal markers *VIM* and *COL1A1,* and decreased gene expression of the epithelial marker *CDH1*. Bands intensities were analysed by the densitometric analysis performed by gel image software, normalized against that of *GAPDH* and are expressed in arbitrary units (panel **B**). Gene expression changes observed in real-time PCR analysis (**c**) are in agreement with the results monitored by semiquantitative RT-PCR. (Data represent mean ± SE; $n = 3$). Asterisks indicate statistical significance $p < 0.01$. CDH1 = E-Cadherin; VIM = Vimentin; COL1A1 = Collagen type; M = Marker

the mesenchymal markers Vimentin and Collagen type I ($p < 0.01$) (Fig. 3). In the light of these data, we further determined, by flow cytometry, whether IL-6 stimulation might determine changes in Vimentin, Collagen type I and E-Cadherin expression in cultured SGEC. As shown in Fig. 3 (panel c) reporting the MFI values, western blotting confirmed the results obtained, providing a clear indication of possible involvement of IL-6 in EMT markers modulation in human SGEC.

Discussion

IL-6 is a multifunctional cytokine with pleiotropic effects affecting the activity of many different cell types (Kishimoto 2006). It is produced at inflammation sites, during infection, trauma or in the tumour microenvironment. IL-6, through linking to the IL-6 receptors (Hirano 2010; Mihara et al. 2012; Wolf et al. 2014) triggers aberrant signalling pathways, inducing the promotion of cancerogenesis and tumour angiogenesis, migration and invasion (Fisher et al. 2014). Numerous biological activities are regulated by the pro-inflammatory cytokine IL-6, and IL-6 overproduction has been implicated in the pathogenesis of several human autoimmune diseases (Nishimoto et al. 2005). As regards SGs expression, IL-6, produced at low levels by healthy SGEC under normal conditions, has been demonstrated to be involved in the chronic inflammatory processes that occur in pSS. Several authors have demonstrated, in fact, that IL-6 levels are elevated in the pSS SGEC cultures medium and IL-6 is also overexpressed at the mRNA level (Boumba et al. 1995; Lisi et al. 2010; Roescher et al. 2009; Sisto et al.

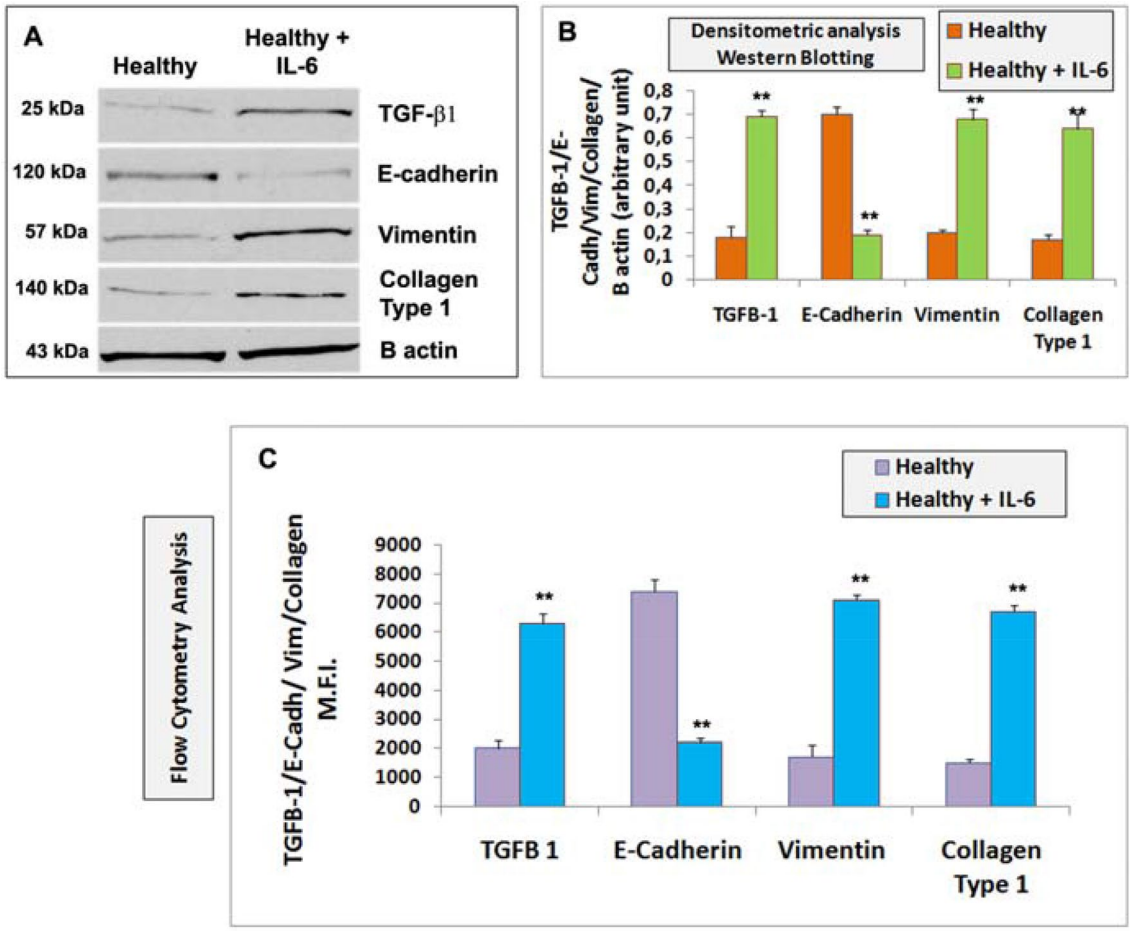

Fig. 3 Panel **a**: Western Blot analysis of TGF-β1, the epithelial marker E-Cadherin and mesenchymal markers Vimentin and Collagen type I, in cultured untreated SGCE, and in healthy SGEC treated with IL-6 (50 ng/ml) for 48 h. Lane 1: healthy control SGEC; Lane 2: IL-6-treated healthy SGEC. Protein expressions were quantified using ImageJ software (panel **b**). The data are expressed as relative intensity of proteins normalized to β-actin expression. Values are considered statistically significant at $*p < 0.05$ or $**p < 0.01$. Interleukins treatment determined a significant increase in TGF-β1, mesenchymal Vimentin and Collagen type I bands intensity, while epithelial marker E-Cadherin expression was reduced compared with untreated control SGEC. All western blots were repeated a minimum of three times. Panel **c**: summary of flow cytometry analysis for TGF-β1, E-Cadherin, Vimentin and Collagen type I SGEC expression in the presence of IL-6. The mean fluorescence intensity (MFI) is shown. Treatment with IL-6 mostly interferes with E-Cadherin basal expression in healthy SGEC, while Vimentin and Collagen type I expression was increased following IL-6 treatment in comparison with untreated control cells ($**p < 0.01$). Data summarize three independent experiments (mean ± SE)

2010). Interestingly, IL-6 secretion by healthy SGEC was involved in the activation of the Furin-TNF-α-converting enzyme (TACE)-amphiregulin (AREG)-IL-6/IL-8 pathway following stimulation with anti-Ro/SSA autoantibodies, emphasizing the pro-inflammatory role of IL-6 in pSS (Lisi et al. 2010; Sisto et al. 2010).

Recent studies have linked IL-6 with the start of the EMT program in cancer cells. IL-6 promoted EMT activation in non-small cell lung cancer via STAT3 signalling and insulin-like growth factor-1 receptor expression (Zheng et al. 2019). IL-6 may promote migration in vitro through the classic and trans-signalling pathways, triggering the EMT process in craniopharyngiomas, benign epithelial tumours of the sellar region in which the inflammatory condition leads to the promotion of tumour metastatic growth (Zhou et al. 2017). From these previous studies, IL-6 is also known to be involved in inflammation-related EMT-cancerogenesis, and the persistent dysregulation of IL-6 secretion affects several organs, including breast, lung, ovaries, pancreas, prostate, colon, kidney and blood (Trikha et al. 2003). In addition, IL-6 has a prominent, crucial role in EMT-associated fibrosis of biliary epithelial cells, characterized by cytoskeletal rearrangements during chronic liver injury, inducing a morphological change versus the mesenchymal phenotype and thus promoting liver cancer and metastatic events (Jiang et al. 2016).

Based on these assumptions, the present study was conducted to analyse a possible role of IL-6 on EMT activation

in pSS, since the effect of IL-6 on EMT induction in SGEC has not yet been studied, to the best of our knowledge. The hypothesis of IL-6 involvement in EMT program activation in pSS was based exclusively on the observation made by our research group, that TGFβ1-EMT-dependent fibrosis occurs in pSS SGs through the Smad canonical and -Erk non-canonical pathways (Sisto et al. 2018a, b, 2019, 2020), determining the transition from SG chronic inflammation to fibrotic disease. In fact, the loss of E-Cadherin and acquisition of Vimentin and Collagen type I resulted detectable in SGEC, and was -correlated with the growing inflammatory grade of pSS. Along these lines, analysing the effect of -dysregulation of the overexpressed cytokines IL-17 and IL-22 in pSS, we demonstrated their contribution to triggering the EMT-dependent fibrotic condition in SGECs. Furthermore, combined treatment with IL-17 and IL-22 induces and accentuates EMT progression, demonstrating that a number of different cytokines can collaborate, increasing the possibility of a fibrotic evolution of pSS (Sisto et al. 2019, 2020).

To further evaluate this scenario, the effects of IL-6 stimulation on inducing the EMT in healthy SGEC were assessed in vitro, and the underlying mechanisms were investigated. The present study revealed the activation of the TGF-β1-dependent EMT program in healthy SGEC following incubation with IL-6. Although the precise molecular mechanisms remain relatively undefined, it is likely that IL-6 activates EMT signalling pathways affecting the delicate balance between the expression of epithelial versus mesenchymal markers in healthy SGEC. In IL-6-treated cells, an enhanced TGF-β1 expression was detected, together with decreased E-Cadherin. Since E-Cadherin is a cell–cell adhesion protein for homotypic cell contacts and is a cell-surface marker of epithelial cells, the loss of E-Cadherin disrupts the epithelial integrity and promotes the EMT. Furthermore, an increased expression of Vimentin and Collagen type I was observed, which is a hallmark of EMT; also on morphological observation, changes in cell shape, motility and adhesion characteristics of the EMT were evident. An illustrative scheme is shown in Fig. 4. Our results are in accordance with other findings revealing a close relationship between IL-6 overexpression and EMT activation, characterized by E-Cadherin repression followed by an increase in Vimentin expression. This was observed, for example, in human breast cancer cells after IL-6 stimulation, indicating a potential role of IL-6 in the induction of EMT resulting in a mesenchymal phenotype in breast cancer cells (Jiang et al. 2016; Sullivan et al. 2009; Xie et al. 2012). In addition, incubation of biliary epithelial cells with IL-6 resulted in a significant decrease in mRNA and protein expression of aberrant E-Cadherin and a significant increase in Vimentin, indicating a role of IL-6 in the EMT in these hepatic cells (Jiang et al. 2016).

In conclusion, the present study demonstrated that, among the multiple roles played by IL-6 in pSS, it seems most likely to be involved in the EMT activation program in SGEC, as indicated by the aberrant mRNA and protein expression of the EMT markers TGF-β1, Vimentin, and Collagen type I, as well as by the acquisition of the typical features of mesenchymal cells. The demonstration of the acquisition, through inflammatory stimuli, of a mesenchymal phenotype by SGEC, while it clarifies that the therapeutic targeting of multiple pro-fibrotic pathways is likely to be more successful than focusing on a single pathway, also provides further leads into identifying key points that may be qualified as vulnerability spots in the dedifferentiation process that occurs in response to chronic inflammation.

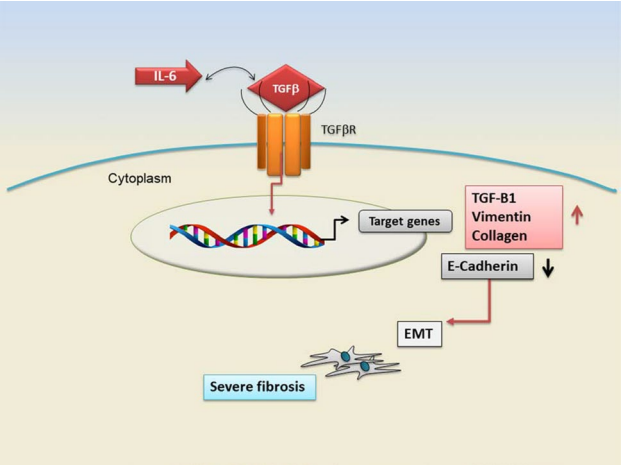

Fig. 4 Postulated mechanism of the IL-6-TGF-β1-induced EMT process in healthy SGEC

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Compliance with Ethical Standards

Conflict of Interest All the authors have no conflicts of interest to disclose.

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