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Abscisic acid ameliorates the systemic sclerosis fibroblast phenotype in vitro

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ABSTRACT

The phytohormone abscisic acid (ABA) has been recently identified as an endogenous hormone in humans, regulating different cell functions, including inflammatory processes, insulin release and glucose uptake. Systemic sclerosis (SSc) is a chronic inflammatory disease resulting in fibrosis of skin and internal organs. In this study, we investigated the effect of exogenous ABA on fibroblasts obtained from healthy subjects and from SSc patients. Migration of control fibroblasts induced by ABA was comparable to that induced by transforming growth factor-β (TGF-β). Conversely, migration toward ABA, but not toward TGF-β, was impaired in SSc fibroblasts. In addition, ABA increased cell proliferation in fibroblasts from SSc patients, but not from healthy subjects. Most importantly, presence of ABA significantly decreased collagen deposition by SSc fibroblasts, at the same time increasing matrix metalloproteinase-1 activity and decreasing the expression level of tissue inhibitor of metalloproteinase (TIMP-1). Thus, exogenously added ABA appeared to revert some of the functions altered in SSc fibroblasts to a normal phenotype. Interestingly, ABA levels in plasma from SSc patients were found to be significantly lower than in healthy subjects. UV-B irradiation induced an almost 3-fold increase in ABA content in SSc cultures. Altogether, these results suggest that the fibrotic skin lesions in SSc patients could benefit from exposure to high(er) ABA levels.

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1. Introduction

Abscisic acid (ABA) is a phytohormone regulating fundamental physiological functions in plants [1]. ABA has been also reported to be an endogenous hormone in humans, regulating different cell responses and functions, including activation of innate immune cells and stimulation of insulin release and glucose uptake [2–5]. ABA released from human and murine innate immune cells behaves as an autocrine signal stimulating their functional activation: (i) in human granulocytes, ABA stimulates phagocytosis, Reactive Oxygen Species (ROS) and Nitric Oxide (NO) production, and cell migration [2]; (ii) in human monocytes, ABA induces cell migration and release of proinflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α), monocyte chemoattractant protein-1 (MCP-1), metalloprotease 9 (MMP-9), and prostaglandin E-2 (PGE_2) [3]; and (iii) in murine microglia, ABA stimulates NO and TNF-α production and cell migration [6]. The signaling cascade of ABA in mammalian cells involves ABA binding to lanthionine synthetase C-like protein 2 (LANCL2) and cAMP production [7,8].

Systemic sclerosis (SSc) is a chronic inflammatory disease resulting in fibrosis of skin and internal organs. The current understanding of SSc pathogenesis indicates that inflammation, autoimmune responses and vascular damage lead to fibroblast activation, with an uncontrolled, excessive deposition of extracellular matrix, mainly type I collagen, causing dermis thickening [9]. In culture, SSc fibroblasts differ in terms of migration, proliferation and especially collagen deposition [10–14], compared to fibroblasts isolated from healthy subjects. Thus, cultures of SSc fibroblasts have been used as an experimental model to investigate the molecular mechanism(s) involved in the dysregulation of type I collagen

Abbreviations: ABA, abscisic acid; TNF-α, tumor necrosis factor-α; LANCL2, lanthionine synthetase C-like protein 2; SSc, systemic sclerosis; FCS, fetal calf serum; TGF-β, transforming growth factor-β; CFDA-SE, carboxyfluorescein succinimidyl ester; FGF, fibroblast growth factor; PBS, phosphate-buffered saline; HBSS, Hanks' balanced salt solution; ECM, extracellular matrix; MMP, matrix metalloproteinases; TIMP-1, tissue inhibitor of metalloproteinase-1.

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deposition [15]. Clarification of these mechanism(s) should lead to the development of new therapeutic strategies for SSc treatment. The role of ABA in the regulation of inflammatory cell function prompted us to explore the effect of the hormone on skin fibroblasts from SSc patients.

2. Materials and methods

2.1. Cell culture

Dermal fibroblasts were obtained after outgrowth from 2 mm skin punch biopsies of patients with SSc ($n = 11$) and matched healthy controls ($n = 9$). In SSc patients biopsies were performed on perilesional area of skin. Briefly, the dermal portion of the skin was cut into small pieces, and spread onto culture flasks. Culture medium, DMEM (Invitrogen, Milano, Italy) containing 20% heat-inactivated fetal calf serum (FCS), 1 mM pyruvate, 2 mM glutamine, 2.5 μg/ml amphotericin B, 100 U/ml penicillin and 100 μg/ml streptomycin, was added. Fibroblast outgrowth started at day 3–7; the skin pieces were removed after 1 week and cultures were grown to confluence, treated with trypsin (2 min at 37 °C) and re-cultured in DMEM.

2.2. Migration assays

Fibroblasts were cultured until approximately 80% confluence was reached, then recovered and resuspended at 1×10^6/ml in DMEM medium supplemented with 1% FBS (chemotaxis medium). Chemotaxis assays were performed using 96-well ChemoTx system microplates (Neuro Probe, Gaithersburg, MD) with a 8-μm pore size polycarbonate filter. Filters were precoated with superfibronectin (1 μg/ml). For chemotaxis assays, ABA (10 μM) or transforming growth factor-β TGF-β (10 ng/ml) were added to the bottom wells. Cell suspensions (25 μl) were then placed on top of the filter and allowed to migrate for 15 h at 37 °C. Counting of transmigrated cells was performed with SITOX green staining, as described previously [2]. Results were expressed as chemotaxis index (number of cells migrated toward chemoattractant/number of cells migrated toward medium).

2.3. Proliferation

Proliferation of control and SSc fibroblasts was investigated using two different methods. In some experiments the proliferation was performed by dye dilution assay in flow cytometry. Briefly, cells were stained with carboxyfluorescein succinimidyl ester (CFDA-SE; Invitrogen, Milan, Italy), a lipophilic dye that reacts with amino groups on peptides and proteins forming a stable amide bond [16]. Specifically, cells were seeded in six-well plates (2×10^5 cells/well), washed three times with phosphate-buffered saline (PBS), and incubated with 5 μM CFDA-SE in PBS in the dark at 37 °C in 5% CO_2 for 5 min. At the end of incubation, the cells were again washed three times with PBS supplemented with 1% FBS and then exposed to the specific treatments. The intensity of cellular CFDA-SE fluorescence was estimated at 1, 2, 3 and 5 days using a FACSCanto II flow cytometer equipped with FACS Diva software (Becton–Dickinson Italia (BD), Milan, Italy). The proliferation of CFDA-SE-labeled cells was estimated by the progressive halving of cellular fluorescence as every cell division was completed. The cytometric data files were analyzed using the Proliferation Wizard module of the ModFit LT 3.2 software (Verity Software House, Topsham, ME, USA) to estimate the frequencies of proliferating cells exposed to treatments. The results were expressed as proliferation index. Proliferation index is the ratio between the total number of cells at the considered time and the respective number of parental cells back-calculated by the mathematical software.

In other experimental settings, SSc and control fibroblasts were seeded in 96-well plates (5×10^3 cells/well) and cultured for 7 days in complete medium in the presence or absence of 10 ng/ml fibroblast growth factor (FGF) or 10 μM ABA. Cell quantification was obtained with SITOX green staining.

2.4. Evaluation of collagen type I deposition, MMP-1 activity and TIMP-1 expression

Control and SSc fibroblasts were cultured in 24 wells (20×10^3/well), and treated or not with 10 μM ABA for 24 h. Supernatant was recovered for quantification of: (i) collagen type I concentration, using an ELISA kit (Collagen type I ELISA, MD Bioproducts, Zurich, Switzerland); (ii) MMP-1 activity, with the SensoLyte® 520 MMP-1 Assay Kit (AnaSpec, Fremont, CA), and (iii) TIMP-1 concentration, by ELISA (Life Technologies, Milano, Italy), following manufacturers' instructions.

2.5. UV-B irradiation

Control and SSc fibroblasts were cultured in 100 mm dishes until 80% confluence was reached. Supernatants were removed, cells washed once with Hanks' balanced salt solution (HBSS) and 2 ml of the same buffer were added. Cells were then irradiated for 5 min using a 0.1 mW/cm^2 UV-B lamp at a distance of 45 cm (corresponding to a dose of 27 mJ/cm^2), and incubated for further 45 min at 37 °C. Four volumes of methanol were then added to stop the incubation. The lamp used in this study emits most of its energy within the UV-B range (280–315 nm).

2.6. ABA content

Total ABA content in extracts from UV-B-irradiated cells was determined by a specific ELISA kit and by HPLC-coupled mass spectrometry [17]. ABA levels in plasma from healthy and SSc patients were evaluated as previously described [5].

2.7. Statistical analysis

Data were compared by means of the Student's unpaired t-test. Statistical significance was set at $p < 0.05$.

3. Results and discussion

Cell migration, proliferation and collagen deposition are key cell functions known to be modified in SSc fibroblasts [9–14]. Thus, the effect of exogenously added ABA on these cell functions was investigated in SSc and control fibroblasts.

Fibroblasts were challenged to migrate toward medium, in the presence or absence of ABA or TGF-β. The spontaneous migration of SSc fibroblasts toward the medium was slightly increased in comparison to that recorded in control fibroblasts (1014 ± 129 and 856 ± 103 migrated cells, respectively, $n = 9$, $p < 0.05$), in line with the already reported enhancement of migratory ability of SSc fibroblasts [10]. As shown in Fig. 1, migration toward TGF-β was comparable in control and in SSc fibroblasts. Migration of control fibroblasts induced by ABA was comparable to that induced by TGF-β. Indeed, ABA has been described to act as a chemoattractant for different mammalian cell types, including granulocytes, monocytes, microglia and mesenchymal stem cells [2,3,6,18]. Interestingly, migration toward ABA, but not toward TGF-β, was impaired in SSc fibroblasts, suggesting a specific unresponsiveness of these cells to the ABA-induced migration.

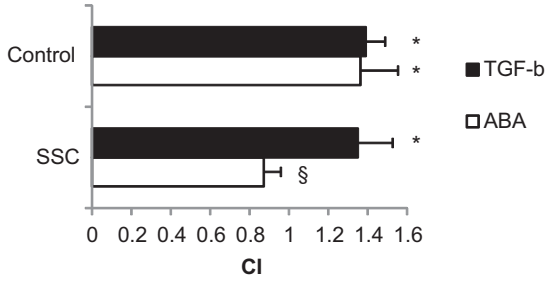

Fig. 1. Skin fibroblasts from SSC patients do not migrate toward ABA. Skin fibroblasts from healthy subjects (control) and from SSc patients were resuspended at 10^6/ml in chemotaxis medium. Chemotaxis assays were performed as reported under Section 2. Cell suspensions were placed on top of the filter and cells were allowed to migrate for 15 h at 37 °C toward chemotaxis medium containing, or not, ABA (10 μM) or TGF-β (10 ng/ml). Counting of transmigrated cells was performed with SITOX green staining. Results are expressed as chemotaxis index (CI) and are the mean ± SD of measurements of three different experiments with fibroblasts from $n = 3$ healthy subjects and from $n = 9$ SSc patients. $^*p < 0.05$ compared to the corresponding CI values of cells migrated toward medium; $^§p < 0.05$ compared to CI values of control fibroblasts migrated toward ABA.

Next, the effect of ABA on fibroblast proliferation was investigated. As shown in Fig. 2A, under basal culture conditions the proliferation rate was significantly lower in SSc than in control fibroblasts, being approximately reduced by 50% after a 5-day culture. Interestingly, ABA significantly increased cell proliferation in SSc, but not in control, fibroblasts (Fig. 2B and C), suggesting that ABA-dependent signaling could interfere with SSc fibroblastspecific metabolic pathways controlling cell proliferation. ABA-induced proliferation was also reported to occur in human mesenchymal stem cells [18] and hemopoietic progenitors [19]. Future studies are required to identify the specific metabolic pathway(s) exploited by ABA in SSc fibroblasts for modulating proliferation. However, ABA related metabolic pathway seems to be different from that triggered by FGF, since proliferation in response to this growth factor is impaired in SSc fibroblasts, while FGF exerted a stimulatory effect on control fibroblast growth (Fig. 2B).

Excess extracellular matrix (ECM) deposition is the leading process determining skin fibrosis [9,15]. The production of different ECM components, mainly type I collagen, is up-regulated in SSc fibroblasts [15]. In order to analyze ABA effects on this process, control and SSc fibroblasts were cultured in the presence or not of ABA and collagen type I concentrations in culture media were measured after 24 h. As shown in Fig. 3A, collagen levels were almost 10 times higher in SSc cultures than in control cultures, in line with previous reports [12–14]. Interestingly, presence of ABA significantly decreased collagen deposition by SSc fibroblasts. Conversely, collagen levels were not modified in control cultures.

The increased deposition of collagen by SSc fibroblasts is known to be at least in part due to a reduced activity of matrix metalloproteinases (MMP) [9,15]. In particular, expression and activity of the collagenase MMP-1 are decreased in SSc fibroblasts, as a consequence of higher expression levels of TIMP-1 (tissue inhibitor of metalloproteinase-1), a MMP-1 inhibitor [15,20]. Thus, to investigate the mechanism whereby ABA reduces collagen deposition in SSc fibroblasts, we analyzed MMP-1 activity and TIMP-1 levels in control and in SSc culture media. As shown in Fig. 3B, MMP-1 activity was increased in the supernatants of SSc fibroblasts treated with ABA, in line with a decreased collagen deposition by these cells (Fig. 3A). TIMP-1 levels, as detected by ELISA, were slightly decreased in the supernatants from ABA-treated SSc fibroblasts compared to untreated SSc cells (47.28 ± 9.46 and 59.10 ± 16.35 ng/ml, respectively, $n = 9$, $p < 0.05$), possibly explaining the ABA-induced increase in MMP-1 activity.

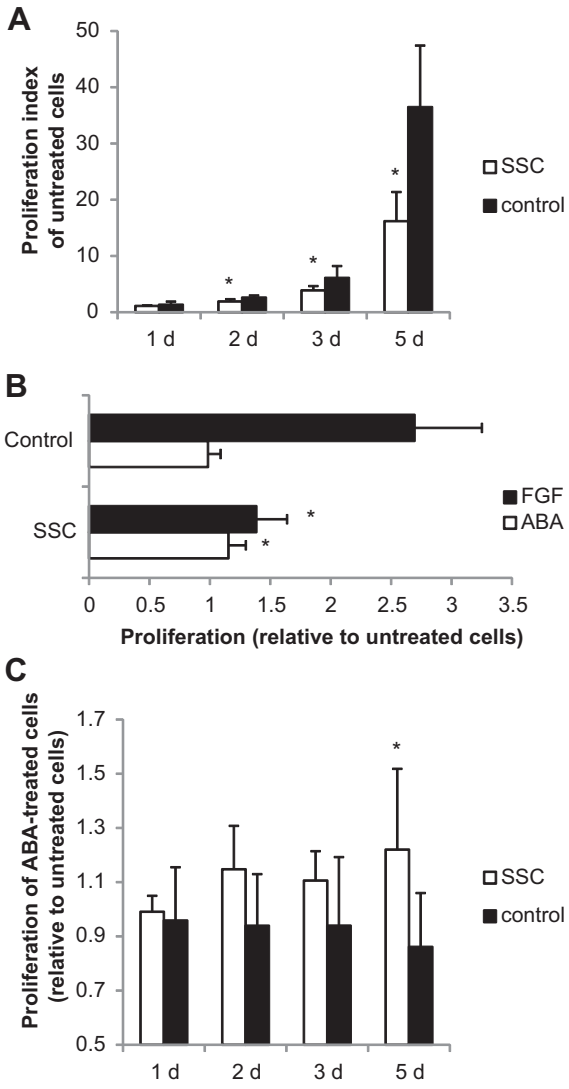

Fig. 2. Skin fibroblasts from of SSC patients proliferate in response to ABA. (A and C) Control and SSc fibroblasts, seeded in six-well plates (2×10^5 cells/well), were incubated with 5 μM CFDA-SE at 37 °C for 5 min. Cells were then washed three times with 10 mM PBS supplemented with 1% FBS and exposed (or not) to 10 μM ABA. The intensity of cellular CFDA-SE fluorescence was estimated by flow cytometry at 1, 2, 3 and 5 days after treatment in cells washed and scraped in PBS–1 μM EDTA. Results are expressed as proliferation index (A) or as proliferation index in ABA-treated cells, relative to untreated cells (C) and are mean ± SD of $n = 6$ and 5 control and SSc fibroblasts, respectively. (B) Control and SSc fibroblasts were seeded in 96-well plates (5×10^3 cells/well) and cultured for 7 days in complete medium, in the presence or absence of 10 ng/ml FGF or 10 μM ABA. Cell quantification was obtained with SITOX green staining. Results are expressed as number of cells in treated samples, relative to the number of cells in untreated samples, and are mean ± SD of three experiments from fibroblasts from $n = 4$ healthy subjects and $n = 6$ SSc patients. $^*p < 0.05$ compared to the corresponding control fibroblasts.

These results indicate that exogenously added ABA appears to revert some of the functions altered in SSc fibroblasts to a normal phenotype. Indeed, ABA reduces cell migration and collagen deposition, and stimulates cell proliferation. These findings suggest that SSc fibroblast alterations might be in part dependent on ABA deficiency. In order to verify this hypothesis, ABA plasma levels were comparatively analyzed in SSc patients and in healthy controls. Interestingly, ABA levels in plasma from SSc patients were found to be significantly lower than in healthy subjects (0.53 ± 0.50 nM ($n = 16$) and 1.08 ± 0.46 nM ($n = 19$), respectively, $p < 0.005$). The decrease of plasma ABA in SSc patients could be related, at least

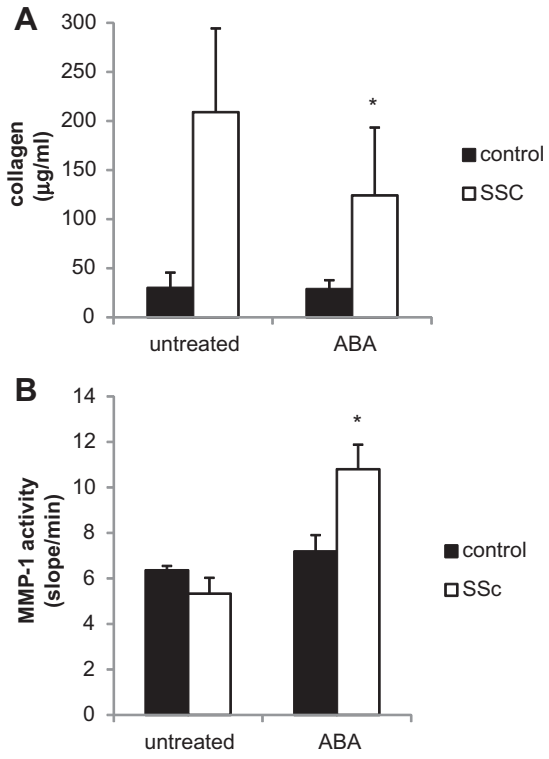

Fig. 3. ABA decreases collagen deposition by skin fibroblasts from SSC patients. Control and SSc fibroblasts were seeded in 24-well plates (20×10^3 cells/well) and treated or not with 10 μM ABA for 24 h. Supernatant was recovered for quantification of: (A) collagen type I concentration, using an ELISA kit (Collagen type I ELISA, MD Bioproducts, Zurich, Switzerland), and (B) MMP-1 activity, with the SensoLyte® 520 MMP-1 Assay Kit. Results are mean ± SD of determinations with $n = 4$ control fibroblasts and $n = 9$ SSc fibroblasts. $^*p < 0.05$ compared to the corresponding untreated fibroblasts.

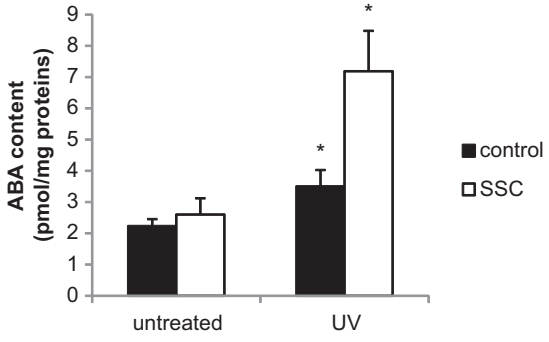

Fig. 4. UV-B irradiation increases ABA content in SSc fibroblasts. Control and SSc fibroblasts were irradiated (or not, untreated) with 27 mJ/cm^2 UV-B and then incubated for additional 45 min at 37 °C in the dark. ABA content was measured by ELISA. Results are the mean from determinations with $n = 8$ control fibroblasts and $n = 5$ SSc fibroblasts. $^*p < 0.05$, compared to corresponding values from un-irradiated cells.

in some patients, to the pancreatic alterations that can be present in SSc patients [21], similarly to what occurs in type I diabetes mellitus, where the destruction of pancreatic β cells correlates with a severe reduction of plasma ABA levels [5]. Whatever the cause of inhibition of ABA production, increasing ABA concentration in the skin could prove advantageous against skin fibrosis in SSc.

UV-B has been recently reported to stimulate ABA production in human granulocytes and keratinocytes [5]. To test whether this effect could be replicated on skin fibroblasts, SSc and control fibroblasts were exposed to UV-B irradiation (27 mJ/cm^2) and their ABA content was determined. Indeed, UV-B irradiation induced an almost 3-fold increase in ABA content in SSc cultures (Fig. 4). Thus, it is possible that the therapeutic effects observed with phototherapy in SSc could be due to stimulation of ABA production [22].

Altogether, these results suggest that the fibrotic skin lesions in SSc patients could benefit from exposure to high(er) ABA levels. This could be obtained through different strategies: (i) exogenous ABA administration to patients; (ii) recommendation of a specific diet for patients, aimed at increasing the intake of ABA-rich food (such as apples, avocado, (Ref. [23])); and (iii) exposure to UV-B irradiation. Indeed, phototherapy has been already suggested as a possible therapy for SSc [22].

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