Baicalein Alleviates Fibrosis and Inflammation in Scleroderma Through Regulating B Cell Abnormalities

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Keywords: Baicalein, Scleroderma, B cell, Autoimmunity, Fibrosis, Inflammation

Posted Date: November 23rd, 2021

DOI: <https://doi.org/10.21203/rs.3.rs-1082925/v1>

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Abstract

Background

Scleroderma (Systemic sclerosis, SSc) is an autoimmune disorder characterized by multisystem extensive fibrosis, vascular changes and immunological dysregulation. B cell abnormalities play an essential role in the fibrotic pathogenesis of scleroderma by promoting autoantibodies and cytokines release as well as modulating the inflammatory processes. Baicalein, a phenolic flavonoid derived from the Chinese herb *Scutellaria baicalensis* Georgi, has been used in the treatment of the pathological processes of various fibrotic and inflammatory diseases. Here, we aimed to investigate the effect of baicalein on the major pathological characteristics of SSc both *in vitro* and *in vivo*, including fibrosis, B cell abnormalities and inflammation.

Methods

The effect of baicalein on collagen accumulation and expression of fibrogenic markers in human dermal fibroblasts were analyzed. The antifibrotic features of baicalein and its mechanisms were investigated in the bleomycin (BLM)-induced dermal fibrosis mice model by histologic examination, hydroxyproline assay, enzyme-linked immunosorbent assay and flow cytometry.

Results

5-120 μ M baicalein significantly abrogated total collagen deposition, decreased soluble collagen secretion, and inhibited the expression of various fibrogenesis molecules, including collagen(COL)1A1, COL1A2, COL3A1, connective tissue growth factor (CTGF), fibronectin, transforming growth factor β 1 (TGF- β 1) and alpha-smooth muscle actin (α -SMA) in both TGF β 1- and PDGF-induced human CCC-ESF-1 dermal fibroblasts. In BLM-induced dermal fibrosis mice model, 25-100 mg/kg baicalein markedly attenuated dermal thickness and collagen accumulation in dose-dependent manners. Baicalein reduced the proportion of B220⁺ B cells, while increased the percentage of CD27⁺ memory B cells in the spleen of BLM-induced mice. Consistent with reversing B cell abnormalities, baicalein treatment potently attenuated serum levels of cytokines(IL-1 β , IL-2, IL-4, IL6, IL-17A, TNF- α), chemokines (MCP-1, MIP-1 β) and autoantibodies (anti-Scl-70, anti-PM-Scl, anti-centromeres and anti-dsDNA).

Conclusions

Baicalein inhibits TGF- β 1 and PDGF- mediated ECM accumulation in human dermal fibroblasts and alleviates the development of experimental dermal fibrosis by reversing B cell abnormalities, reducing autoantibody production and ameliorating inflammation. Baicalein may have the potential to be further developed as a therapeutic candidate against SSc.

Background

Scleroderma (systemic sclerosis, SSc) is an autoimmune disorder characterized by extensive fibrosis, vascular and inflammatory alterations, release of autoantibodies against nuclear/cellular antigens. SSc involves skin and various internal organs, including lung, heart, gastrointestinal tract, and kidney [1]. It remains an incurable disease with mean disease duration of 11.7 years [2] and has very high case-specific mortality among the autoimmune diseases, with 55% of patients dying as a direct consequence of the disease [3]. Despite evidence of improved overall survival and treatment options, the scant effective disease-modifying treatment is a serious clinical challenge for SSc. Therefore, identification of promising therapeutic agents toward SSc is urgently needed [4].

Baicalein is a primary phenolic flavonoid derived from the Chinese herb *Scutellaria baicalensis* Georgi. It has been widely demonstrated multiple functions, including anticancer [5], anti-inflammatory, antioxidant[6], anxiolytic and anti-infection effects[7]. Previous studies have reported that it has been increasingly used against pulmonary, hepatic, cardiac and renal fibrosis as potential therapeutic agents [8–11]. The suppression of fibrosis by baicalein was partially attributed to the attenuation of collagen and ECM production[12–15], inhibition of cell proliferation and induction of apoptosis of fibroblasts[16], inhibition of myofibroblast differentiation[8] and downregulation of fibrogenic markers expression[9, 13, 14]. Collectively, baicalein modulates pro-fibrotic and inflammatory pathways, then results in the inhibition of fibrosis in different organs. However, the effect of baicalein against SSc and the exact molecular mechanisms responsible for its effect have not been elucidated.

SSc is dominated by immunological abnormality, which involves infiltration and activation of multiple immune cells followed by autoimmunity, secreting pro-inflammatory cytokines and releasing chemokines [17]. Continuously activated immune cells cause endothelial cells activation and upregulation of adhesion molecules to recruit immune cells [18]. Furthermore, immune cells activation initiates the expression of profibrotic markers, activate fibroblasts and transform myofibroblasts[17]. B cells are a heterogeneous immune cell population with different subsets which play important roles in fibrotic pathogenesis of SSc as highlighted by the production of autoantibodies and cytokines, elevated serum IgG levels and B-cell infiltration in the dermis[19]. Mounting evidences showed that the percentage and absolute amount of CD19⁺ B cells increased in SSc, with higher expression of CD19[20, 21]. Decreased memory B cells, especially pre-switched memory B cells in SSc patients has been consistently described in recent studies[19, 22, 23]. Some previous data have confirmed these results and reported augmented spontaneous apoptosis in CD27⁺ memory B cells, upregulation of apoptotic regulators CD95 (Fas) and downregulation of antiapoptotic regulators Bcl-2 in memory B-cell subsets, which add evidences for the continuous loss of memory B cells in SSc[19, 24]. Therefore, B cells have potential as an attractive therapeutic target for SSc that be exploited to result in decreased production of autoantibodies by B cell signaling and deactivated fibroblasts by pro-fibrotic pathways[25]. Recent studies have shown the relationship between baicalein and immune cells for its anti-fibrosis, such as downregulation of pro-inflammatory cytokines and chemokines (IL-1 β , IL-6, TNF- α and MCP-1)[15], inhibition of the infiltration of inflammatory cells (macrophages and lymphocytes) [26]. Previous observations led us to postulate that

baicalein might modulate B cell abnormalities and the downstream factors, including autoantibodies, cytokines and chemokines, for its efficacy on scleroderma.

In the present study, we aimed to investigate the effect of baicalein on the major pathological characteristics of scleroderma both *in vitro* and *in vivo* with bleomycin-induced skin fibrosis mice model, including fibrosis, B cell abnormalities and inflammation.

Methods

Cell line and culture

CCC-ESF-1 normal human [embryonic](#) dermal fibroblasts were obtained from the Cell Resource Center, Peking Union Medical College and were used between passages 10 and 14. Cells were maintained in DMEM supplemented with 10% fetal bovine (Gibco) in a humidified atmosphere of 5% CO₂ at 37 °C.

Drug

Baicalein (Cat# 111595-201607) was obtained from National Institutes for Food and Drug Control (Beijing, China) and prepared in stock at the dose of 20 mM in dimethyl sulfoxide (DMSO) and the working concentrations were prepared by diluting the stock in medium. TGF-β1 and PDGF were obtained from Peprotech (Rochy Hill, NJ, USA). The inhibitor of TGF β receptor type I (LY364947) and the selective PDGFRα/β inhibitor ([crenolanib](#)) were obtained from [MedChemExpress](#).

Measurement of collagen accumulation and secretion

CCC-ESF-1 cells were seeded in collagen I-coated plates for *in vitro* models of fibrosis and cultured 48 h to 80-100% confluence. After a washing with PBS, the medium was then changed to a serum-free DMEM supplemented with 1% insulin-transferin-selenium (ITS), which was the basic medium used throughout this study, for 24 h and then cells were incubated in the medium with or without TGF-β1(5ng/mL) or PDGF(40ng/mL) for additional 48h in the presence or absence of vehicle, different concentrations of baicalein. Some inhibitors (1 μM LY364947, 2 μM [crenolanib](#)) were used as positive controls. The deposits of collagen were assessed by Picro-Sirius red (PSR) staining and quantitative analysis as previously reported[12]. Briefly, cells were fixed with methanol overnight at -20°C and incubated in 0.1% PSR staining solution for 4 h. Then cells were washed twice with 0.1% acetic acid and PSR was eluted in 0.1 M sodium hydroxide. The optical density (OD) value was measured at 540nm. Collagen secretion was detected by measuring soluble collagen cell culture supernatant using the Sircol Soluble Collagen Assay (Biocolor, Country Antrim, UK) according to the manufacturer's instructions.

Cytotoxicity assay

Cytotoxicity was assessed using lactate dehydrogenase release (LDH) assay. 120 μL supernatant from each well was collected and detected for LDH release according to the manufacturer's instructions (Beyotime, China). Cytotoxicity was determined by the optical density (OD) value of LDH release by cells.

ELISA assays of collagen type I and α-SMA

CCC-ESF-1 cells were lysed with three freeze-and-thaw cycles between -80°C and 37°C in PBS and centrifuged at 12000g for 15min at 4°C. Collagen type I and α-SMA in the supernatants were measured using ELISA kits according to the manufacturer’s manual (CUSABIO, China). Results were expressed as the ratio of collagen I or α-SMA per total protein.

Reverse transcriptase quantitative polymerase chain reaction (qPCR) analysis

Total RNA in cells was isolated by the RNeasy Mini kit (Qiagen). cDNA was prepared from 2 µg of total RNA using the RevertAid First strand cDNA Synthesis Kit (Thermo Scientific™) and then subjected to qPCR to quantify the gene expression using SYBR Green SuperReal Premix Plus (Tiangen Co. China). The primer pairs used for analyses were listed in Table 1. GAPDH was used to normalize for the amounts of loaded cDNA. Relative expression of the target genes was calculated using 2^{-ΔΔCt} method.

Table 1 Primer sequence

Genes	Primer Sequence	Length
human COL1A1	Forward: 5'-TCAAGAGAAGGCTCACGATGG-3'	69
	Reverse: 5'- TCACGGTCACGAACCACATT -3'	
human COL1A2	Forward: 5'- GGTCAGCACCAACCGATGTC -3'	51
	Reverse: 5'- CACGCCTGCCCTTCCTT -3';	
human COL3A1	Forward: 5'- GCTGGCTACTTCTCGCTCTG -3'	97
	Reverse: 5'- TCCGCATAGGACTGACCAAG -3'	
human CTGF	Forward: 5'- CAGCATGGACGTTTCGTCTG -3'	115
	Reverse: 5'- AACCACGGTTTGGTCCTTGG-3';	
human fibronectin	Forward: 5'- TTCTAAGATTTGGTTTGGGATCAAT -3'	51
	Reverse: 5'- TCTTGATTGGCTGCATATGC -3'	
human TGF-β1	Forward: 5'- CAATTCCTGGCGATACCTCAG -3'	86
	Reverse: 5'- GCACAACTCCGGTGACATCAA -3'	
human α-SMA	Forward: 5'- GACAATGGCTCTGGGCTCTGTAA -3'	147
	Reverse: 5'- CTGTGCTTCGTCACCCACGTA -3'	
human GAPDH	Forward: 5'- GACCACTTTGTCAAGCTCATTTCC -3'	151
	Reverse: 5'- GTGAGGGTCTCTCTCTTCCTCTTGT -3'	

Mouse model of scleroderma

Six to seven -week old female C57BL/6J mice were obtained from Beijing Vital Laboratory Animal Technology (Beijing, China) and maintained in specific pathogen free lab, with food and water *ad libitum*. All animal procedures were in accordance with the Institutional Animal Care and Use Committee (IACUC) guidelines with the approval of the Institutional Animal Ethical and Welfare Committee (Approval Number: protocol #20162032).

The bleomycin-induced skin fibrosis model was initially described by Yamamoto et al[27]. Bleomycin (BLM, Nippon Kayaku, Tokyo, Japan) was dissolved in 0.9% NaCl at a concentration of 1 mg/mL and sterilized by filtration. Mice were randomly divided in 5 groups with at least 9 mice in each group. 100µL of BLM or 0.9% NaCl were injected subcutaneously into the shaved back well defined areas (1cm²) of the mice daily and orally gavaged with vehicle control (0.5% [carboxymethylcellulose sodium](#)), D-penicillamine (125mg/kg) or baicalein (25,50 and 100 mg/kg) once a day for 4 weeks.

Quantification of fibrosis in mice

The injected skin areas were fixed in 4% paraformaldehyde in PBS, embedded in paraffin and stained with Masson's Trichrome staining. Dermal thickness was determined by measuring the largest distance between the epidermal–dermal junction and the dermal-subcutaneous fat junction. The collagen content in lesional skin was analyzed by hydroxyproline assay according to the manufacturer's instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) and was expressed as µg hydroxyproline per mg of tissue wet weight.

ELISAs for serum autoantibodies and cytokines

Serum levels of anti-Scl70, anti-dsDNA, anti-centromeres and anti-PM-Scl were measured using ELISA kits (Euroimmun Medizinische Labordiagnostika AG, Germany) according to the manufacturer's instructions with minor modifications. A 1:5 serum dilution was used for the determination of all autoantibodies. The secondary antibody was a goat polyclonal anti-mouse IgG H&L conjugated to horseradish peroxidase (HRP) (Abcam, UK). The optical density (OD) value was measured at 450/650nm. Serum levels of IL-1β, IL-2, IL-4, IL-6, IL-17A, TNF-α, MCP-1 and MIP-1β were detected using MILLIPLEX MAP kits (Merck Millipore, Germany) according to the manufacturer's instructions.

Flow cytometric analysis

Single-cell suspensions were obtained from mouse spleen in PBS. Erythrocytes were lysed with RBC lysis buffer. Spleen cells were labeled with mAb specific for cell surface markers B220 (PE-Cy7, RA3-6B2) from BD Pharmingen and CD27 (PE, LG.3A10) from Miltenyi Biotec. All samples were assessed by CytoFLEX flow cytometry system (Beckman Coulter Inc., CA, USA). At least 30000 lymphocyte-gated cells were routinely collected for each sample. B cells were identified as B220⁺ lymphocytes. Memory B cells were identified as CD27⁺B220⁺ lymphocytes.

Statistical Analysis

Data were expressed as mean $\pm$ SD or mean $\pm$ SEM. All statistical analyses were performed using GraphPad Prism 8.0.2 Software. Statistical significant differences of multiple groups were determined by ordinary ANOVA followed by Dunnett's test for post-hoc comparisons. In each case, P value $\leq$ 0.05 was considered statistically significant.

Results

Baicalein attenuates TGF- β 1 and PDGF-induced fibrosis in dermal fibroblasts.

As a proof of principle that TGF- β 1 and PDGF are the central mediators of tissue fibrosis in SSc[28, 29], we selected these two stimulators to establish *in vitro* models for analyzing the antifibrotic activity of baicalein. The total collagen deposition, soluble collagen in the supernatant and cytotoxicity of CCC-ESF-1 cells were analyzed. As shown in Figure 1, addition of 5 ng/mL TGF- β 1 or 40 ng/mL PDGF potently induced total collagen accumulation and collagen secretion. Baicalein could significantly abrogate total collagen deposition with ED₅₀ of 18.73 $\pm$ 3.02 μ M in TGF- β 1-induced model and 18.07 $\pm$ 2.67 μ M in PDGF-induced model, and markedly decrease soluble collagen secretion with ED₅₀ of 27.90 $\pm$ 5.86 μ M in TGF- β 1-induced model and 22.54 $\pm$ 5.83 μ M in PDGF-induced model. LDH assay showed that exposure to baicalein ranged from 5 μ M to 120 μ M resulted in relatively low cytotoxicity against TGF- β 1 or PDGF treated CCC-ESF-1 cells.

Baicalein suppresses the expression of molecular markers of fibrogenesis.

In order to further verify whether the antifibrotic activities of baicalein was due to the direct effect on molecular markers of fibrogenesis, mRNA and protein expression analyses were performed by qPCR and ELISA. In TGF- β 1-induced model, treatment with 5-120 μ M baicalein resulted in dose-dependent downregulation of the gene expression of fibrotic-related markers, including COL1A1, COL1A2, COL3A1, CTGF, fibronectin, TGF- β 1 and α -SMA (Fig. 2A,2B), as well as the protein expression of collagen type I and α -SMA (Fig. 2C). Similar results were observed in PDGF-induced cells, baicalein potently reversed the stimulatory effects of PDGF on all the markers of fibrogenesis in dose-dependent manners (Fig. 2A-2C).

Baicalein Ameliorates The Skin Fibrosis Of Bleomycin-induced Scleroderma

Given that baicalein inhibited collagen deposition and secretion *in vitro*, we further investigated the effect of baicalein on the dermal fibrosis in bleomycin-induced mice model. Compared to NaCl-treated mice, subcutaneous injection with bleomycin significantly induced cutaneous fibrosis as represented by the increased dermal thickening and markedly accumulation of hydroxyproline content (P<0.05), whereas the mice administrated with 25-100 mg/kg baicalein showed significantly decreased dermal thickness and collagen accumulation in dose-dependent manners, which indicated the antifibrotic effect of baicalein *in vivo*.

Baicalein inhibits bleomycin-induced serum autoantibodies overproduction and cytokine production.

The pathogenesis in SSc is highly associated with the inflammatory abnormalities, and bleomycin-induced scleroderma has demonstrated the dysregulation of inflammatory cytokine levels [30, 31]. Here, we examined T helper and B cell cytokines and chemokines in the serum of bleomycin-induced mice. Notably, significant increase of IL-1 β , IL-2, IL-4, IL6, IL-17A, TNF- α , MCP-1 and MIP-1 β levels were demonstrated in BLM-treated mice, significant reduction of serum levels due to baicalein treatment was observed in all these eight cytokine/chemokine markers (Fig. 4). Meanwhile, it has been widely reported that the presence of autoantibodies (Abs) is a central feature of immune activation in human SSc. The production of specific antibodies, such as anti-nuclear and anti-centromere Abs, is detectable and associated with the pathological processes in BLM-induced murine model[32]. To further elucidate if baicalein affects the secretion of auto-antibodies, we detected the Abs levels in BLM-induced mice serum. Baicalein diversely suppressed the serum anti-Scl-70, anti-PM-Scl, anti-centromeres and anti-dsDNA levels in dose-dependent manners compared to BLM-induced mice (Fig. 5).

Baicalein modulates bleomycin-induced alteration of B cells distribution.

Abnormalities of B cell homeostasis are thought to play an essential role in SSc [19]. Here, we detected the proportion of B cells and memory B cells in spleen of mice. As shown in Figure 6, the BLM-induced mice exhibited a potentially increase of B220⁺ B cell subpopulation, whereas the percentage of CD27⁺ memory B cells markedly attenuated in spleen. As compared to bleomycin treatment only, exposure to baicalein markedly reduced the proportion of B220⁺ B cells and increased the proportion of CD27⁺ memory B cells in dose dependent manners.

Discussion

SSc is a systemic autoimmune disorder featured by early inflammatory reaction followed by diffuse fibrosis and vascular changes of skin and organs. Progressive fibrosis caused by excessive accumulation of extracellular matrix (ECM) and collagen is an essential hallmarks of SSc[1, 17]. It is widely accepted that TGF- β 1 [28] and PDGF [29] are the central mediators of tissue fibrosis in SSc, which induce collagen deposition and mesenchymal cell proliferation, migration, accumulation and activation. Many researches have reported that baicalein exerted certain therapeutic effects on pulmonary fibrosis, hepatic fibrosis, cardiac fibrosis and renal interstitial fibrosis[8–11]. 150 μ M baicalein suppressed TGF- β 1-induced collagen deposition as well as the expression of collagen I and α -SMA by activating p38MAPK/JNK pathway in NIH-3T3 cells [14]. Baicalein was also found to attenuate the production of type I collagen, downregulate expression of CTGF and α -SMA and retard α -SMA filament formation in TGF- β 1-stimulated human lung fibroblasts[8, 13]. In renal fibroblasts, 20-80 μ M baicalein specifically inhibited TGF- β 1-induced ECM accumulation and the expression of collagen I in mRNA and protein levels [9]. In line with these observations, we found that after treatment with 5-120 μ M baicalein for 48 h, TGF- β 1-induced collagen accumulation and secretion in skin fibroblasts were significantly attenuated at dose-dependent manners in present study (Fig. 1). Additionally, qPCR and ELISA assays showed that

incubation with baicalein resulted in fairly potent suppression of mRNA expression of fibrogenic markers (COL1A1, COL1A2, COL3A1, CTGF, fibronectin, TGF- β 1 and α -SMA)(Fig. 2A,B), as well as the decreased collagen type I and α -SMA levels (Fig. 2C). Moreover, in agreement with the observations from TGF- β 1-induced model, treatment of PDGF-induced CCC-ESF-1 cells with baicalein under identical conditions showed the similar effective trend on fibrosis, as evident from skin fibroblast deactivation, reduced collagen release, deregulation of fibrogenesis-related mRNA expression and suppressed myofibroblast differentiation with decreased mRNA and protein levels of α -SMA (Fig. 2A,C). These results suggest that baicalein exerts direct antifibrotic activity by specifically inhibiting TGF- β 1 and PDGF-mediated ECM accumulation.

In order to develop the relevance of above *in vitro* findings, the *in vivo* efficacy of baicalein on the progression of scleroderma was validated in present study. It has been reported that 50 and 100 mg/kg baicalein treatment significantly ameliorated kidney fibrosis in unilateral ureteral obstruction mice [15]. Administration of 25 and 100 mg/kg baicalein remarkably reversed angiotension II-induced and aortic banding induced cardiac fibrosis, respectively [26, 33]. 50 and 100 mg/kg of baicalein attenuated bleomycin-induced pulmonary fibrosis[34]. Here, we used the widely accepted bleomycin-induced experimental skin fibrosis model to detect the antifibrotic effect of baicalein on SSc. We found that treatment with 25-100 mg/kg of baicalein inhibited the bleomycin-induced skin fibrosis as evident from the reduced dermal thickness and hydroxyproline content in dose-dependent manners (Fig. 3). Immunological dysregulation which includes autoimmunity, infiltration and activation of immune cells is the essential feature of SSc[17, 18]. B cells have been involved in the pathogenesis of SSc directly as well as indirectly through producing autoantibodies and releasing proinflammatory/profibrotic cytokines and chemokines[17, 35, 36]. Previous studies reported that SSc patients showed altered B-cell homeostasis and activation with increased B-cells but decreased memory B-cells[19, 22, 23]. Many antinuclear antibodies, such as topoisomerase I, ACA, RNA polymerase III, ribonuclear protein U1, centromere and nucleolar antigen PM/Scl etc, were detected in 43% of scleroderma population[37, 38]. Additionally, some cytokines and chemokines, such as IL-4, IL-6, IL-10, MCP-1 and MIP-1, have high levels in the blood, skin and lungs. They were reported to correlate with distinct clinical features and disease severity[39–41]. They were also identified as the fibrogenic markers by activating fibroblasts and enhancing collagen and ECM production in the fibrotic pathogenesis of some SSc mouse models[30–32, 42]. In present mice model, subcutaneous injection of bleomycin resulted in increased level of B220⁺ B cell subpopulation and reduced percentage of CD27⁺ memory B cells, which associated with autoantibodies production and overexpressed levels of cytokines or chemokines (Fig. 4,5). All these data suggested that B cells were hyperactivated and played important roles in fibrotic pathogenesis of BLM-induced mice, which is comparable with the data from other SSc mice models[43–45]. Baicalein treatment potently attenuated serum levels of cytokines, chemokines and autoantibodies produced by T or B cells in bleomycin-induced mice (Fig. 4,5). Under the same condition, the splenic B220⁺ B cells were decreased and CD27⁺ memory B cells were increased, suggesting that baicalein attenuated the inflammatory and immunological state induced by bleomycin via regulating the B cell distribution (Fig. 6).

Conclusions

In summary, our pronounced results have identified for the first time that baicalein exerts dose-dependently anti-fibrotic effects in scleroderma via suppressing TGF- β 1 and PDGF-mediated ECM accumulation *in vitro* and alleviating fibrosis, regulating B cell abnormalities, reducing autoantibody production and attenuating inflammation in bleomycin-induced scleroderma *in vivo*. It is tempting to suggest that baicalein may have the potential to be further developed as a therapeutic candidate against SSc.

Declarations

Ethics approval and consent to participate

All animal procedures were in accordance with the Institutional Animal Care and Use Committee (IACUC) guidelines with the approval of the Institutional Animal Ethical and Welfare Committee (Approval Number: protocol #20162032) of Institute of Chinese Materia Medica, China Academy of Chinese Medical Sciences, Beijing.

Consent for publication

All authors were concerned and agreed to publish before the submission.

Availability of data and materials

The datasets used in present study are available from the corresponding author on reasonable request.

Competing interests

All authors declare that they have no conflict of interest.

Funding

This work was supported by National Key R&D Program of China (2018YFC1708100, 2018YFC1708105) and International Science and Technology Cooperation Program of China (2013DFG31080)

Authors' contributions

JL, GZ and XH critically designed this research and revised this manuscript. BP carried out the experiments and wrote the manuscript. QH, RH and HH performed part of the experiments. YC created part of the figures. All authors have read and agreed to the final version of the manuscript.

Acknowledgements

We thank Mr. Baoqiang Dai and Ms. Lifang Wang for their helps in animal care.

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Figures

Figure 1

Baicalein inhibits TGF-β1 (A) or PDGF (B) induced fibrosis in CCC-ESF-1 cells. Cells were seeded in collagen-I-coated plates and treated with or without 5 ng/mL TGF-β1 or 40 ng/mL PDGF in the presence or absence of 5-120 μM baicalein for 48 h. Total collagen deposition was visualized by PSR staining and

quantified by spectrophotometric analysis. Soluble collagen in the supernatant was detected by Sircol Soluble Collagen Assay. Cytotoxicity was assessed using lactate dehydrogenase release (LDH) assay. Representative images were shown, scale bar=200 μ m. All quantitative data were presented as mean $\pm$ SD from one representative experiment out of at least three with similar results, N=6 wells per group. * $P<0.05$, ** $P<0.01$ verse TGF- β 1 or PDGF only group.

Figure 2

Baicalein suppresses the expression of fibrogenesis markers in TGF- β 1 or PDGF-induced CCC-ESF-1 cells. Cells were treated as described in Figure 1 and collected for qPCR (A, B) and ELISA (C) analysis. Data were expressed as mean $\pm$ SD of 3 replicates. Each experiment was repeated for 3 times with similar results. * $P<0.05$, ** $P<0.01$ verse TGF- β 1 or PDGF only group, respectively.

Figure 3

Baicalein attenuates the skin fibrosis in bleomycin-induced scleroderma mice. Bleomycin (1 mg/mL, 100 μ L/mice) was injected subcutaneously into the shaved back well defined areas (1cm²) of the mice daily for 4 weeks. Mice were orally gavaged with vehicle control (0.5% carboxymethylcellulose sodium), D-penicillamine (125mg/kg) or baicalein (25, 50 and 100 mg/kg) once a day for 4 weeks. Representative images of Masson's Trichrome staining of skin of mice in each group were shown (A), scale bar=200 μ m. Dermal thickness and hydroxyproline content were quantified (B). All quantitative data were presented as mean $\pm$ SEM from 9 mice in each group. * $P < 0.05$, ** $P < 0.01$ vs. bleomycin (BLM)-treated mice.

Figure 4

Baicalein decreases serum levels of cytokines and chemokines in bleomycin-induced scleroderma mice. Mice were treated as described in Figure 3 and serum was collected for MILLIPLEX MAP Human cytokine/chemokine analysis. All quantitative data were shown as mean $\pm$ SEM from 9 mice in each group. * $P < 0.05$, ** $P < 0.01$ vs. bleomycin (BLM)-treated mice.

Figure 5

Baicalein inhibits bleomycin-induced serum autoantibodies overproduction. Mice were treated as described in Figure 3 and serum was collected for ELISA analysis. All quantitative data were shown as mean $\pm$ SEM from 9 mice in each group. * P<0.05, ** P<0.01 vs. bleomycin (BLM)-treated mice.

Figure 6

Baicalein inhibits the abnormalities of B cells distribution in bleomycin-induced scleroderma model. Mice were treated as described in Figure 3 and spleen cells were collected to analyze the proportion of B cells (A) and CD27+ memory B cells (B). All quantitative data were shown as mean $\pm$ SEM from 9 mice in each group. * $P<0.05$, ** $P<0.01$ vs. bleomycin (BLM)-treated mice.