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Original article

Modulation of collagen type I, fibronectin and dermal fibroblast function and activity, in systemic sclerosis by the antioxidant epigallocatechin-3-gallate

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Abstract

Objectives. SSc is characterized by the overproduction of extracellular matrix (ECM) proteins, such as collagen and fibronectin, by activated fibroblasts, as well as oxidative stress. This study investigates the anti-fibrotic potential of the antioxidant epigallocatechin-3-gallate (EGCG) on activated dermal fibroblasts from SSc patients.

Methods. Dermal fibroblasts from a cell line (AG), healthy individuals (CON) and SSc patients were treated with EGCG, TGF-β, PDGF-BB or other antioxidants [antioxidants superoxide dismutase (SOD), catalase, *N*-acetyl-L-cysteine (NAC) and diphenyleneiodonium (DPI)]. Collagen type I, fibronectin, connective tissue growth factor (CTGF), α-smooth muscle actin and mitogen-activated protein (MAP) kinases were measured by ELISA and western blot. Fibroblast contractile forces were measured by collagen gel contraction. Reactive oxygen species (ROS) were assessed by dichlorofluorescein assay and nuclear factor κ beta (NF-κB) activity by DNA binding assay.

Results. EGCG (1–100 μM) dose-dependently decreased collagen type I secretion in culture medium after 24 h in AG fibroblasts. Collagen type I protein expression in cell lysates was also significantly reduced by 40% in EGCG-treated cells (40 μM). Furthermore, EGCG also down-regulated TGF-β-induced collagen type I, fibronectin and CTGF. Similarly, in CON fibroblasts EGCG decreased basal and stimulated collagen type I, fibronectin and CTGF after 24 h, while in SSc the effects of the antioxidant were apparent after 48 h. Fibroblast-mediated contraction of collagen gels was inhibited by EGCG as early as 1 h in AG fibroblasts, and in the CON and SSc fibroblasts. Additionally, EGCG also inhibited TGF-β-stimulated gel contraction similar to other antioxidants DPI and NAC, but not SOD or catalase. EGCG suppressed TGF-β-induced ROS production in all fibroblasts. Furthermore, EGCG inhibited TGF-β or PDGF-BB-induced phospho-extracellular signal-regulated kinase (ERK)1/2 MAP kinase and NF-κB activity in SSc fibroblasts.

Conclusion. The results suggest that the antioxidant, EGCG, can reduce ECM production, the fibrotic marker CTGF and inhibit contraction of dermal fibroblasts from SSc patients. Furthermore, EGCG was able to suppress intracellular ROS, ERK1/2 kinase signalling and NF-κB activity. Taken together, EGCG may be a possible candidate for therapeutic treatment aimed at reducing both oxidant stress and the fibrotic effects associated with SSc.

Key words: Epigallocatechin-3-gallate, Fibroblast, Collagen type I, Fibronectin, Systemic sclerosis, Scleroderma, Antioxidant, Reactive oxygen species, Extracellular matrix proteins.

BASIC SCIENCE

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Introduction

SSc (scleroderma) is a multi-system CTD characterized by increased deposition of extracellular matrix (ECM) proteins such as collagen and fibronectin. The main features of SSc include elevated levels of reactive oxygen species (ROS), vasculopathy, autoimmunity and progressive fibrosis of the skin and other internal organs [1]. The prevalent connective tissue fibrosis is especially prominent in the diffuse cutaneous form of the disease, where excessive connective tissue accumulation is due to ECM overproduction by fibroblasts and myofibroblasts, activated by soluble factors such as TGF-β [2, 3] and connective tissue growth factor (CTGF) [4]. The development of contractile forces, stress fibres and expression of α-smooth muscle actin (α-SMA) are also crucial for the differentiation of fibroblasts into myofibroblasts in SSc and the process coincides with an increase in cell proliferation [5, 6]. Fibroblast activation has been reported to be linked to the abnormal production of ROS by the increased expression of stimulatory PDGF receptor autoantibodies in SSc [7–9], and the differentiation of myofibroblasts induced by TGF-β [10, 11].

ROS that play a role in inflammatory diseases include superoxide anions ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and peroxynitrite ($ONOO^-$). In scleroderma, the sources of oxidative stress are complex and so far have been shown to result from ischaemic–reperfusion injury, dysregulated metabolism of the free radical nitric oxide ($NO^\bullet$) [12, 13] and increased levels of $O_2^{\bullet -}$ in fibroblasts and monocytes via the nicotinamide adenine dinucleotide phosphate-oxidase (NADPH) oxidase system [7, 9, 14]. Previously, we have shown clear evidence for oxidative stress in SSc by the enhanced oxidation of lipids and lipoproteins [15], subsequently confirmed by others [16], and also the presence of modified, nitrated proteins (a marker of $ONOO^-$ production) in SSc plasma and skin [13]. Furthermore, our group and others have recently studied the metabolism of $NO^\bullet$ in SSc, and the tight-skin 1 (Tsk-1/$^+$) mouse model, a natural genetic mutation predisposing to the development of a connective tissue-like disease and systemic fibrosis, where it appears to be profoundly disturbed [12, 13, 17]. Moreover, we have demonstrated a failure of correct regulation of collagen type I synthesis by $NO^\bullet$ in SSc dermal fibroblasts [18].

Indeed, in scleroderma what appears less certain is the potential counteractive role of the antioxidant defence system, the exact stage of the disease at which increases in reactive species occur and how both elements are linked to the main events of the disease. Therefore, in earlier work our laboratory and others have discovered that there is reduced antioxidant capacity in SSc since antioxidants in the plasma such as ascorbic acid, α-tocopherol, β-carotene and selenium were found at lower concentrations in patients than in controls [15, 19]. While we have demonstrated the beneficial effects of the antioxidant probucol in patients with RP secondary to SSc [20], recent clinical trials showed only limited success in the treatment of patients with the antioxidant α-tocopherol or ascorbic acid, which did not decrease urinary markers of oxidative stress such as F(2)-isoprostanes nor did this treatment improve microvascular perfusion after cold exposure [21, 22]. More promising has been the use of the potent antioxidant *N*-acetyl-L-cysteine (NAC), which has improved the vascular symptoms of RP in patients with SSc [23, 24]. The type of antioxidant used, phase of the disease and duration of use are likely to be the key factors in successful treatment therapies involving antioxidants in SSc, the associated RP and indeed other inflammatory diseases such as atherosclerosis [25].

Natural dietary antioxidants, such as polyphenols from green tea (*Camellia sinensis*) extracts, a popular beverage consumed worldwide, have recently attracted attention, particularly epigallocatechin-3-gallate (EGCG). The prominent antioxidant effects of EGCG derive from the phenol rings that act as electron traps to scavenge free radicals, inhibit the formation of ROS such as $O_2^{\bullet -}$, $ONOO^-$ and reduce oxidative stress [26]. Indeed, we have recently demonstrated that EGCG is an effective inhibitor of oxidative stress-induced protein tyrosine nitration during the isolation of blood platelets [27]. In addition to antioxidant properties, EGCG has been shown to possess anti-fibrotic, anti-cancer and anti-inflammatory activities. It regulates both TGF-β and PDGF-induced α1(I) collagen, fibronectin, α-SMA and proliferation in stellate, smooth muscle cells [28–33] and keloid fibroblasts [34]. Subsequently, studies have shown that EGCG may directly inhibit molecular targets and regulate multiple signal transduction pathways such as the mitogen-activated protein (MAP) kinases [extracellular signal-regulated kinase (ERK), Jun-N-terminal kinase (JNK) and p38] and the redox-sensitive transcription factor nuclear factor κ beta (NF-κB) [35–37]. Therefore, the protective inhibitory actions of the natural antioxidant EGCG, along with a history of consumption without adverse side effects, and higher potent antioxidant capability (than α-tocopherol or ascorbic acid) [38], make it a good candidate for therapeutic treatment aimed at reducing both oxidative stress and fibrogenesis in patients with SSc. Here, we explore the potential therapeutic role of EGCG on collagen type I and fibronectin biosynthesis, fibroblast function and activity, and other key parameters associated with fibrogenesis in SSc.

Materials and methods

Materials

All chemicals unless otherwise indicated were purchased from Sigma-Aldrich (Poole, UK) or Invitrogen (Paisley, UK).

Fibroblast culture

Scleroderma dermal fibroblasts (SSc) were obtained from punch biopsies (4 mm^3) taken from the affected (lesional) skin of the forearm of eight patients with early-onset dcSSc (within 2 years from the first non-RP manifestation of SSc) and fulfilled the ACR preliminary criteria for the classification of SSc. All study subjects were women and skin biopsy samples were taken from patients before treatment with immunosuppressive agents. Normal dermal fibroblasts (CON) were also taken from equivalent sites in eight healthy control individuals matched for age and sex. Informed consent was obtained from all study subjects and the study was approved by the Royal Free Hampstead local research ethics committee.

The biopsies were cut into 1-mm^3 pieces, and cultured as previously detailed [2, 3, 18]. In experiments, control and scleroderma fibroblasts were used between passages 2 and 6 to avoid changes in their original phenotype during subculture.

The human dermal fibroblast cell line AG1518 (AG) was also used in the current study. This cell line was established from fetal foreskin (Coriell Cell Repositories, NJ, USA) and not used after passage 20. Cells were cultured in identical medium to the control and scleroderma dermal fibroblasts.

Treatment of fibroblast cultures

Briefly, dermal fibroblasts were further cultured in 96-, 24- and 6-well plates, or 150-mm dishes to a confluent monolayer, and the medium replaced with serum-free (0% foetal calf serum) DMEM for 24 h. Cells were then incubated with the antioxidant (−)-EGCG (1–100 μM; Alexis Biochemicals, Nottingham, UK), with or without TGF-β (10 ng/ml; R&D Systems, Oxford, UK) or PDGF-BB (PDGF-BB, 20 ng/ml; R&D Systems) in serum-free DMEM for 0–48 h. Other antioxidants superoxide dismutase (SOD, 150 U/ml), catalase (300 U/ml), NAC (1–10 mM), the NADPH oxidase inhibitor diphenyleneiodonium (DPI, 1–20 μM; Alexis Biochemicals) were also used. As further controls, ROS stimulants H_2O_2 (500 μM) and 3-morpholinosydnonimine (SIN-1) (500 μM; Alexis Biochemicals) in serum-free DMEM were used as appropriate, or fibroblasts were pre-incubated for 1 h with the ERK kinase inhibitor U0126 (10 μM; Cell Signalling, Hertfordshire, UK) or EGCG before addition of TGF-β or PDGF-BB for 1 h where indicated.

MTT cell survival assay

After experimental incubation, cells were washed with PBS, and MTT [(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] was added at a final concentration of 0.5 mg/ml serum-free DMEM medium for 80 min. Briefly, MTT precipitate produced by viable cells was dissolved in dimethyl sulfoxide and measured at a wavelength of 550 nm by an automated microplate reader (Dynex Technologies, West Sussex, UK). Survival is defined in terms of optical density units. The relationship of the absorbance of MTT to cell number was verified in separate experiments in which 2.5×10^3 to 1.3×10^6 fibroblasts were added to different wells of a microtitre plate. There was a significant ($P < 0.001$) linear relationship between cell number and absorbance with a correlation coefficient of 0.96.

Extracellular collagen type I and fibronectin ELISA

After experimental treatment, culture medium was harvested from each well and assayed for collagen type I protein using a competitive ELISA assay as previously described [18]. In brief, 96-well microtitre plates were coated with 2 μg/ml human collagen type I (Southern Biotechnology Associates, Alabama, UK). Collagen in the sample culture medium was digested by mixing with pepsin solution (0.6 mg/ml) in acetic acid (150 mM) overnight at 4°C, and then neutralization solution (200 mM Tris, 150 mM NaCl). Wells were then incubated for 2 h at 37°C with either a human collagen type I standard (0.04–10 μg/ml) or sample culture medium, together with goat anti-collagen type I (1 : 600; Southern Biotechnology Associates) in blocking buffer. After washing, wells were incubated with rabbit anti-goat immunoglobulin G conjugated to alkaline phosphatase (1 : 2500) for 1.5 h and colour development initiated by addition of *p*-nitrophenyl phosphate (1 mg/ml) for 3 min at room temperature. The reaction was stopped by adding NaOH (6 M) and the absorbance read at 410 nm by an automated microplate reader (Dynex Technologies).

Fibronectin was measured in the fibroblast culture medium after experimental treatment by using a commercial sandwich ELISA (Takara, Otsu, Japan), according to the protocols provided by the manufacturer. In brief, 96-well microtitre plates were coated with mouse anti-human fibronectin (Takara) and immunoreactive wells revealed with a peroxidase-conjugated secondary antibody. The signal was developed with tetra-methylbenzidine chromogen solution (Takara) and stopped with sulphuric acid (1 N). The absorbance was read at 450 nm by an automated microplate reader (Dynex Technologies). The culture medium was used for total protein determination by the Coomassie Plus protein assay (Pierce, IL, USA).

Western blotting for intracellular collagen type I, fibronectin, CTGF, α-SMA and MAP kinases

After experimental treatment, confluent fibroblasts grown in 6-well plates were washed with PBS, homogenized in radioimmunoprecipitation assay lysis buffer containing protease inhibitors and western blot analysis performed as previously described [18]. Briefly, nitrocellulose membranes were incubated overnight with goat anti-collagen type I antibody (1 : 1000; Southern Biotechnology Associates), mouse anti-fibronectin (1 : 1000; Calbiochem, Nottingham, UK), goat anti-CTGF (1 : 1000; Santa Cruz, California, USA), mouse anti-α-SMA (1 : 1000; DAKO, Cambridge, UK), rabbit anti-ERK1/2 (1 : 500; Cell Signalling), rabbit anti-phospho-ERK1/2 (1 : 500; Cell Signalling), rabbit anti-JNK (1 : 500; Cell Signalling), rabbit anti-phospho-JNK (1 : 500; Cell Signalling), rabbit anti-p38 (1 : 500; Cell Signalling), rabbit anti-phospho-p38 (1 : 500; Cell Signalling) and goat anti-β-actin antibody (1 : 1000; Abcam, Cambridge, UK) or mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 1 : 1000; Abcam), as a loading control. The membranes were probed with the appropriate horseradish peroxidase-conjugated secondary antibody, detected by ECL (Amersham, Buckinghamshire, UK) and quantified using a camera imager (Biospectrum AC Imaging; UVP, Cambridge, UK). Arbitrary integrated density values were recorded.

Fibroblast-mediated collagen gel contraction

Fibroblasts were trypsinized and suspended in DMEM mixed with collagen solution as described previously [2, 3]. After polymerization of the collagen/cell suspension, gels were gently detached from the wells by adding 1 ml of DMEM in the presence or absence of the antioxidant

EGCG, TGF-β or other antioxidants for 24 h. For time-course experiments (0–24 h), fibroblasts were exposed to EGCG for the indicated time, and the medium then changed to DMEM for the remainder of the 24-h time period. Gel contraction was quantified according to loss of gel weight over a 24-h period.

Measurement of intracellular ROS

Measurement of intracellular ROS was determined by the procedure of Suzukawa *et al.* [39]. Briefly, fibroblasts were plated (5×10^6) into 96-well plates and pre-incubated with EGCG, DPI or NAC for 1 h. Cells were then incubated with 2-7-dichlorodihydrofluorescein diacetate (DCF-DA, 10 μM) with EGCG, DPI or NAC, with or without TGF-β for 30 min. DCF-DA is incorporated into cells and emits fluorescence when oxidized. As further controls, ROS stimulants H_2O_2 and SIN-1 were also used. The resultant DCF fluorescence intensity was measured using a microplate fluorimeter (Biolite F1; Labtech, East Sussex, UK) with excitation and emission at 485 and 538 nm, respectively, and arbitrary units of DCF fluorescence recorded.

DNA binding assay for NF-κB p65, CREB-1 and c-Fos

After experimental treatment for 4 h, confluent fibroblasts grown in 150-mm dishes were washed with PBS, scraped into 1.5-ml ice-cold PBS and pelleted at 500 *g* for 5 min at 4°C. The cell pellet was further separated into nuclear and cytoplasmic fractions using the NucBuster Protein Extraction Kit (Novagen, Nottingham, UK) according to the manufacturer's instructions. Protein concentrations of the nuclear extract were determined using the Coomassie Plus protein assay (Pierce). The nuclear extract was further analysed for DNA binding by using a commercial transcription factor profiling assay (BD Transfactor Profiling Assay; BD Biosciences, Oxford, UK) according to the manufacturer's instructions. Briefly, nuclear extracts (30 μg) were incubated in 96-well plates coated with the DNA consensus binding sequence for NF-κB p65, cyclic-AMP response element binding protein (CREB-1) or c-Fos. After further incubation with the appropriate primary antibody, immunoreactive wells were revealed with a peroxidase-conjugated secondary antibody. The signal was developed with tetramethylbenzidine chromogen solution (BD Biosciences) and stopped with sulphuric acid (1 N). The absorbance was read at 450 nm by an automated microplate reader (Dynex Technologies).

Data analysis

Data were expressed as mean (S.E.M.) where *n* was the number of independent experiments each of which was assayed in duplicate or triplicate. Data were analysed for statistical significance by unpaired Student's *t*-test or comparisons of multiple means were made by using one-way analysis of variance (ANOVA) followed by the Student's Newman–Keuls test. $P < 0.05$ (either by ANOVA or unpaired Student's *t*-test) was considered statistically significant.

Results

Effect of EGCG on fibroblast viability and markers of fibrogenesis

After 24 h, the effects of the antioxidant EGCG on the ECM proteins collagen type I and fibronectin were assessed by ELISA and western blotting. EGCG (1–100 μM) dose-dependently decreased collagen type I secretion in fibroblast medium after 24 h in AG fibroblasts. Maximal reduction of extracellular collagen production was 81% in EGCG-treated cells (80 μM) compared with untreated control fibroblasts ($P < 0.05$), while EGCG was effective at as low as 10 μM (Fig. 1A). There were no significant effects on total protein levels or cell viability (Fig. 1A). Western blot analysis for intracellular collagen type I protein expression in cell lysates was also significantly reduced by 40% in EGCG-treated cells (40 μM) compared with controls ($P < 0.05$), and in a dose-dependent manner (EGCG; 10–40 μM), with no significant effect on β-actin as an internal control (Fig. 1B). Time-course experiments showed that the effects of EGCG (40 μM) on collagen type I were apparent after a 24-h incubation (Fig. 1C). Furthermore, EGCG (40 μM) also down-regulated TGF-β-induced collagen type I (Fig. 1B), fibronectin and the fibrotic marker CTGF (Fig. 1D).

Similarly, EGCG (40 μM) significantly decreased basal collagen type I (Fig. 2A and B) and fibronectin (Fig. 2A) after 24 h in fibroblasts from healthy controls ($P < 0.05$), with no significant effects on total protein levels (Fig. 2A). Interestingly, while EGCG (40 μM) also significantly reduced collagen type I and fibronectin in medium from SSc fibroblasts after 24 h (Fig. 2A), intracellular collagen type I expression was decreased after 48 h and indicated that SSc-derived fibroblasts were more resistant to the effects of the antioxidant (Fig. 2B and C). Additionally, EGCG (40 μM) reduced TGF-β-induced collagen type I and CTGF (Fig. 2C). Overexpression of the contractile protein α-SMA has also been shown to be a marker of myofibroblasts. Surprisingly, basal α-SMA expression (as well as β-actin and GAPDH as internal controls) was unaffected by EGCG (40 μM) in both control and SSc fibroblasts (Fig. 2D), after 48 h (Fig. 2C), and also in AG fibroblasts (Fig. 1D).

Effect of EGCG and other antioxidants on fibroblast-mediated collagen gel contraction

Fibroblastic contractile forces were measured by collagen gel contraction where activated fibroblasts and myofibroblasts have been shown to exert greater contractile forces than resting fibroblasts (as measured indirectly by reduced gel weight and shrinkage of gel diameter). Fibroblast-mediated contraction of collagen gels was inhibited in a dose-dependent manner by EGCG (20–100 μM) after 24 h in AG fibroblasts (Fig. 3A). Additionally, EGCG (40 μM) significantly inhibited gel contraction 2-fold compared with controls ($P < 0.05$; Fig. 3A).

FIG. 1 Effect of EGCG on collagen type I and fibroblast viability in the dermal fibroblast cell line (AG). (**A**) Dermal fibroblasts were incubated with EGCG (1–100 μM), or medium control for 24 h. Culture supernatants were collected for ELISA measurement of extracellular collagen type I or total protein in the AG dermal fibroblast cell line ($n = 8$). Alternatively, cell survival was assessed using the MTT assay in AG fibroblasts ($n = 12$), as described in the 'Materials and methods' section. The inset phase-contrast picture shows the branched morphology of AG cell survival with EGCG (40 μM). (**B**) Dermal fibroblasts (AG) were incubated with or without EGCG (10–40 μM) or additionally TGF-β (10 ng/ml) for 24 h ($n = 4$); (**C**) EGCG (40 μM) for 0, 0.5, 1, 4, 7 or 24 h ($n = 3$); or (**D**) with or without EGCG (40 μM) or additionally TGF-β (10 ng/ml) for 24 h ($n = 3$–4). Whole-cell lysates (25 μg of protein) were prepared and immunoblotted onto a nitrocellulose membrane and antibodies against collagen type I, fibronectin, CTGF, α-SMA or β-actin (as an internal control) used. Data [mean (S.E.M.)] were expressed as percentage of the medium control, or fold increase over control from the indicated number of independent experiments (each performed in triplicate). $P < 0.05$ was considered statistically significant *compared with control, **compared with TGF-β control.

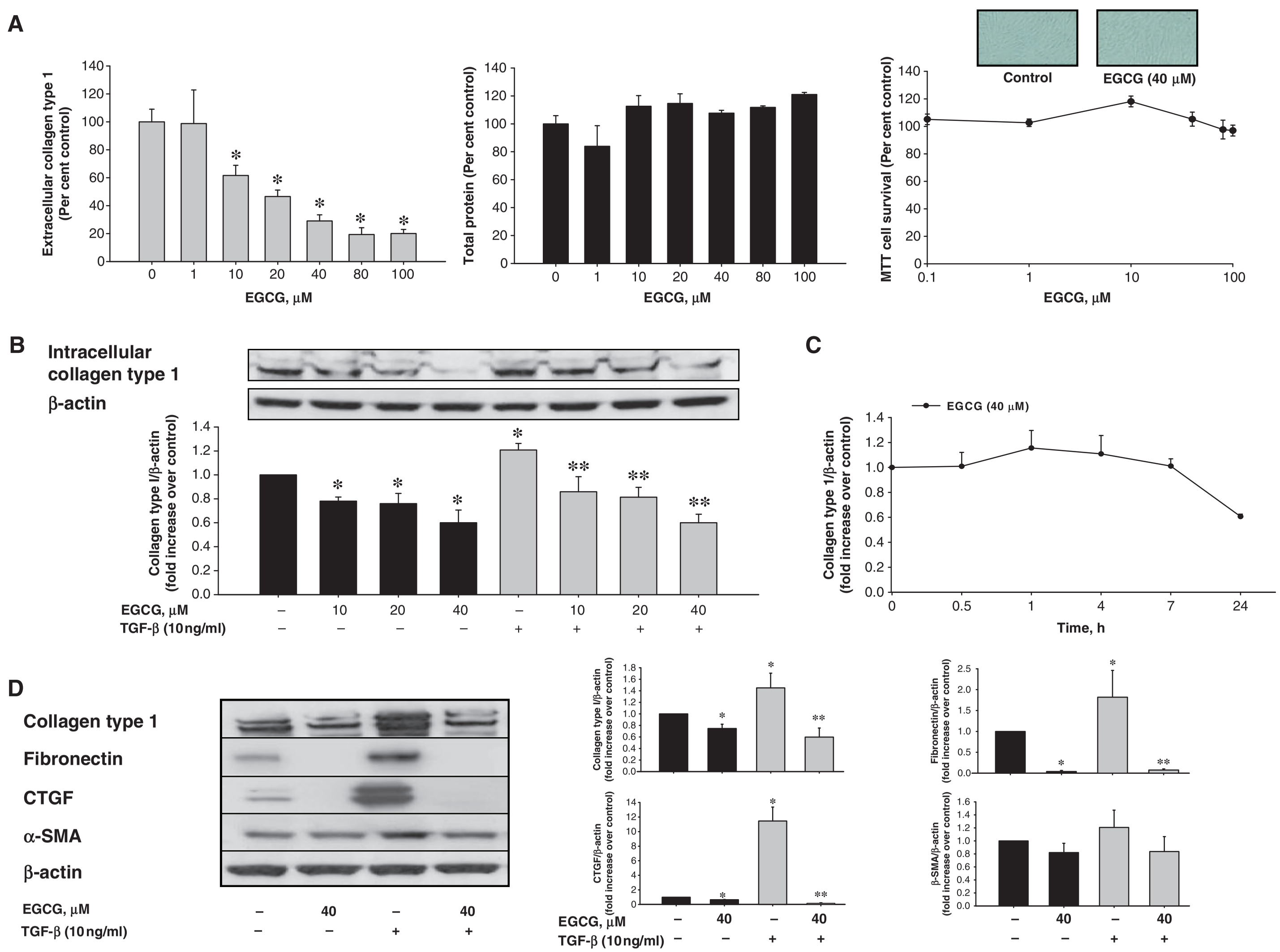


Fig. 2 Effect of EGCG on markers of fibrogenesis in dermal fibroblasts from patients (SSc) and healthy controls (CON). (**A**) Culture supernatants were collected for ELISA measurement of extracellular collagen type I, fibronectin or total protein in CON: dermal fibroblasts from healthy controls ($n = 4$); and SSc: affected dermal fibroblasts from patients with SSc ($n = 5$) incubated with or without EGCG (40 μM). (**B**) Dermal fibroblasts from four healthy controls (C1–C4); and five SSc patients (S1–S5) were incubated with or without EGCG (40 μM) for 24 h. (**C**) Alternatively, SSc dermal fibroblasts ($n = 3$) were incubated with or without EGCG or additionally TGF-β (10 ng/ml) for 48 h. Whole-cell lysates (25 μg of protein) were prepared and immunoblotted onto a nitrocellulose membrane and antibodies against collagen type I, CTGF, α-SMA or β-actin (as an internal control) used. (**D**) Dermal fibroblasts from four healthy controls (C1–C4); and five SSc patients (S1–S5) were incubated with or without EGCG (40 μM) for 24 h. Whole-cell lysates were immunoblotted to membrane and antibodies against α-SMA and GAPDH (as internal control) used. Data [mean (s.e.m.)] were expressed as fold increase over control or percentage of medium control. $P < 0.05$ was considered statistically significant *compared with control, **compared with TGF-β control.

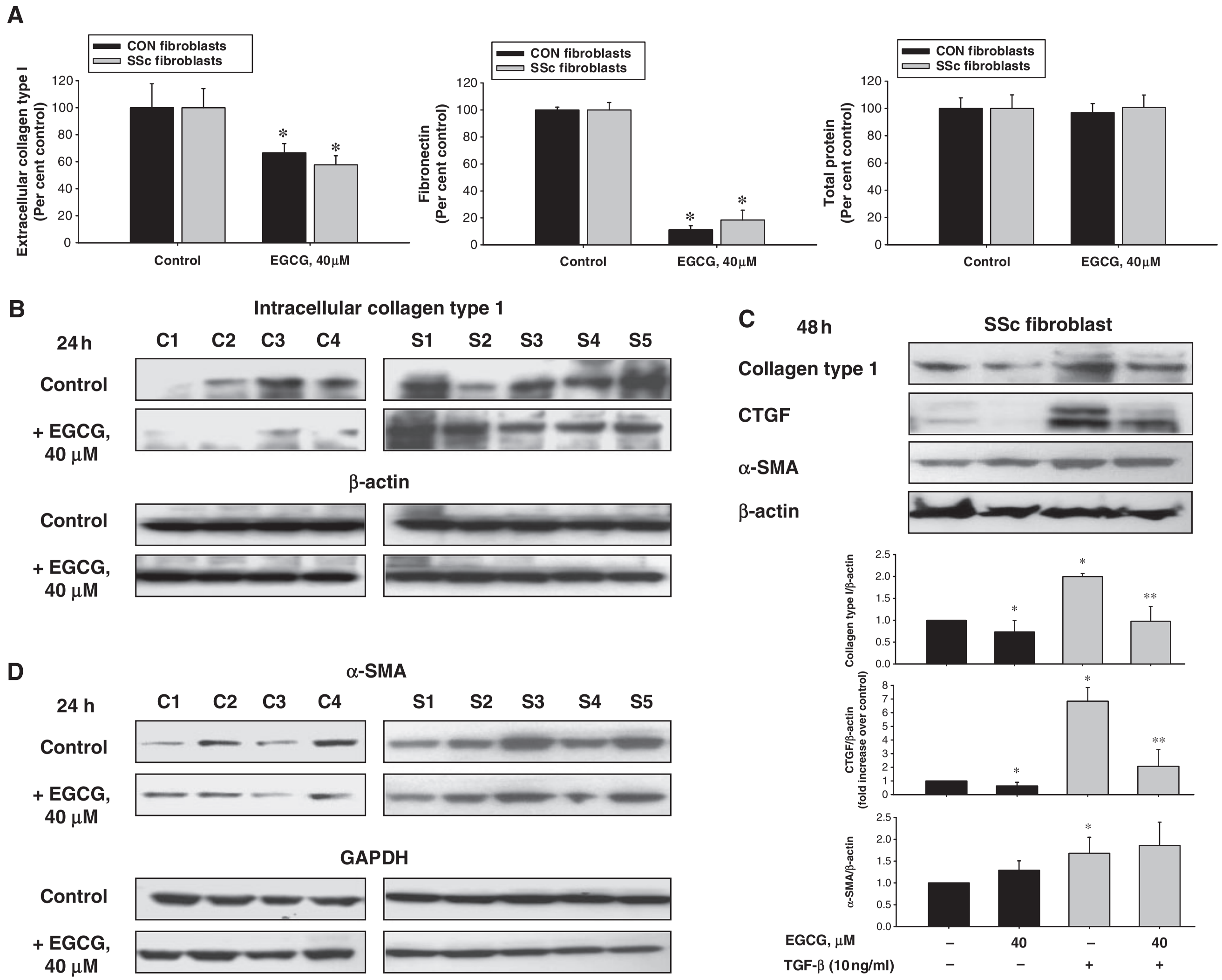
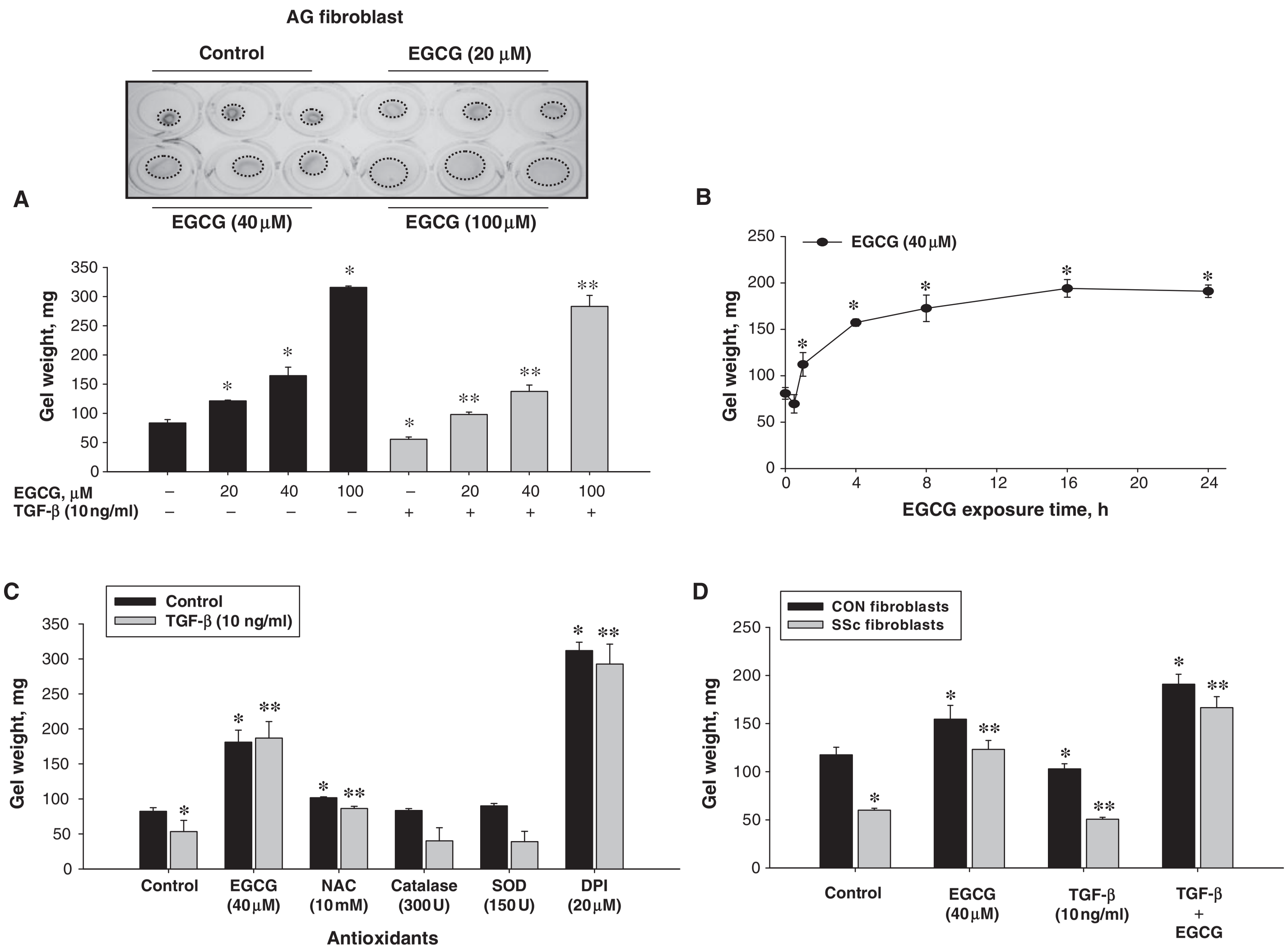


FIG. 3 Effect of EGCG, and other antioxidants, on fibroblast-mediated collagen gel contraction in dermal fibroblasts from patients (SSc), healthy controls (CON) and the dermal fibroblast cell line (AG). (**A**) Dermal fibroblasts (AG) were seeded into a 3D collagen gel matrix as described in the 'Materials and methods' section, and incubated with or without, EGCG (20–100 μM) or additionally TGF-β (10 ng/ml) ($n = 3$) for 24 h, or (**B**) incubated with EGCG (40 μM) for 0, 0.5, 1, 4, 8, 16 or 24 h ($n = 3$). (**C**) Dermal fibroblasts (AG) were seeded into a 3D collagen gel and incubated with or without TGF-β (10 ng/ml), or additionally EGCG (40 μM), NAC, (10 mM), catalase (300 U/ml), SOD (150 U/ml) or the NADPH oxidase inhibitor DPI (20 μM) ($n = 3$) for 24 h; (**D**) dermal fibroblasts from CON healthy controls ($n = 4$), and SSc patients ($n = 4$) were incubated with or without TGF-β (10 ng/ml) or additionally EGCG (40 μM). Data [mean (S.E.M.)] were expressed as gel weight (mg) from the indicated number of independent experiments (each performed in triplicate). $P < 0.05$ was considered statistically significant *compared with control, **compared with TGF-β control or SSc control.



Time-course experiments showed that the gradual inhibitory effect of exposure to EGCG (40 μM) on fibroblast gel contraction was as early as 1 h (1.4-fold *vs* controls) and reached a plateau after 16 h (>2-fold *vs* controls; Fig. 3B). EGCG (20–100 μM) also inhibited TGF-β-stimulated fibroblast gel contraction (Fig. 3A and C), and the effect was similar to that of the NADPH oxidase inhibitor DPI (20 μM), but not SOD (150 U/ml) or catalase (300 U/ml). The antioxidant NAC (10 mM) significantly inhibited TGF-β-stimulated fibroblast gel contraction but to a much lower extent than EGCG or DPI (Fig. 3C).

SSc fibroblasts showed greater collagen gel-mediated contraction compared with control fibroblasts [60.1 (2.0) mg in SSc *vs* 117.5 (7.9) mg in control] (Fig. 3D). As in AG fibroblasts, EGCG (40 μM) also inhibited both basal and TGF-β-induced contraction of collagen gels by fibroblasts from both healthy individuals and SSc patients (Fig. 3D).

Effect of EGCG, NAC and DPI on the generation of intracellular ROS

In order to assess the effectiveness of EGCG as an antioxidant on dermal fibroblasts, intracellular ROS were induced and measured by DCF-2A oxidation. After pre-incubation of EGCG (40–100 μM) for 1 h, ROS were significantly reduced in TGF-β, H_2O_2 or SIN-1-stimulated fibroblasts (Fig. 4A). Similarly, both the antioxidants NAC (1–20 mM) and DPI (1–20 μM) also reduced TGF-β-stimulated induction of ROS (Fig. 4B and C, respectively).

ROS have also been shown to be increased in SSc fibroblasts and proposed to play a role in fibroblast activation [7, 9, 14]. As was the case for the AG fibroblasts, EGCG (40 and 100 μM) reduced TGF-β-stimulated ROS in control fibroblasts from healthy individuals (Fig. 4D). Interestingly, compared with control fibroblasts, SSc-derived fibroblasts were again more resistant to the effects of the antioxidant and EGCG reduced TGF-β-stimulated ROS only at a higher concentration of 100 μM (Fig. 4D).

Effect of EGCG on MAP kinase signalling

The intracellular MAP kinase signalling pathways (ERK1/2, JNK and p38) have been shown to play a central role in many disease systems, as well as in SSc [9, 40]. Also, MAP kinases have been shown to be a target for EGCG regulation in various cell types [35–37]. The effect of EGCG on MAP kinases was further analysed by western blot in dermal fibroblasts. After pre-incubation of EGCG (40 μM) for only 1 h, both basal and TGF-β-inducted phospho-ERK1/2 were reduced in AG fibroblasts (Fig. 5A). The inhibitory effects of EGCG (40 μM) on phospho-ERK1/2 also paralleled the effect of the ERK1/2 inhibitor U0126 (10 μM) after 2 h (Fig. 5A). Furthermore, the reduction in phospho-ERK1/2 by EGCG (40 μM) was still apparent after 24 h, as was the case for phospho-JNK, but not phospho-p38 MAP kinase (Fig. 5A). EGCG (10–40 μM) had no effect on the total forms of ERK1/2, JNK and p38 MAP kinases as an internal control (Fig. 5A).

Similarly, EGCG (40 μM) decreased basal phospho-ERK1/2 after 24 h in both control fibroblasts from healthy individuals and SSc fibroblasts from patients (Fig. 5B). Moreover, in SSc fibroblasts, after only 1 h, EGCG (40 μM) was also able to reduce PDGF-BB-stimulated phospho-ERK1/2 (Fig. 5C).

Effect of EGCG on NF-κB activity

The activity of the transcription factor NF-κB has been reported to be regulated by the MAP kinases ERK, JNK and p38 through different mechanisms, and redox-sensitive NF-κB has been shown to be a target for EGCG regulation in various cell types [35–37]. The effect of EGCG on NF-κB p65 activity was further analysed by DNA binding in dermal fibroblasts. EGCG (40 μM) significantly decreased NF-κB p65 DNA binding activity in both control and SSc fibroblasts, while other transcription factors such as c-Fos and CREB-1 were not significantly affected (Fig. 5D).

Discussion

The present study demonstrates for the first time that the natural green tea antioxidant, EGCG, reduces ECM proteins such as collagen type I and fibronectin, in human dermal fibroblasts from normal healthy individuals, SSc patient biopsies and in a dermal fibroblast cell line. SSc fibroblasts, however, were more resistant to the effects of the antioxidant. In addition, EGCG also inhibited the fibrotic marker CTGF and fibroblastic contractile forces, without affecting cell viability. Furthermore, EGCG was able to counteract induced intracellular ROS, and suppress TGF-β- or PDGF-BB-stimulated ERK1/2 and JNK signalling pathways, as well as NF-κB activity, whereas other transcription factors such as CREB-1 and c-Fos were not significantly affected. Taken together, the results suggest that EGCG may be a possible candidate for therapeutic treatment aimed at reducing both oxidant stress and the fibrotic effects associated with the SSc disease, which is characterized by excessive collagen production and deposition by activated fibroblasts in both internal organs and skin.

There is considerable evidence implicating EGCG in the modulation of the ECM in several different types of cells. EGCG has been shown to possess anti-fibrotic, anti-cancer and anti-inflammatory activities regulating both TGF-β and PDGF-induced α1(I) collagen, fibronectin, α-SMA and proliferation in activated human and rat hepatic stellate cells [28–30], rat pancreatic stellate cells [31, 32], rat aortic smooth muscle cells [33] and human keloid fibroblasts [34]. Our data on the inhibitory effects of EGCG on collagen type I and fibronectin secretion, and no observed toxic effects on cell viability in human dermal fibroblasts from control subjects, SSc patients and a dermal fibroblast cell line, are consistent with previous studies. Additionally, we have shown that EGCG can effectively reduce expression of the fibrotic marker CTGF, which was previously demonstrated in our study to be a key factor in the enhanced activation of fibroblasts by the growth factor TGF-β in SSc [2–4]. Similarly, in SSc

FIG. 4 Effect of EGCG, NAC and DPI on the generation of intracellular ROS in dermal fibroblasts from patients (SSc), healthy controls (CON) and the dermal fibroblast cell line AG. Intracellular ROS were assessed using DCF-2A fluorescence as described in the 'Materials and methods' section in (**A**), fibroblasts from a dermal cell line (AG) pre-incubated with or without EGCG (20–100 μM) for 1 h, and further stimulated with or without TGF-β (10 ng/ml), H_2O_2 (500 μM), SIN-1 (500 μM) or additionally EGCG (20–100 μM) for 30 min ($n = 4$). Or in (**B**), pre-incubated with or without NAC (1–10 mM) or additionally TGF-β (10 ng/ml) ($n = 4$), or in (**C**), pre-incubated with or without DPI (1–20 μM) or additionally TGF-β (10 ng/ml) ($n = 4$). (**D**) Intracellular ROS were assessed in fibroblasts from CON healthy controls ($n = 4$), and SSc patients ($n = 4$) pre-incubated with or without EGCG (20–100 μM) for 1 h, and further stimulated with or without TGF-β (10 ng/ml), or additionally EGCG (20–100 μM) for 30 min. Data [mean (S.E.M.)] were expressed as relative light units (RLUs) from the indicated number of independent experiments (each performed in triplicate). $P < 0.05$ was considered statistically significant *compared with control, **compared with TGF-β, H_2O_2, SIN-1 or SSc control.

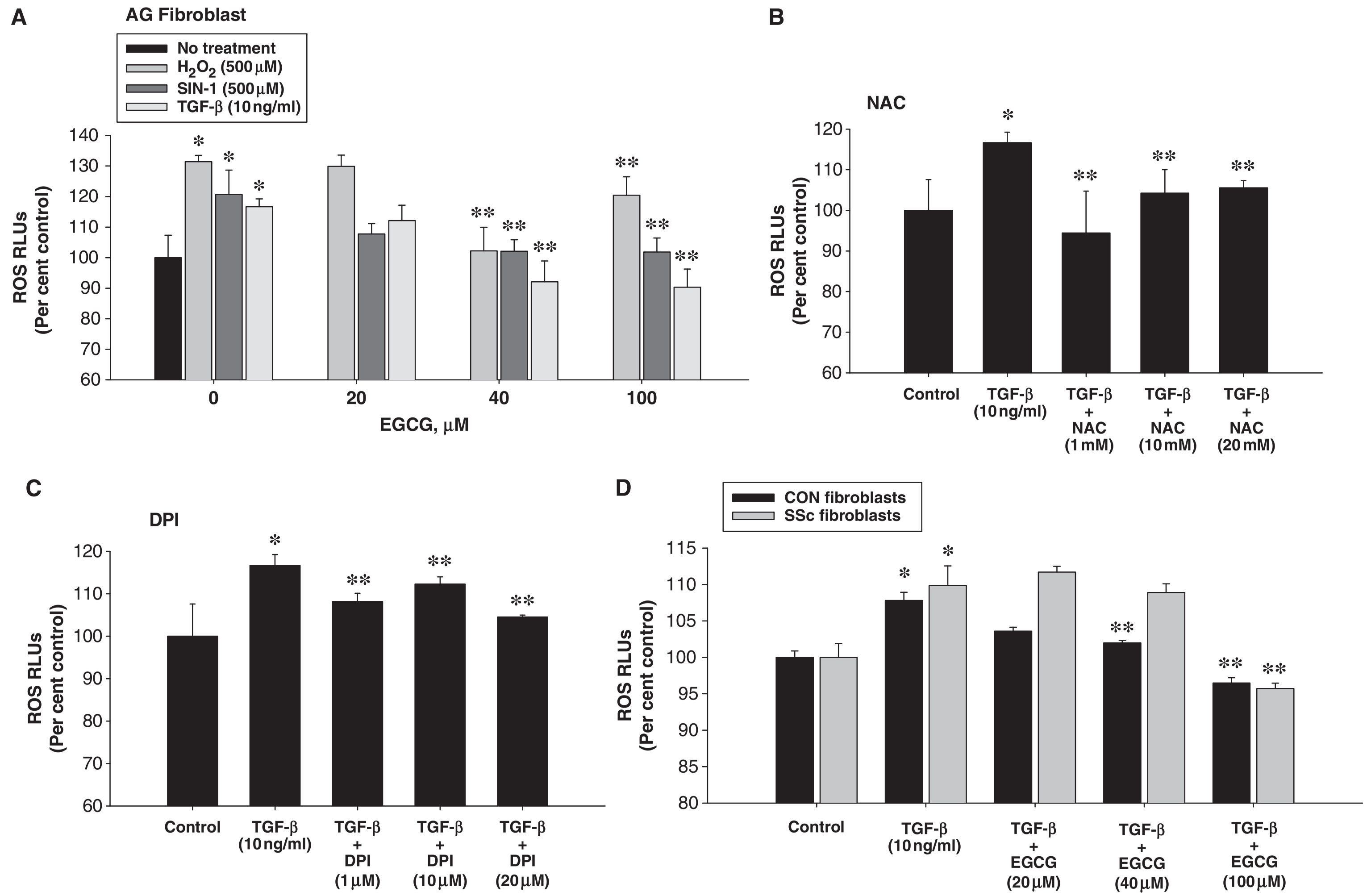
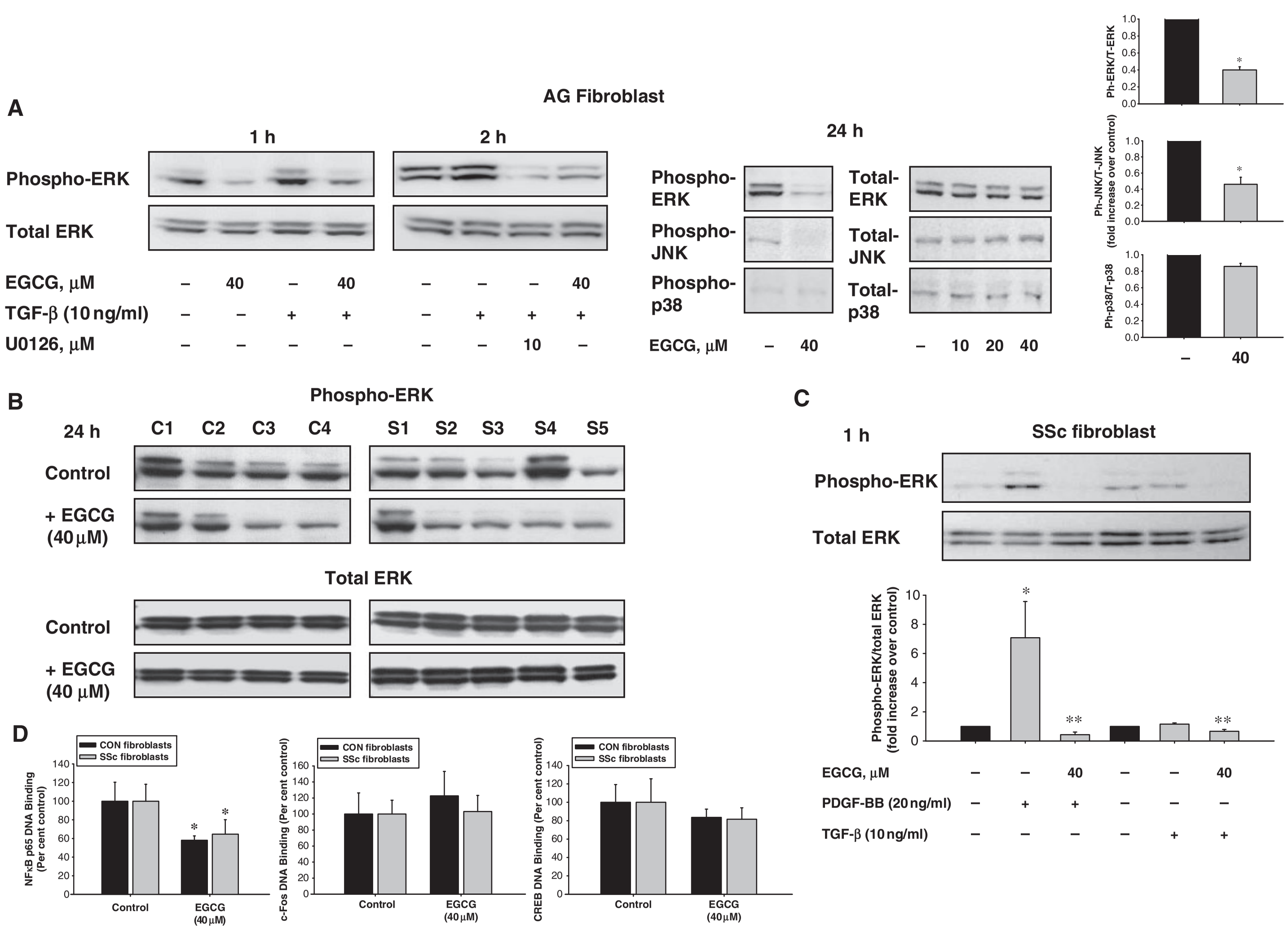


FIG. 5 Effect of EGCG on MAP kinase signalling pathways and NF-κB p65 activity in dermal fibroblasts from patients (SSc), healthy controls (CON) and the dermal fibroblast cell line (AG). Dermal fibroblasts (AG) were (**A**) pre-incubated with or without EGCG (40 μM) or the ERK kinase inhibitor U0126 (10 μM) for 1 h, and further stimulated with or without TGF-β (10 ng/ml), or additionally EGCG (40 μM) or U0126 (10 μM), for 1 and 2 h ($n=3$); or incubated with or without EGCG (10–40 μM) for 24 h ($n=3$); Whole-cell lysates (25 μg of protein) were prepared and immunoblotted onto a nitrocellulose membrane and antibodies against the MAP kinases phospho-ERK1/2, total ERK1/2, phospho-JNK, total JNK, phospho-p38 or total p38 used as shown in representative blots. In (**B**), dermal fibroblasts from four healthy controls (C1–C4); and five SSc patients (S1–S5) were incubated with or without EGCG (40 μM) for 24 h. (**C**) Alternatively, SSc dermal fibroblasts ($n=3$) were pre-incubated with or without EGCG (40 μM) for 1 h, and further stimulated with or without PDGF-BB (20 ng/ml), TGF-β (10 ng/ml) or additionally EGCG (40 μM) for 1 h. (**D**) Dermal fibroblasts were incubated with or without EGCG (40 μM) for 4 h in CON fibroblasts from healthy individuals ($n=3$); and SSc fibroblasts from patients ($n=3$). Nuclear extracts (30 μg) were prepared and used for NF-κB p65, c-Fos or CREB-1 DNA binding activity as described in the ‘Materials and methods’ section. Data [mean (S.E.M.)] were expressed as fold increase over control or per cent control from the indicated number of independent experiments (each performed in duplicate). $P < 0.05$ was considered statistically significant *compared with control, **compared with PDGF-BB or TGF-β control.



disease other investigators have demonstrated the potential anti-fibrotic effects of other antioxidants, with NAC also reported to decrease collagen type I synthesis in dermal fibroblasts from SSc patients [14].

These findings, to our knowledge, are the first to report the effects of EGCG on fibroblast-mediated contraction of collagen gels by dermal fibroblasts from SSc patients. The increased number and enhanced contractile forces of activated fibroblasts and myofibroblasts in SSc are a key feature of the disease and greatly contribute to the characteristic ECM overproduction [5, 6]. Our data that EGCG can effectively inhibit fibroblast-mediated gel contraction, agree with an earlier study that reported the inhibitory effect of EGCG on a human dermal fibroblast cell line [41]. Furthermore, our findings show that the effects of EGCG on fibroblast-mediated collagen gel contraction were similar to those of other antioxidants, such as NAC and the NAPDH oxidase inhibitor DPI, but not SOD or catalase. The results suggest that the inhibitory effect of EGCG on fibroblast-mediated gel contraction may be due to inhibition of ROS. These findings highlight that the type of antioxidant used, which each target different antioxidant defence mechanisms, and their potency, should be the key considerations in deciding potential therapeutic treatments as has become clear for other diseases such as atherosclerosis [25]. The prominent antioxidant effects of EGCG derive from the phenol rings, which can act as electron traps to scavenge free radicals, inhibit the formation of ROS such as $O_2^{\bullet-}$, $ONOO^-$ and reduce oxidative stress [26]. Indeed, EGCG has been reported to possess a higher potent antioxidant capability than other dietary antioxidants such as α-tocopherol or ascorbic acid [38], and has a history of safe beverage consumption without adverse side effects. Unsurprisingly, while the use of NAC has shown promising results in clinical trials to improve the vascular symptoms of RP in patients with SSc [23, 24], the use of α-tocopherol or ascorbic acid did not decrease urinary markers of oxidative stress such as F(2)-isoprostanes, nor improve microvascular perfusion after cold exposure [21, 22].

ROS have been reported to be linked to enhanced fibroblast activation by the increased expression of stimulatory PDGF receptor autoantibodies in SSc fibroblasts [7–9], and TGF-β-induced differentiation of myofibroblasts [10, 11]. In particular, both these studies have further highlighted the role of the NADPH oxidase subunits located within the membrane of the cell. Previously, however, we were the first to show clear evidence for oxidative stress in the disease SSc by the enhanced oxidation of lipids and lipoproteins [15], subsequently confirmed by others [16], and also the presence of modified, nitrated proteins (a marker of $ONOO^-$ production) in SSc plasma and skin [13]. Our data that EGCG can counteract TGF-β-induced ROS, similar to both NAC and the NADPH oxidase inhibitor DPI, in human dermal fibroblasts from healthy controls, SSc patients and in a dermal fibroblast cell line, indicate its potential effectiveness as an antioxidant to reduce oxidant stress in the disease scleroderma. In previous studies, we have highlighted the potential effectiveness of EGCG as an antioxidant at concentrations as low as 2 μM in the inhibition of oxidative stress-induced protein tyrosine nitration during the isolation of platelets [27]. It is also interesting to note that other studies have shown data that EGCG can beneficially inhibit ROS through attenuating NADPH oxidase expression [42, 43].

EGCG, in addition to a role as a potent and effective antioxidant, has also been shown by numerous studies to directly inhibit molecular targets and regulate multiple signal transduction pathways such as MAP kinases (ERK, JNK and p38) and also the redox-sensitive transcription factor NF-κB [35–37]. Our findings showed that EGCG could suppress phosphorylation of both the ERK and JNK signalling pathways and also decrease NF-κB activity in human dermal fibroblasts from healthy controls and SSc patients, which have been reported to be increased in the disease SSc [9, 40]. Moreover, several studies have highlighted other potential mechanisms by which EGCG could exert anti-fibrotic effects such as blocking the binding of PDGF-BB to its receptor, or inhibiting PDGF receptor expression by blocking the activation of NF-κB and AP-1 [29, 44, 45]. Further studies are required to determine whether EGCG could also block binding to the TGF-β receptor, reduce TGF-β receptor expression or even affect collagen degradation by regulation of MMPs [30]. Furthermore, EGCG could regulate phosphorylation of contractile proteins, such as myosin light chain kinase, which has been shown to be important in fibroblast-mediated gel contraction [46, 47].

Perhaps the most challenging and unique aspect of the potential therapeutic use of EGCG will be issues relating to the bioavailability of the drug in the bloodstream after ingestion. Typically, in *in vivo* studies human plasma EGCG levels (after drinking the equivalent of ∼2–3 cups of green tea or ingestion of 2 mg/kg pure form of EGCG) is usually between 0.3 and 4 μM [37, 48], although less is known about intracellular concentrations. The bioavailability is important since most of the published studies in cell culture systems have demonstrated the effectiveness of EGCG at much higher concentrations of between 10 and 100 μM [28–34, 37, 48]. Our present findings additionally have shown that SSc fibroblasts were more resistant to the effects of the antioxidant (particularly on reducing collagen type I and ROS), and would require either a higher concentration of EGCG or longer treatment time compared with normal fibroblasts. An effective solution may be to increase the bioavailability of EGCG to the skin by topical or intra-lesional administration, which would enable higher doses to be directly applied. Hence, topical administration of EGCG has successfully been shown to inhibit ultraviolet radiation-induced oxidative stress and tumorigenesis in human and animal skin models [49, 50]. Furthermore, in human keloid fibroblasts, studies have shown that intra-lesional injections of EGCG (∼62 mg/kg) inhibited keloid development without inducing systemic health damage, whereas other investigators observed that a high dose of EGCG (200 mg/kg) caused no toxicity [34]. Further *in vivo* studies are required to determine the optimal dosage, duration of use and treatment methods to effectively administer EGCG as a therapeutic agent to the skin.

In conclusion, the present study demonstrates for the first time that the natural green tea antioxidant, EGCG, can reduce both collagen type I and fibronectin production in fibroblasts derived from SSc patients, control subjects and a dermal fibroblast cell line. SSc fibroblasts, however, were more resistant to the effects of the antioxidant. In addition, EGCG also inhibited the fibrotic marker CTGF and fibroblastic contractile forces, without affecting cell viability. Furthermore, EGCG was able to suppress intracellular ROS, ERK1/2 and JNK signalling pathways, as well as NF-κB activity. Taken together, EGCG, which has a long history of safe beverage consumption and higher potent antioxidant capability than α-tocopherol or ascorbic acid [38], may be a possible candidate for therapeutic treatment aimed at reducing both oxidant stress and the fibrotic effects associated with the disease SSc.

Rheumatology key messages

- The findings suggest that the antioxidant EGCG can reduce ECM production and markers of fibrosis in scleroderma fibroblasts.
- EGCG is a possible candidate for therapeutic treatment aimed at reducing both oxidant stress and fibrosis in scleroderma.

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