Cannabinoids inhibit fibrogenesis in diffuse systemic sclerosis fibroblasts

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Objective. It has been demonstrated that the endocannabinoid system is up-regulated in pathologic fibrosis and that modulation of the cannabinoid receptors might limit the progression of uncontrolled fibrogenesis. The aim of this study was to investigate whether the synthetic cannabinoid receptor agonist WIN55,212-2 could modulate fibrogenesis in an in vitro model of dcSSc.

Methods. The expression of cannabinoid receptors CB1 and CB2 was assessed in dcSSc fibroblasts and healthy control fibroblasts. To investigate the effect of WIN55,212-2 on dcSSc fibrogenesis, we studied type I collagen, profibrotic cytokines, fibroblast transdifferentiation into myofibroblasts, apoptotic processes and activation of the extracellular signal-related kinase 1/2 pathway prior to and after the treatment with the synthetic cannabinoid at increasing concentrations.

Results. Both CB1 and CB2 receptors were over-expressed in dcSSc fibroblasts compared with healthy controls. WIN55,212-2 caused a reduction in extracellular matrix deposition and counteracted several behavioural abnormalities of scleroderma fibroblasts including transdifferentiation into myofibroblasts and resistance to apoptosis. The anti-fibrogenic effect of WIN55,212-2 was not reverted by selective cannabinoid antagonists.

Conclusions. Our preliminary findings suggest that cannabinoids are provided with an anti-fibrotic activity, thereby possibly representing a new class of agents targeting fibrosis diseases.

Key words: Systemic sclerosis, Cannabinoids, Fibrogenesis, Fibroblasts.

Introduction

SSc is a CTD characterized by excessive extracellular matrix (ECM) synthesis and deposition with consequent damage of the affected tissues. This disruption of organ architecture results in high associated morbidity and mortality. Although the pathogenesis is still unclear, autoimmunity, small vessel vasculopathy and inflammation represent the main features that precede fibrosis, due to inappropriate fibroblast activation and transdifferentiation into myofibroblasts. At present, SSc is not a curable disease and current treatments aim to limit disease manifestations; available drugs, including immunosuppressive agents, are only partially capable of modifying the progressive tissue damage.

A growing body of literature has been highlighting the immunomodulating properties of cannabinoids, including anti-inflammatory activity, vasomotor response and cell death regulation [1, 2]. Apart from the naturally occurring extracts of marijuana plants (Cannabis sativa), cannabinoids include a group of synthetic derivatives and many endogenous fatty acids capable of binding and modulating the activities of the two cloned cannabinoid receptors CB1 (CB1r) and CB2 (CB2r) [3, 4]. However, not all of the biological activities of cannabinoids can currently be fully explained by CB1r/CB2r-mediated mechanisms, which is consistent with the hypothesis that, besides receptor-independent pathways [5], additional receptorial pathways could be involved in signal transduction including the transient receptor potential vanilloid type-1 (TRPV1) [6], the peroxisome proliferator-activated receptors (PPARs) [7], G-protein receptor 55 (GPR55) [8], α-7 nicotinic receptors and 5-HT3 receptors [9].

Recently, it has also been demonstrated that the pleiotropic cannabinoid system is involved in both liver and pancreatic fibrosis. Furthermore, cannabinoids may play a pro- or anti-fibrogenic role depending on their interaction with CB1r or CB2r [10–12]. This raises the possibility that pharmacologic modulation of the endocannabinoid system could be a target to limit tissue damage in pathologic fibrosis [13]. Accordingly, in 2009 Akhmetshina et al. [14] published very interesting results demonstrating that CB2r is involved in murine bleomycin-induced dermal fibrosis by reducing leucocyte infiltration and dermal thickening.

Considering the potential anti-fibrotic properties of cannabinoid receptor modulation, as well as the various pathogenetic similarities among fibrotic diseases, we investigated whether the synthetic cannabinoid receptor agonist, WIN55,212-2, was able to modulate fibrogenesis in dcSSc fibroblasts.

Our results suggest that treatment with the synthetic cannabinoid WIN55,212-2 down-regulates the synthesis of type I collagen, probably involving the phosphorylation pathways of the mitogen-activated protein kinases (MAPKs), extracellular signal-related kinase (p-ERK), thereby modulating profibrotic cytokines [TGF-β, connective tissue growth factor (CTGF) and IL-6] and myofibroblast transdifferentiation, as well as favouring programmed cell death. Although the over-expression of CB1r and CB2r was observed in dcSSc fibroblasts, these receptors seem not to be directly involved in WIN55,212-2-induced collagen reduction.

Materials and methods

Skin biopsies

Dermal fibroblasts were obtained by a 5-mm punch biopsy, from the leading edge of the affected skin, on the forearm of five patients affected by dcSSc [15] and from five healthy volunteers matched for sex and age. All subjects gave their informed consent, and the study was approved by the University of Siena’s institutional review board. None of the patients was receiving immunosuppressive medication or corticosteroids, nor taking cannabinoids for either recreational or therapeutic use at the time of biopsy (Table 1).
Cannabinoids and scleroderma fibroblasts

Dermal fibroblast isolation and culture
Skin specimens were digested using 1 mg/ml clostridial collagenase in phosphate buffered saline. The cell suspensions were plated out in 10 ml of DMEM supplemented with 1-glutamine (2 mM), fetal calf serum (FCS) (10%), penicillin (200 U/ml) and streptomycin (200 μg/ml) in 100-mm culture dishes and incubated in a humidified atmosphere containing 5% CO2. The experiments were conducted at the third passage in order to avoid changes in the original phenotype.

Culture stimulation
Dermal fibroblasts were plated out in 500 μl of complete medium (2.5% FCS). After 24 h, fibroblasts were incubated with WIN55,212-2 (1 and 10 μM) for 24 h, except for p-ERK1/2 assay where cells were incubated for 1 h. Experiments with the synthetic cannabinoid receptor antagonists AM281 (20 μM) and AM630 (20 μM) were performed by incubating for 10 min before cannabinoid receptor agonist treatment. Cannabinoids were solubilized in dimethyl sulfoxide (DMSO). Basal fibroblast cultures were run along with solvent control.

Supernatant assay
The supernatant was collected and stored at −20°C prior to and after synthetic cannabinoid treatment. An ELISA kit (Euroclone, Lugano, Switzerland) and EIA kit (Takara Bio, Otsu, Japan) were used for the measurement of type I collagen and procollagen type I carboxy-terminal peptide (procollagen type I PIP) supernatant levels, respectively.

Western blot analysis
Equal amounts of protein were separated from the samples using SDS on 12% polyacrylamide gel (SDS-PAGE). Protein lysates were then transferred onto a nitrocellulose membrane for 2 h at room temperature and subsequently incubated with primary antibodies: 1:100 dilution for anti-CB1r (N-15), anti-CB2r (S-16), anti-collagen α1 type I (coll1a1) and 1:1000 for anti-α-smooth muscle actin (α-SMA) (B4-Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-phospho-p44/42 MAPK (Thr202/Tyr204) (Cell Signalling Technology, Irving, CA, USA). After several washes in Tris-buffered saline Tween-20 (TBST), the membrane was incubated with the appropriate secondary antibody for 45 min (TBST milk 1%) and then washed again. Proteins were visualized using an ECL Western Blotting kit (Amersham Biosciences, Buckinghamshire, UK).

Semi quantitative RT–PCR
Total RNA from treated and untreated fibroblasts was isolated using a RNeasy plus mini kit (Qiagen, Milan, Italy), then converted to cDNA using omniscript reverse transcriptase (Qiagen) and oligo dT primers; 0.5 μg of RNA were used for each reverse transcription. Then 2 μl of cDNA were used as a template in 50 μl of mix reactions containing gene-specific primers, reagent for PCR and HotStarTaq Plus DNA Polymerase (Qiagen). Gene-specific primer pairs (designed by using the PRIMER3 program) were as follows: 5′-ggt cta aag gtt cca atg gt-3′ (forward) and 5′-acc agg ttc acc gtt gtt ac-3′ (reverse) for coll1a1; 5′-ggg act atc ctc gca gaa-3′ (forward) and 5′-cct cct tgt agg cgt cgt-3′ (reverse) for TGFR-β; 5′-gga aat gtc cca ccc at-3′ (forward) and 5′-ttc tgg tca aac acc ccc tt-3′ (reverse) for CTGF; 5′-ttc aat gtc cca gcc atg ta-3′ (forward) and 5′-gaa gta ata gcc acg ctg-3′ (reverse) for α-SMA; and 5′-ggt gaa ggt cgg aat cca cgg-3′ (forward) and 5′-ggt cag gac ttc ttc cag gtt-3′ (reverse) for GAPDH. A preliminary experiment was performed using a range of cycle numbers to ensure that PCR amplification was within the linear range for each of the genes tested. Aliquots of 1 μl of PCR product added to 5 × DYE were fractioned on a 1.2% agarose gel.

Immunocytochemistry
Cytocentrifuged monolayers were air dried, fixed with acetone and incubated with the primary antibody for 60 min at room temperature. An EnVision-TM Kit (Dako, Glostrup, Denmark) was used for the conjugation of peroxidase. The monolayers were then incubated for 5 min with a pre-diluted diamino- benzidine solution (Dako). The α-SMA-positive fibroblasts were counted blindly by two of us (E.S. and E.G.-G.) at 200× magnification on the basis of the first 300 cells in three non-contiguous microscope fields.

Cell viability
Cell metabolic activity was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The dcSSc and healthy fibroblasts were plated out at a density of 1 × 104 cells/well in 96-well plates with the addition of WIN55,212-2 (1 and 10 μM), AM281 (20 μM) and AM630 (20 μM) for 24 h. MTT stock solution was added to each well to a final concentration of 3 mg/ml. After 4 h of incubation at 37°C, the dark blue formazan crystals that formed from MTT cleavage in actively metabolizing cells were dissolved in DMSO. Optical density was measured at 570 nm using a spectrophotometer [16].

Cell viability was evaluated in dcSSc and healthy fibroblast cultures before and after treatment (discussed earlier), using a trypan blue exclusion test and measurement of apoptotic and necrotic cells. In particular, fibroblasts were stained with fluorescein isothiocyanate-labelled annexin V and propidium iodide (PI). The number of annexin V and PI-positive cells was quantified by flow cytometry (Annexin V-FITC, APOAF-50TST; Sigma-Aldrich, Milan, Italy).

Statistical analysis
All values presented are expressed as the mean ± s.d. of the five separate experiments. Analysis of variance (ANOVA) was used for a comparison of multiple means, followed by the Student–Newman–Keuls method post hoc test. A statistical significance level of 95% (P < 0.05) was used. All data analyses were performed with the Sigma Stat 3.5 statistical package.

Results
Cannabinoid receptor expression in scleroderma and healthy human dermal fibroblasts
CB1r and CB2r were present in whole cell lysates. Both cannabinoid receptors were found to be over-expressed in dcSSc fibroblasts compared with healthy fibroblasts (Fig. 1). After a 24-h exposure of fibroblasts to WIN55,212-2 (1–10 μM), the cannabinoid was removed and CBrs protein expression was analysed at 0, 24, 48 and 72 h. Agonist-induced inhibition of CBrs was observed to be maximal without a drug-free interval, and pre-treatment levels were restored within 72 h from agonist withdrawal (Fig. 1).
Effect of WIN55,212-2 on type I collagen production

One of the hallmarks of dcSSc is the uncontrolled production and deposition of ECM, and of type I collagen as its principal component. In basal conditions, an over-expression of col1a1 mRNA and protein was observed in dcSSc fibroblasts compared with healthy controls. Upon incubation with WIN55,212-2, a reduction in col1a1 mRNA and protein was observed in dcSSc fibroblasts and healthy lysates at 10 μM (Fig. 2A).

Type I collagen is newly synthesized from procollagen by the cleavage of the carboxy-terminal peptide (PIP). A quantitative evaluation of procollagen type I PIP was carried out in order to further confirm the inhibitory effect of WIN55,212-2 on ECM neosynthesis. In basal conditions, dcSSc procollagen type I PIP was observed to be significantly increased (36%) as compared with healthy controls (mean ± s.d.: 175.88 ± 39.4 vs 129.52 ± 12.24 ng/ml; P < 0.05). After 10 μM WIN55,212-2 incubation, a significant mean reduction in procollagen type I PIP supernatant levels was observed in both dcSSc (53%) and healthy fibroblasts (56%) (mean ± s.d.: 82.78 ± 27.74 and 56.46 ± 11.09 ng/ml; P < 0.05) (Fig. 2B).

Moreover, we tested whether the effect of the synthetic cannabinoid receptor agonist WIN55,212-2 was mediated by the classic cannabinoid receptors CB1r and/or CB2r. The pre-incubation of dcSSc and healthy fibroblasts with the selective CB1r and CB2r antagonists (20 μM AM281 and 20 μM AM630, respectively) did not significantly revert the effect of WIN55,212-2. Similar inhibition was observed in fibroblasts from healthy controls. Results are mean ± s.d. of five separate experiments (*P < 0.05).

Influence of WIN55,212-2 on TGF-β and CTGF mRNA levels

TGF-β is considered to be one of the promoters of the early profibrotic response orchestrated by scleroderma fibroblasts,
which is subsequently maintained by the profibrogenic cytokine CTGF [17, 18].

As expected, cytokine mRNA expression was observed to be up-regulated in dcSSc fibroblasts compared with healthy controls. Exposure of dcSSc fibroblasts to WIN55,212-2 (10 μM) resulted in a substantial decrease in TGF-β and CTGF mRNA expression (Fig. 3A).

**Inhibition of IL-6 by WIN55,212-2**

IL-6 is a pleiotropic cytokine supposed to be involved in the initiation and promotion of fibrosis in dcSSc [19, 20].

In our experimental model, IL-6 was significantly up-regulated in dcSSc fibroblast supernatant compared with healthy controls (mean ± s.d.: 182.5 ± 29.09 ng/ml vs 58.5 ± 13.9 pg/ml; P < 0.001). WIN55,212-2 (1 and 10 μM) treatment of dcSSc fibroblasts resulted in a significant mean inhibition of IL-6 levels up to 93% at 10 μM (mean ± s.d.: 94.79 ± 9.2 pg/ml (1 μM), 12.12 ± 4.76 pg/ml (10 μM); P < 0.001). Similarly, results were obtained on healthy fibroblasts upon WIN55,212-2 treatment with a significant mean decrease in IL-6 supernatant levels up to 83% at 10 μM (P < 0.001). The pre-incubation of fibroblasts with the synthetic cannabinoid receptor antagonists AM281 (20 μM) and AM630 (20 μM) did not significantly reverse the reduction of IL-6 supernatant levels promoted by 10 μM WIN55,212-2 (Fig. 3B).

**Effect of WIN55,212-2 on myofibroblasts**

Myofibroblasts are specialized fibroblasts characterized by the cytoskeletal protein α-SMA. In pathologic fibrotic processes, they are considered to be resistant to apoptosis, representing one of the main sources of ECM and profibrotic cytokines [10, 21, 22]. In order to evaluate the effect of WIN55,212-2 on fibroblast transdifferentiation in a profibrotic phenotype, confluent skin fibroblast cultures from dcSSc and healthy individuals were grown to be analysed for α-SMA by western blotting, RT–PCR and immunocytochemistry. As expected, dcSSc fibroblasts showed an intense protein expression of α-SMA, strongly differentiating them from healthy fibroblasts, which proved negative by western blotting. A reduction in α-SMA protein expression was observed after the treatment with 10 μM WIN55,212-2, which was not reverted by the synthetic cannabinoid receptor antagonists AM281 (20 μM) and AM630 (20 μM) (Fig. 4A). Parallel findings were observed at mRNA levels, indicating an over-expression of α-SMA mRNA in dcSSc fibroblasts compared with healthy controls. Similarly, α-SMA mRNA levels were dramatically reduced after the treatment with 10 μM WIN55,212-2 (Fig. 4A).

A quantitative evaluation conducted on cytoketrifuged fibroblasts obtained from the same culture plates demonstrated an intense immunostaining of dcSSc fibroblasts for α-SMA (mean ± s.d.: 35.2 ± 13.2% compared with healthy controls, which did not show detectable α-SMA-positive cells (P < 0.001). A significant reduction in α-SMA-positive fibroblasts (up to 43%) was observable following 10 μM WIN55,212-2 incubation (mean ± s.d.: 20 ± 11.6%; P < 0.001) compared with baseline levels (Fig. 4B).

**Effect of WIN55,212-2 on apoptosis and cell viability**

One of the hallmarks of the fibroblast abnormalities found in fibrotic diseases, including dcSSc, is increased resistance to apoptosis. Annexin V and PI were used to identify the rate of primarily apoptotic and primarily necrotic fibroblasts, respectively, by flow cytometry. In basal conditions, Annexin V-positive cells were significantly less common in dcSSc cultures compared with healthy controls (mean ± s.d.: 19.96 ± 2.57% vs 52.35 ± 3.13%; P < 0.001). Exposure of dcSSc fibroblasts to 10 μM WIN55,212-2 led to a significant (2-fold) increase in the number of apoptotic cells compared with the DMSO basal control (mean ± s.d.: 44.7 ± 11.54%; P < 0.05). WIN55,212-2 did not significantly modify the number of apoptotic cells present in healthy cultures. Again, pre-incubation with the antagonists did not affect the rate of cells undergoing apoptosis (Fig. 5).

The number of primarily necrotic cells (PI+) did not differ between dcSSc and healthy fibroblasts in basal conditions. Similarly, we did not record any significant changes after 24 h of cannabinoid incubation (1, 10 μM) (data not shown). A Trypan blue exclusion test confirmed that WIN55,212-2 (1, 10 μM) does not significantly alter cell viability in healthy and dcSSc fibroblast cultures (data not shown).

An MTT assay excluded a metabolic impairment as 96 ± 4 and 85 ± 2% of fibroblasts were metabolically active at WIN55,212-2 concentrations of 1 and 10 μM, respectively. Similarly, neither of the synthetic cannabinoid receptor antagonists showed any significant toxicity at the concentrations used in this study (data not shown). Control experiments run with the same amounts of solvent (DMSO) did not significantly alter cell metabolism or viability (data not shown).

**Inhibition of ERK-1/2 phosphorylation by WIN55,212-2**

Recent evidence supports the notion that the MAPK-related kinase ERK-1/2 could be involved in dcSSc fibrogenesis [22–24]. On this basis, we sought to clarify whether WIN55,212-2 might modulate the activation of this signalling pathway by evaluating the activated phosphorylated form of ERK-1/2 (p-ERK-1/2).
In basal conditions, p-ERK-1/2 levels appeared to be mildly increased in dcSSc compared with healthy fibroblasts. Incubation of 10 μM WIN55,212-2 of both groups resulted in a decrease in p-ERK-1/2 protein expression. The results obtained were similar after pre-incubation with the CB1r/CB2r antagonists (Fig. 6).

Discussion

The first experiment described herein showed that sclerodema fibroblasts clearly overexpress CB1r and CB2r. Since these observations suggest the intriguing hypothesis of endocannabinoid system involvement in this model of pathologic fibrosis, this study was undertaken to investigate the in vitro effect of the synthetic cannabinoid receptor agonist WIN55,212-2 on dcSSc fibrogenesis.

In the present study, we demonstrated that WIN55,212-2 induced a robust inhibition of type I collagen as well as procollagen type I PIP supernatant levels, which is also associated with changes in skin score adding validity to this measure in dcSSc [25]. The reduction in collagen production was accompanied by a decrease in mRNA levels of TGF-β and its downstream mediator CTGF. These growth factors are clearly involved in the fibrotic process of dcSSc and, consistently with this concept, we found an over-expression of both cytokines in dcSSc samples suggesting that cannabinoids might counteract pathogenetically relevant features of ECM production. On this basis, the inhibitory effect of WIN55,212-2 on such profibrotic cytokines might account for the inhibition of fibroblast transdifferentiation, as shown by the reduction of α-SMA in dcSSc cultures and, therefore, in ECM neosynthesis. However, we could also hypothesize that the inhibition of the over-expressed IL-6 in dcSSc cultures might play a role in this anti-fibrogenic effect. This assumption is supported by the evidence indicating that IL-6 is critically involved in scleroderma pathogenesis [26] and able to induce fibroblast transdifferentiation in α-SMA-positive myofibroblast phenotype [27]. The synthetic cannabinoid receptor agonist WIN55,212-2 acted similarly on dcSSc and healthy fibroblasts, although the extent of the effects on scleroderma cells seems to be more pronounced.

It is noteworthy that WIN55,212-2 significantly increased the number of dcSSc fibroblasts undergoing apoptosis. These observations are consistent with the anti-proliferative effect of cannabinoids described elsewhere [28], also suggesting that the anti-fibrogenic effect of WIN55,212-2 should be addressed for the enhancement of dcSSc fibroblast apoptosis on one hand and for the reduction of ECM production on the other hand. Furthermore, we would like to comment that WIN55,212-2 did not demonstrate a specific toxicity at the concentrations used in this study (1, 10 μM), as no increase in the number of necrotic cells nor metabolic impairment were found after the cannabinoid treatment.

In this study, the anti-fibrogenic effect of WIN55,212-2 did not seem to be mediated by the ‘classic’ cannabinoid receptors CB1r and CB2r. In line with this observation is the notion that other transducing systems may be involved in cannabinoid activity, in addition to these well-defined receptor coupled signalling pathways, including inhibition of ERK signalling [29, 30]. The ERK pathway is also an attractive target for anti-fibrotic strategies, since it seems to be constitutively active in dcSSc [31] and its inhibition is thought to hold promise for the treatment of scleroderma [32–34], as well as other pathologic fibrotic conditions [10]. Additionally, since the ERK pathway had been suggested to play a crucial inhibitory role in cell survival and proliferation, its inhibition by WIN55,212-2 might be the upstream trigger of the enhanced apoptosis of fibroblasts.

Taken together, our results suggest that WIN55,212-2 is able to interfere with dcSSc fibroblasts at the multiple cellular levels involved in orchestrating fibrogenesis, either by inhibiting profibrotic chemokines, by disrupting the induction of α-SMA expression or by facilitating the activation of endocellular pathways leading to cell death. Although our findings are in accordance with a likely involvement of the endocannabinoid
FIG. 6. The cannabinoid receptor agonist WIN55,212-2 reduces ERK-1/2 activation. Expression of the phosphorylated ERK-1/2 MAP kinase in whole cell lysates, as determined by western blot. Incubation of dcSSc and healthy fibroblasts with WIN55,212-2 (10 μM) for 1 h resulted in a reduced expression of p-ERK-1/2. Cannabinoid antagonists AM281 (20 μM) and AM630 (20 μM) did not modify p-ERK-1/2 levels.

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