

Chondroitin sulphate decreases collagen synthesis in normal and scleroderma fibroblasts through a Smad-independent TGF- β pathway – implication of C-Krox and Sp1

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Abstract

Despite several investigations, the transcriptional mechanisms which regulate the expression of both type I collagen genes (*COL1A1* and *COL1A2*) in either physiological or pathological situations, such as scleroderma, are not completely known. In this study, we determined the effects of both native ichtyan chondroitin sulphate (CS) and its derived hydrolytic fragments (CSf) on human normal (NF) and scleroderma (SF) fibroblasts. Here, we demonstrate for the first time that CS and CSf exert an inhibitory effect on type I collagen protein synthesis and decrease the corresponding mRNA steady-state levels of *COL1A1* and *COL1A2* in NF and SF. These glycosaminoglycan molecules repress *COL1A1* gene transcription through a -112/-61 bp sequence upstream the start site of transcription and imply hc-Krox and Sp1 transcription factors. In addition, CS and CSf induced a down-regulation of T β RI expression. As a conclusion, our findings highlight a possible new role for CS and CSf as anti-fibrotic molecules and could help in elucidating the mechanisms of action by which CS and CSf exert their inhibitory effect on type I collagen synthesis.

Keywords: collagen I • C-Krox • Sp1 • fibroblasts • scleroderma • transcription • fibrosis • COL1A1 • Smad • TGF- β • T β RII

Introduction

Chondroitin sulphate (CS) is a glycosaminoglycan (GAG) which is composed of an alternating sequence of sulphated and/or unsulphated residues of D-glucuronic acid (GlcA) and D-N-acetylgalactosamine (Gal Nac) linked by $\beta(1\rightarrow4)$ bonds. It belongs to a major class of GAG that is present as an extracellular matrix (ECM) molecule in most tissues. CS plays a great role in the structure and function of proteoglycans in articular cartilage. Their alterations in articular diseases, including osteoarthritis (OA) and rheumatoid

arthritis (RA), are a major event in the damage process affecting the joint [1]. In this context, CS has been proposed as a chondroprotective drug [2] and is presently orally administered for treatment of knee OA [3]. This compound has been shown to exert anti-inflammatory [4] and anabolic effects on chondrocyte matrix molecules, including aggrecan synthesis [5].

Much less is known about the properties of CS biological functions in other tissues, where CS proteoglycans are majoritarily represented by mesenchyme proteoglycan (PG-M)/versican and smaller molecules such as decorin, biglycan and fibromodulin. CS GAGs have been implicated in cell division, neuronal development and spinal cord injury [6–9]. However, the complexity and heterogeneity of CS composition and structure have hampered efforts to understand its precise biological roles. Nevertheless, it has been well established through several approaches that CS clearly displays anti-adhesive properties [10]. In this respect, CS can be considered as one of the ECM molecules that inhibit cell adhesion.

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Members of this group include tenascin, thrombospondin, laminin, mucin, SPARC and CS-containing proteoglycans [11, 12]. This property has been tentatively used in the prevention of post-surgical adhesions in patients who undergo laparotomy. Indeed, intraperitoneal administration of a CS solution at the completion of surgery, in rabbit or rat models, reduced the formation of adhesions between the intestine and the parietal peritoneum [13, 14]. However, the cellular mechanisms responsible for the anti-adhesive properties of CS are presently poorly understood. Annexin 6 has been proposed to act as a cell surface receptor for CS chains in human fibroblast WI38 and chick embryonic fibroblasts [15]. The downstream signalling pathways by which CS may exert its action on focal contacts, stress fibres state and gene expression are hardly known. Data have shown that the anti-adhesive protein tenascin induces focal adhesion disassembly through its FN type III repeats (TnfnA-D) which bind to annexin 2 and activates cyclic guanosine-3', 5'-monophosphate (GMP)-dependent protein kinase [16]. However, the results obtained with inhibitors of signalling suggest that the inhibitory mechanism for CS chains may be different from that of tenascin [15]. Since annexin 6 has been found to make a complex with p120GAP (Ras GTPase-activating protein), Fyn (Src family kinase) and Pyk2 (focal adhesion kinase family member), it is hypothesized that the interaction of CS and annexin 6 causes the state change of these signalling molecules and induces the anti-adhesion activity [17].

The data available about the preventing effect of CS on post-operative adhesions suggest that, besides its anti-adhesive mechanism, this GAG could also reduce the production of ECM, especially collagen, which generally accompanies the formation of adhesions. It has been found that there was an increased amount and synthesis of type I collagen in post-operative intra-abdominal adhesion [18, 19]. Interestingly, in experiments on a rat model of cecal serosa abrasion, it has been observed that the deposition of fibrin and collagen type I was reduced significantly by 73% and 89% in the injured serosa, respectively, in animals treated with intraperitoneal CS solution [14]. We, therefore, hypothesized that CS molecules could exert an inhibitory effect on collagen synthesis in mesenchymal cells, including dermal fibroblasts, and be considered as both an anti-adhesive and anti-fibrotic agent. In this study, we have investigated the effects of both native ichtyan CS (MW 44 kD) and its derived hydrolytic fragments (MW range : 13–23 kD) on human normal and scleroderma fibroblasts (SF). Scleroderma is a complex autoimmune disease characterized by pathological re-modelling of connective tissues [20]. A unique feature of SF in culture is the retention of an abnormal phenotype, characterized by enhanced ECM synthesis and the expression of the myofibroblast marker, α -smooth muscle actin (α -SMA) during serial passage *in vitro*. Although the molecular basis for the persistently activated phenotype of SF at sites of fibrogenesis remains unclear, recent findings demonstrate that the multi-functional cytokine transforming growth factor- β (TGF- β) and its intracellular signalling pathway have crucial roles in the initiation and propagation of the fibrotic response [21]. The present data demonstrate for the first time that CS is capable of reducing the synthesis of types I and III

collagens by both normal and SF. This effect is exerted at the transcriptional level and, for type I collagen, involves the -112/-61 bp sequence upstream the initiation start site of transcription and the transcription factors C-Krox and Sp1.

Materials and methods

Cell culture

Human dermal normal (NF) and SF were obtained by explant cultures, using biopsies of adult patients, with their official consent (Department of Dermatology, Caen University Hospital, Dr Anne Domp Martin) and the approval of the local Ethical Committee. SF were taken from the affected area. They were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with antibiotics and 10% heat foetal calf serum (FCS) and used between the fourth to ninth passages. They were grown at 37°C in a 5% CO₂ humidified atmosphere, with medium change every 2–3 days.

Preparation of ichtyan CS fragments

CS fragment samples (molecular weight range *Mr* 13,000–23,000) from shark cartilage were a gift from Pierre-Fabre Laboratories (Castres, France). They were obtained by hydrolysis in HCl solution pH 1.1, for 2.5 hrs at 70°C, followed by cooling and neutralization by NaOH. The samples were precipitated by addition of ethanol, thoroughly washed and dried. The molecular weight of the CS fragments was determined by size-exclusion chromatography on a 30 cm × 7.8 mm TosoH, Japan, TSK Gel 3000 PWXL (TosoHaas), equipped with a 4 cm × 6.0 mm pre-column TSK PWXL (TosoH, Japan). 0.125 M SO₄Na₂ solution was used as the mobile phase and standard references served to calibrate the column (*Mr* : 12,900, 14,500, 17,700, 24,900, 31,500).

Collagen labelling and assay

To assay newly synthesized collagen, NF and SF cultures, at 80% confluency, were pre-incubated in 10% FCS-containing DMEM in 9.6-cm² dishes for 15 hrs in the presence of 50 μ g/ml sodium ascorbate. Then, the medium was replaced by the same fresh medium supplemented with β -amino-propionitrile (β -APN) (100 μ g/ml) and [³H]-proline (2 μ Ci/ml) (Perkin Elmer Life Sciences, Courtaboeuf, France), containing ichtyan CS (MW of 44 kD) or CSf (MW range: 13–23 kD) at 1–100 μ g/ml. After 24 hrs, the culture medium and the cell layer were collected and the amount of labelled collagen was assayed with pure bacterial collagenase [22, 23].

Preparation of cytoplasmic extracts

NF and SF were seeded in 55 cm² Petri dishes, and incubated for 24 hrs at 80% confluency with or without CS or CSf at 1, 5, 10, 25 μ g/ml. Following treatment, fibroblasts were rinsed once with ice-cold phosphate buffered saline (PBS) and lysed for 30 min. at 4°C in RIPA buffer (50 mM Tris-HCl, pH 7.5, 1% NP-40, 150 mM NaCl, 1 mM ethylene glycol tetraacetic acid (EGTA), 1 mM NaF, 0.25% sodium deoxycholate, 0.5 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), Leupeptin, Pepstatin A

and Aprotinin at 10 µg/ml). After centrifugation (30 min., 14,000 × *g*, 4°C), the supernatant was collected for further analysis by SDS-PAGE and Western blot.

Western blotting

The cell layer-associated proteins (15 µg) were resolved on a 8% polyacrylamide gel using 1% SDS/ Tris-glycine buffer. Then the proteins were processed by Western blot as previously described [24]. The primary antibodies used in the experiments were anti-TβRI antibody (1/1000 dilution), anti-TβRII antibody (1/2500 dilution) and anti-Smad 7 antibody (1/1000 dilution), all from Santa Cruz Biotechnology, Inc. (Tebu Bio SA, Le Perray en Yvelines, France). After 2 hrs of incubation, the membranes were rinsed and incubated for 2 hrs with a secondary antibody (horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody, 1:5000 dilution, Santa Cruz Biotechnology, Inc.). TβRI, TβRII and Smad7 proteins were revealed using a Western blot detection kit (West Pico, Perbio Science France S.A.S., Brebières, France). Signals were captured with the Fluor-S Multimager video system (Bio-Rad, France). The TβRI, TβRII and Smad 7 expression was normalized to that of β-actin or β-tubulin. Mouse monoclonal anti-actin (1/300 dilution) and rabbit polyclonal anti-tubulin (1/500 dilution) were purchased from Tebu-Bio SA.

Quantitative real-time RT-PCR

Total RNA was extracted and 2 µg were reverse transcribed into cDNA in the presence of random hexamers as previously described [25]. Real-time PCR amplifications were performed in a SYBR Green PCR Master mix with 5 µl of cDNA diluted 1:100, using sequence-specific primers (between 300 and 800 nM) for *COL1A1*, *COL1A2*, *COL3A1*, *Sp1*, *Sp3* and *hckrox*, defined with the 'Primer Express' software (Applied Biosystems) (Table 1). rRNA 18S primers were purchased from Applied Biosystems (Courtaboeuf, France). Thermal cycling parameters were already described [25] and carried out on an 'ABI Prism 7000 sequence detection system' (Applied Biosystems). Relative gene expression was done by using the $2^{-\Delta\Delta CT}$ method [26].

Transient cell transfection experiments

Dermal fibroblasts were seeded in 150 cm² flasks. At confluency, cells were trypsinized and re-suspended in culture medium. They were divided in aliquotes of 0.8 to 1.2 × 10⁶ cells per nucleofection sample. After centrifugation (200 × *g*, 10 min.), they were suspended in 100 µl of Human Dermal Fibroblast Nucleofector Solution (HDFN) at room temperature. Reporter plasmids (2 µg) and pSV40β-gal plasmid (2 µg) were added. The nucleofection was then performed with the AMAXA's Nucleofector, according to the manufacturer's recommendations (Köln, Germany). Then, 500 µl of pre-warmed culture medium was added and the sample transferred to a 6-well plate. After 12 hrs at 37°C, the culture medium was changed and the cells were incubated for 6 hrs with or without CS or CSf at 50 µg/ml. The plasmids used were *COL1A1*-luciferase reporter vectors, previously described [27]. Then, the samples were harvested and protein content, luciferase and β-galactosidase activities were determined as previously described [24]. Luciferase activities were normalized to transfection efficiency and protein amount, and means ± SD of three independent samples were expressed as relative luciferase units (RLUs).

Nuclear extracts and gel retardation assays

Nuclear extracts were prepared by a mini preparation procedure [28] and gel retardation assays were performed with the following oligonucleotides (Eurogentec, Angers, France): Aα1: 5'-CTTGCGGGAGGGGGGCGTGGGTG-GACA-3' (C-Krox binding site is indicated in italics), -112/-61 wt : 5'-AGGCAGCTCTGATTGGCTGGGGCACGGGC-GGCCGGCTCCCCCTCC-GAGGG-3' (C-Krox, Sp1, Sp3 and CBF sites are indicated in italics), SBE: 5'-GGAGTATGTCTAGACTGACAATGTAC 3'. They were end-labelled with [γ -³²P] dATP (Perkin Elmer Life Sciences [Courtaboeuf, France]) using T4 polynucleotide kinase (Promega, Charbonnières, France). Fibroblasts nuclear extracts (7.5 µg) were incubated for 15 min. at room temperature with the probe (2 fmol) in 20 µl of a specific binding buffer [29–31] in the presence of 2 µg of poly(dIdC)•poly(dIdC) (Amersham Biosciences, Orsay, France) used as a DNA non-specific competitor. Samples were fractionated by electrophoresis for 1.5 hrs at 150 V on a 5% polyacrylamide gel in 0.5× TBE (45 mM Tris borate, 1 mM Na₂ EDTA) and visualized by autoradiography.

Calculation and statistical analysis

Each experiment was repeated at least three times with similar results. Results are expressed as mean ± SD of triplicate determinations. Statistical significance was assessed using the Student's *t*-test.

Results

CS and CSf induce inhibition of type I collagen production in normal and scleroderma fibroblasts

We first determined the effect of increasing concentrations of CS and CSf on collagen biosynthesis in NF and SF primary cultures. Newly synthesized collagens were assayed after [³H]-proline labelling of the fibroblasts and treatment by the CS molecules. In NF, CS and CSf treatments were found to reduce total collagen neosynthesis (Fig. 1A), which is known to be mainly formed of type I isoform (80%) in dermal fibroblasts. As expected, basal collagen neosynthesis of SF was found to be greater than that of NF (approximately 250%) (Fig. 1B). Fig. 1B shows that CS and CSf induced a significant decrease of collagen synthesis in SF.

CS and CSf decrease the COL1A1, COL1A2 and COL3A1 mRNA steady-state levels in NF and SF

To determine whether the decrease induced by CS and CSf on the synthesis of the two major isotypes produced by fibroblasts (*i.e.* type I and III collagens) was accompanied by a similar effect at the transcriptional level, we determined the steady-state levels of *COL1A1*, *COL1A2* and *COL3A1* mRNA. In NF, CS and CSf reduced the steady-state levels of *COL1A1* mRNA (Fig. 2A), *COL1A2* mRNA

(Fig. 2B) and *COL3A1* mRNA (Fig. 2C). Similar results were obtained in SF treated with CS and CSf, excepted for CSf at 1 µg/ml (Fig. 2D–F). These data demonstrate that CS and CSf exert a transcriptional control on type I and III collagen genes.

A -112/-61 bp minimal region of the *COL1A1* gene mediates the inhibitory effect of CS and CSf

To better understand the molecular mechanisms by which CS and CSf down-regulate type I collagen at protein and mRNA levels, the transcriptional activity of the human *COL1A1* gene constructs containing deletions in the promoter region was assayed. A schematic representation of the transcription factors interacting with the *COL1A1* proximal promoter is presented in Fig. 3A, as well as the sequence of the -61/-112-bp region in which are found two GC-rich *cis* elements, a CAAT box and a functional C-Krox binding site (Fig. 3B).

Transient transfections performed on NF cultures showed that CS and CSf inhibit the transcriptional activity of all the constructs, except for the shortest containing 61 bp of the proximal promoter (Fig. 4A–C). The same data were observed in SF (Fig. 4D–F). These results suggest that the -112/-61 bp sequence of the proximal promoter mediates CS and CSf-induced inhibition of *COL1A1* gene transcription, in both NF and SF.

CS and CSf decrease the binding of Sp1, CBF and C-Krox to the -112/-61 bp sequence of *COL1A1*

A previous study performed in our laboratory [31] demonstrated that the transcription factors interacting directly with the -61/-112 bp sequence of the *COL1A1* promoter are C-Krox, Sp1 and CCAAT binding factor (CBF). We next determined whether the C-Krox, Sp1 and CBF-binding activities could be correlated with the amounts of type I collagen produced by dermal fibroblasts. As shown in Fig. 5A, the C-Krox, CBF and Sp1-binding activities were found to be higher in SF compared to adult NF (ANF) which present also a much more higher DNA-binding activity compared to foreskin fibroblasts (FF). These data are strictly correlated with the amounts of collagen produced by the different fibroblast culture models; that is SF synthesize higher amounts of collagen compared to NF, themselves producing much more collagen compared to FF.

To identify the transcription factors that mediate the inhibitory effect of CS and CSf on type I collagen expression in the short *COL1A1* promoter, electrophoretic mobility shift assay (EMSA) analysis was performed using the wild-type oligonucleotide sequences A α 1 (previously used to clone C-Krox by Southwestern in NIH-3T3 fibroblasts, [29]) and -112/-61 bp. These two wild-type double-stranded labelled oligonucleotides were incubated with nuclear extracts from FF, ANF and SF. As shown in Fig. 5B and C, one complex was formed upon incubation with the A α 1 wt probe (that binds specifically C-Krox, [30, 31]) when the binding reaction was performed with control nuclear extracts. DNA-bind-

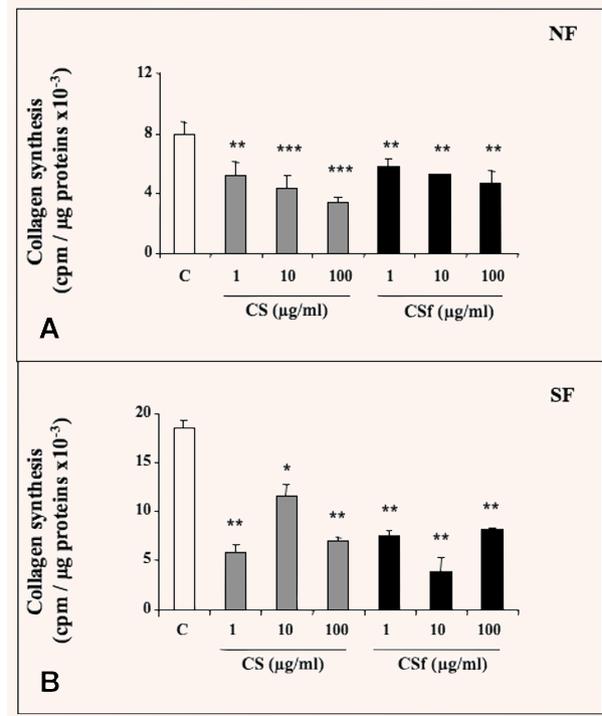


Fig. 1 Effect of chondroitin sulphate (CS) and CSf on collagen neosynthesis in normal (NF) and scleroderma (SF) fibroblasts. Dermal fibroblasts (80% confluency) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) + 10% foetal calf serum (FCS) containing sodium ascorbate (50 µg/ml). 24 hrs later, cells were incubated for 24 hrs in DMEM + 10% FCS containing sodium ascorbate (50 µg/ml), β-aminopropionitrile (β-APN) (100 µg/ml), 2 µCi/ml [³H]-proline, in the presence or absence of CS or CSf, at concentrations ranging from 1 to 100 µg/ml. At the end of the experiment, the amount of radiolabelled collagen was assayed in both medium and cell layer as collagenase-digestible material. The values, normalized to the amount of total protein assayed by the Bradford colometric method, were expressed as cpm/µg protein and represent the mean ± S.D. of triplicate dishes. Statistical significance was evaluated using the Student's t-test. *P < 0.05; **P < 0.01.

(A) Normal fibroblasts (NF).
(B) Scleroderma fibroblasts (SF).

ing activity of the transcription factor involved in this complex, that is C-Krox, is decreased when nuclear extracts from FF or NF treated with CS or CSf were used. With the -112/-61 wt probe (Fig. 5A, D, and E), two major complexes were formed when using control nuclear extracts. Both complexes have been extensively characterized in our previous study [31], where we demonstrated that the slower migrating complex appears as a doublet corresponding to Sp1 and CBF, whereas the higher migrating complex involves C-Krox.

The DNA-binding activity of Sp1, CBF and C-Krox was reduced by CS and CSf in NF (Fig. 5C, D) as well as in SF (Fig. 5E). In

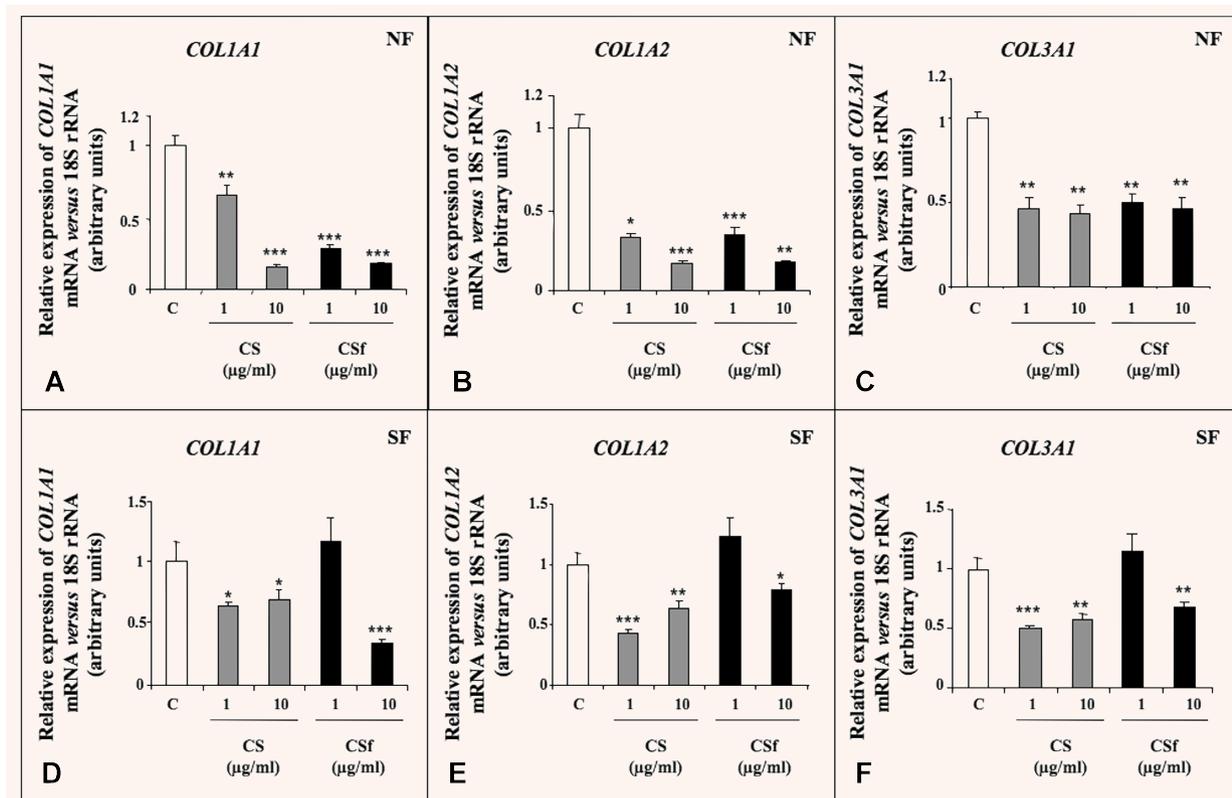


Fig. 2 Effect of CS or CSf on the steady-state levels of type I and III collagen mRNA in cultured fibroblasts. (A, B, C) Normal fibroblasts. (D, E, F) Scleroderma fibroblasts. Total RNAs (2 µg) from fibroblasts treated or not with CS or CSf (1, 10 µg/ml) were reverse-transcribed into cDNAs. The resulting products were diluted (1/100) and analysed by real time RT-PCR using specific primers for *COL1A1* (A, D), *COL1A2* (B, E), *COL3A1* (C, F) mRNA and *18S* rRNA. Results were normalized to *18S* rRNA and presented as relative levels of *COL1A1*, *COL1A2* and *COL3A1* mRNA. Statistical significance was evaluated using the Student's t-test. * $P < 0.05$; ** $P < 0.01$ and *** $P < 0.001$.

conclusion, these experiments indicate that CS and CSf inhibit transcriptional activity of *COL1A1* gene by decreasing the binding activity of Sp1, CBF and C-Krox to the -112/-61 bp sequence of the promoter.

CS and CSf modulate the expression of hc-Krox, Sp1 and Sp3 in NF and SF

Then, we searched for a potential effect of CS and CSf on the expression of three zinc finger proteins hc-Krox, Sp1 and Sp3, all known to be involved in *COL1A1* gene transactivation [31–33]. C-Krox and Sp1 were found to be recruited on the *COL1A1* promoter through a direct interaction with the *cis*-responsive elements, whereas Sp3 interact with the promoter through a protein–protein interaction, at least with C-Krox, as we demonstrated [31].

Real time RT-PCR analysis was performed using total RNA extracted from NF and SF treated or not by the two CS molecular forms. The mRNA steady-state levels of hc-Krox and Sp3 were found

to be decreased by CS and CSf, in NF and in SF (Fig. 6A, C, D and F). However, a difference in the changes of Sp1 mRNA steady-state levels were observed between healthy and pathologic fibroblasts in response to CS and CSf treatments. In NF (Fig. 6B), CS and CSf inhibit Sp1 expression, whereas in SF, CS appears to have no effect on the mRNA steady-state level of Sp1, and CSf increase its expression.

CS and CSf down-regulate TβRI protein expression

Since TGF-β1 has been shown to stimulate type I collagen transcription in NF and that many research groups have described abnormalities within the TGF-β1 signalling pathway in SF, we therefore asked whether CS and its derived low-molecular fragments could down-regulate some of the candidate genes involved in TGF-β1 transduction in our model. Thus, we evaluated the effect of CS and CSf on TβRI protein expression using Western

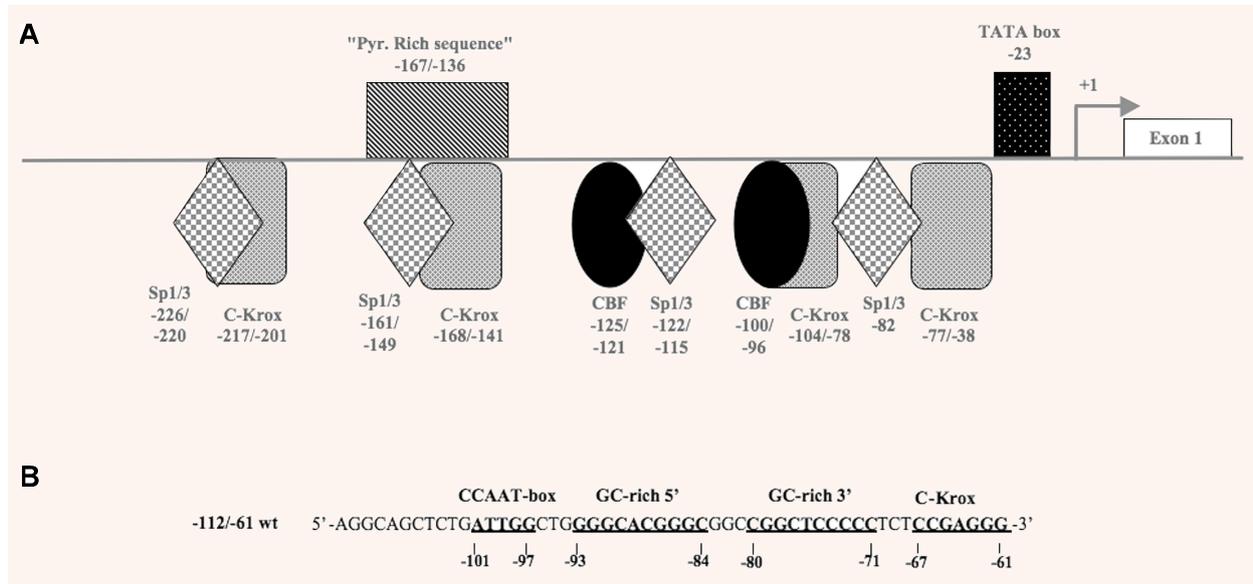


Fig. 3 Schematic representation of the *COL1A1* short promoter. (A) Schematic representation of the transcription factors known to interact with the short promoter region of the *COL1A1* gene. (B) Sequence of the -112/-61-bp region of *COL1A1* gene including the CCAAT box, the 5' and 3' GC boxes and the C-Krox binding site.

blot analysis. We found that the two types of CS molecules decreased T β R1 protein expression in both NF and SF (Fig. 7A and D). This effect resulted from a transcriptional control since RT-PCR experiments demonstrated that CS and CSf decreased the steady-state levels of T β R1 mRNA (data not shown).

Then, same experiments were done on T β RII and Smad7 expression. In fact, in NF and SF, both CS and CSf had no effect, neither on T β RII nor on Smad7 protein expression (Fig. 7B, C, E and F). Similarly, CS and CSf treatment of NF and SF failed to modulate the expression of phosphorylated Smad2/3 from nuclear extracts in Western blot experiments, indicating that this TGF- β signalling pathway is not the target of CS and CSf (data not shown). These results are confirmed by EMSA analysis performed by using the Smad-binding element (SBE) probe. Fig. 7G and H shows that a complex was formed upon incubation with the SBE probe and control nuclear extracts. When the binding reaction was performed with nuclear extracts treated by CS or CSf at 50 μ g/ml, no variation of the DNA-binding activity of the Smads is observed in both NF and SF. Moreover, the competition reaction proved that the Smad-DNA complex is specific.

Discussion

To our knowledge, this is the first report on the effect of CS on the production of types I and III collagens by human dermal fibroblasts, either normal or sclerodermal. We show that the GAG exerts

a transcriptional inhibitory control on both dermal specific collagens, and that the effect on type I collagen synthesis is mediated by a short proximal region of the promoter located between -112 and -61 bp. This region has already been shown to harbour an inverted CCAAT motif and GC-rich repeats, perfectly conserved between mouse and humans, and therefore bind several transcription factors, including Sp1 and Sp3, CBF/NF-1 [31, 34, 35], and c-Krox [29, 31, 36, 37]. Furthermore, it has been shown that this *COL1A1* minimal promoter contains sufficient sequences for its basal and tissue-specific transcription [38]. Using overexpression of these proteins, it has been possible to demonstrate that they induced a potent trans-activation of the minimal *COL1A1* promoter [31]. Additionally, forced expression of Sp1 has been shown to induce increased transcriptional activity of a *COL1A1* promoter (-174 to +42 bp) [35]. However, expression of Sp3, which binds to the same recognition sites as Sp1, stimulated *COL1A1* promoter activity only at higher concentrations, whereas 10-fold excess of Sp3 abrogated the Sp1-induced *COL1A1* promoter activity, suggesting that the ratio Sp1:Sp3 may function as a fine-tuning mechanism on the expression of type I collagen [35], as well as type II collagen [24]. Interestingly, the present data demonstrate that the inhibitory effect of CS on collagen synthesis is accompanied by a decreased expression of the transactivating factors Sp1, Sp3 and c-Krox, the three zinc-finger proteins which bind to the -112/-61 bp *COL1A1* promoter mediating the effect of the GAG. Furthermore, the DNA-binding activity of these factors was inhibited upon chondroitin treatment of the fibroblasts. Thus, the present results confirm the crucial role of the minimal *COL1A1* promoter region -112/-61 bp, and demonstrate that CS is capable of

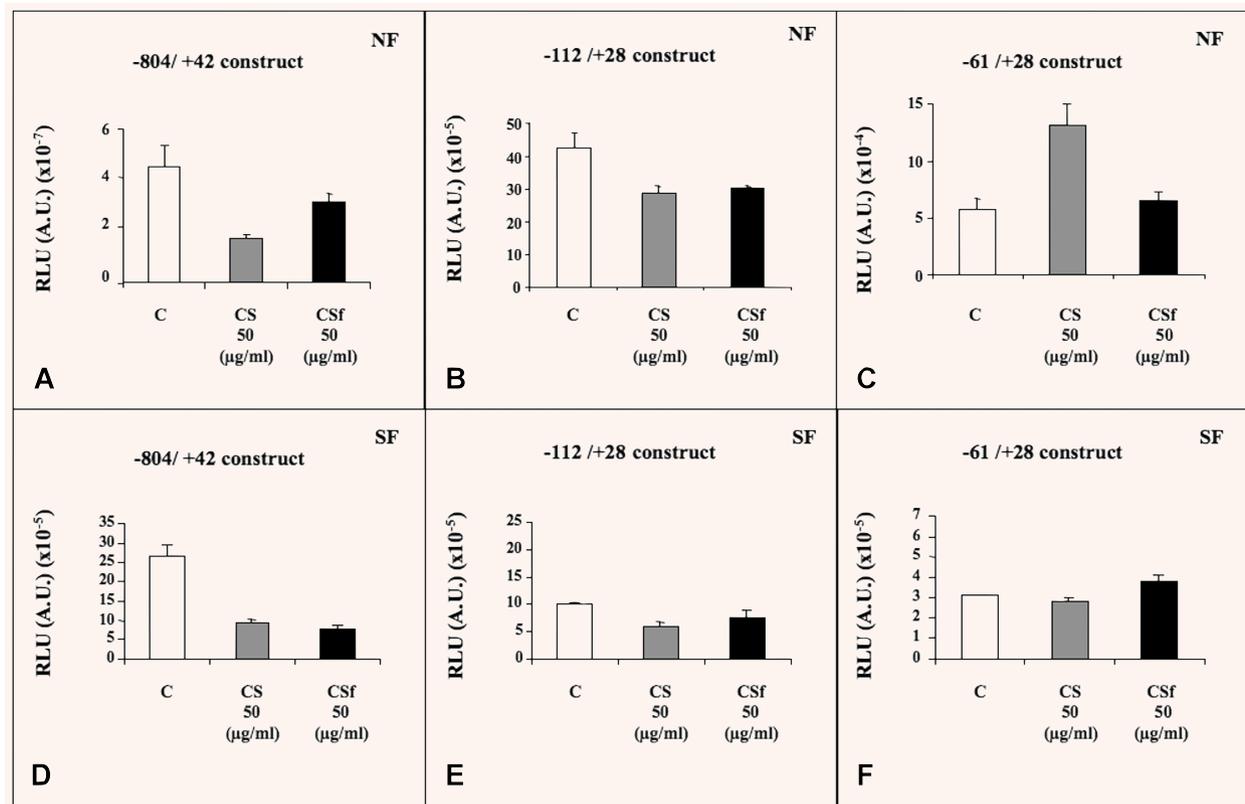


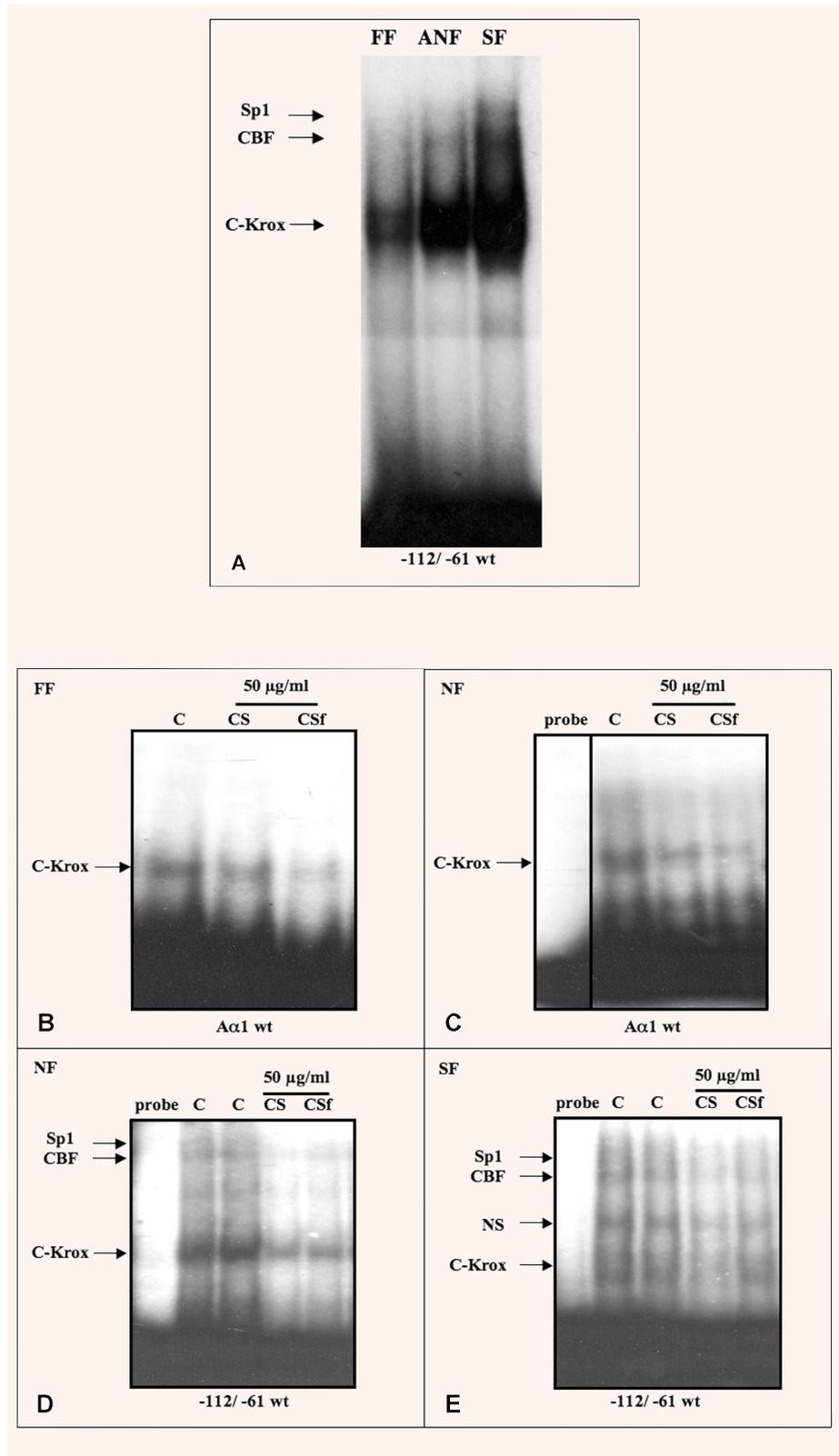
Fig. 4 Effect of CS or CSf on the transcriptional activity of the human *COL1A1* gene in normal and scleroderma fibroblasts. (**A, B, C**) Normal fibroblasts. (**D, E, F**) Scleroderma fibroblasts. Fibroblasts were transiently transfected with 2 µg of different *COL1A1* reporter plasmids, together with the expression vector pSV40-Bgal (2 µg). After 12 hrs of transfection, the medium was changed and the cells were incubated with or without CS or CSf at 50 µg/ml, in DMEM containing 10% FCS. Six hours later, the samples were harvested and protein content, luciferase and β-galactosidase activities were assayed. Each set of transfection was performed in triplicate. Transcriptional activity of each construct was expressed as relative luciferase activity after correction for transfection efficiency and protein amounts.

down-regulating the transcription of type I collagen gene through modulation of both expression and binding activity of the major transcription factors which recognize this promoter sequence.

Fibroblasts from affected scleroderma skin cultured *in vitro* produce excessive amounts of type I and type III collagens [39, 40], and display increased transcription of corresponding genes [41, 42]. It has been suggested that the transcriptional activation of dermal fibroblasts in scleroderma may be a result of stimulation by autocrine TGF-β signalling. In this regard, a Smad-binding element has been found in the *COL1A2* promoter [41]. This sequence (SBE, bp -263 and -258 in the α2(I) collagen promoter) has been proposed as the only *cis*-regulatory element mediating the intrinsic up-regulation of type I collagen in SF [41]. However, a recent study has revealed that the bp -353 to -264 promoter fragment as well as the bp -264 to -186 fragment containing SBE is a *cis*-regulatory element in SF [43]. Furthermore, it has been found that the mutation of SBE in α2(I) collagen promoter still responds to TGF-β in normal fibroblasts [44]. Thus, these

data suggest that, although Smad proteins are key factors in the TGF-β signalling process in SF, other transcription factors are likely to be involved in the up-regulation of collagen gene expression in scleroderma. Our findings further support this hypothesis since no modulation of the DNA-binding activity of the Smads to the SBE probe was observed in both cell types. They rather suggest that the CS-induced down-regulation of *COL1A1* promoter in both normal and SF implies CBF and GC-binding proteins, including Sp1, and c-Krox, acting through a more proximal region of the promoter (-112/-61 bp). This is also in agreement with previous investigations demonstrating that the transcriptional activity of the *COL1A1* promoter is regulated by Sp1 binding both under basal conditions [32, 34] as well as under the stimulation of TGF-β. Furthermore, elevated Sp1 binding has been detected in fibrotic diseases, such as scleroderma, as well as in models of experimentally induced fibrosis during periods of enhanced *COL1A1* transcription [33, 45, 46]. Since hc-Krox and Sp3 can bind to the same *cis*-elements as Sp1 and play important roles in the control of

Fig. 5 Effect of CS or CSf on the DNA-binding of C-Krox and Sp1 to the $\text{A}\alpha 1$ wt and -112/-61 wt probes. DNA-binding was analysed by electrophoretic mobility shift assay (EMSA). Labelled double stranded $\text{A}\alpha 1$ wt and -112/-61 wt probes were incubated with 7.5 μg of nuclear extracts from foreskin fibroblasts (FF) (**A**, **B**), adult NF (ANF) (**A**, **C**, **D**) and SF (**A**, **E**), treated or not with CS or CSf (50 $\mu\text{g}/\text{ml}$). Protein-DNA complexes are indicated by an arrow.



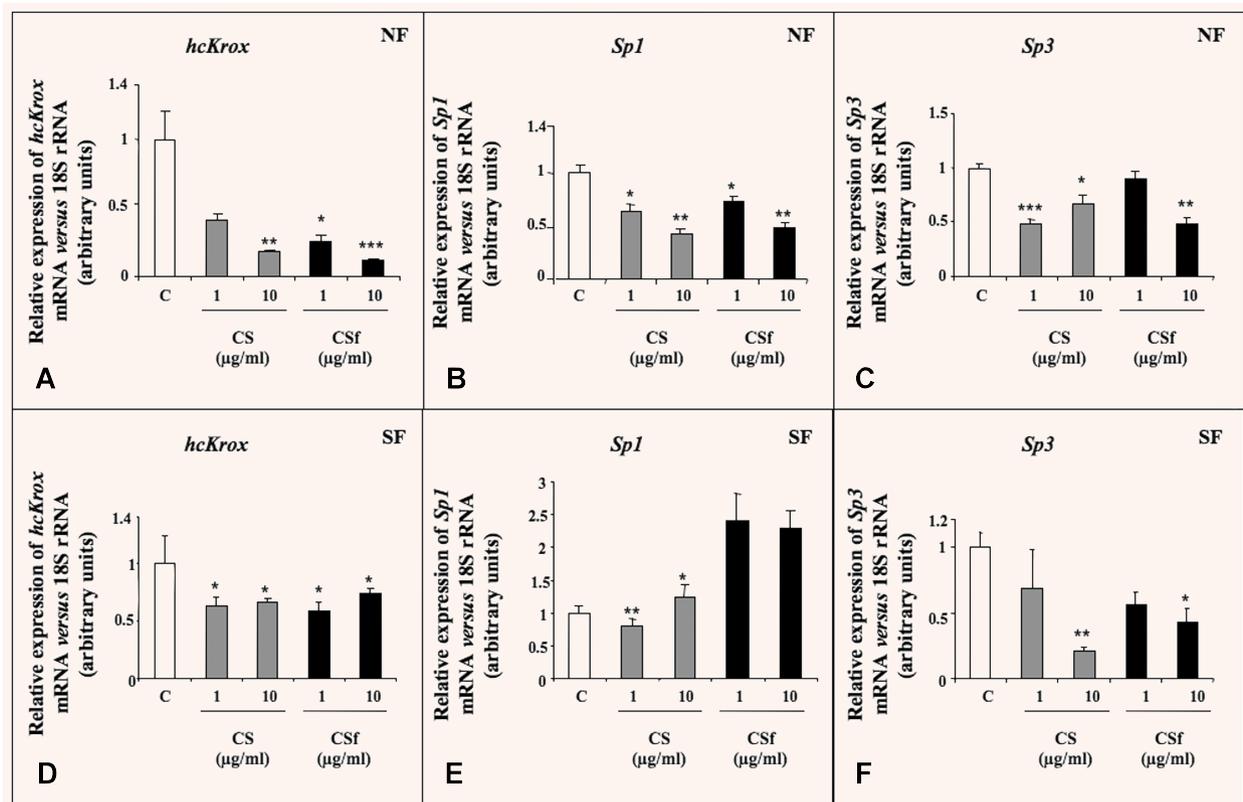


Fig. 6 Effect of CS and CSf on the steady-state levels of C-Krox, Sp1 and Sp3 mRNA in cultured fibroblasts. (A, B, C) Normal fibroblasts. (D, E, F) Scleroderma fibroblasts. Total RNAs (2 µg) from fibroblasts treated or not with CS or CSf (1, 10 µg/ml) were reverse-transcribed into cDNAs. The resulting products were diluted (1/100) and analysed by real time RT-PCR using specific primers for *hc-Krox* (A, D), *Sp1* (B, E), *Sp3* (C, F) mRNA and *18S* rRNA. Results were normalized to *18S* rRNA and presented as relative levels of *hc-Krox*, *Sp1*, *Sp3* mRNA. Statistical significance was evaluated using the Student's t-test. * $P < 0.05$; ** $P < 0.01$ and *** $P < 0.001$.

COL1A1 expression [29, 35, 37], it is likely that these factors may also contribute to modulate the CS down-regulation of type collagen synthesis in both normal and SF. This is supported by the decreased expression and binding activity of these factors in the CS-treated cells.

Although Smad-binding elements do not appear as playing a role in the mechanism of CS down-regulation of *COL1A1* promoter activity in SF, we cannot rule out that Smad proteins, such as Smad3, could interact with the -112/-61 bp complex, possibly by protein-protein affinity. Indeed, it has been reported an increased interaction of Smad3 with Sp1 in SF [47]. Furthermore, it has been recently shown that Smad3, Sp1 and Ets1 form a transcriptionally active complex, leading to the $\alpha 2(I)$ collagen promoter activation in SF [43]. Current characterization of the transcription factors associated with Sp1 at the level of the -112/-61 bp promoter, using immunoprecipitation and ChIP procedures, will probably provide information on this possibility.

Since a number of reports have indicated that TGF- β signalling plays an important role in up-regulation of type I collagen

promoter activity in SF, it was of interest to determine whether the inhibitory effect of CS could involve a down-regulation of the main elements of TGF- β signalling pathway. Our results indicate that there were no significant differences in the expression of the TGF- β receptor II and the inhibitory Smad7 at the protein level. Similarly, Smad2/3 protein amounts were not affected in CS-treated fibroblasts (data not shown). On the other hand, we found a clear down-regulation of the receptor I protein expression, suggesting that the mechanism underlying the effect of CS on collagen synthesis by normal and SF may involve a reduced sensitivity to TGF- β caused by decreased expression of the signalling receptor I. However, potential consequences on the downstream signalling messengers, including Smad proteins, remain to be elucidated. In this regard, it must be noted that the CS effect may not necessarily implicate alterations of the Smad pathway. Recent reports have identified non-Smad pathways including protein kinase C (PKC)- δ , geranyl transferase I or p38 as the participants in the regulation of expression of various matrix genes by TGF- β family [48, 49]. Further research is

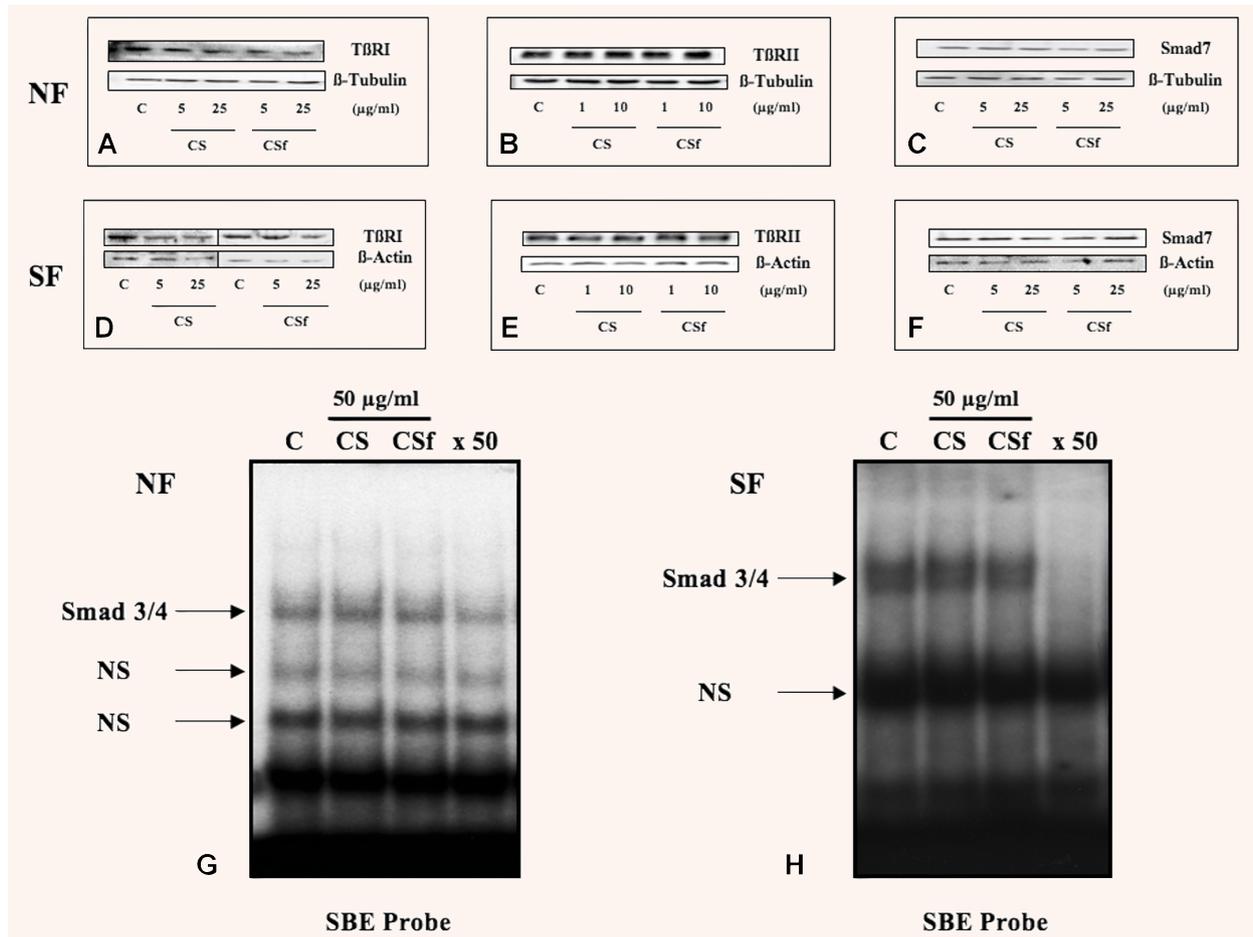


Fig. 7 Effect of CS or CSf on the protein expression of TβRI, TβRII and Smad7 in cultured fibroblasts. (A, B, C, G) Normal fibroblasts. (D, E, F, H) Scleroderma fibroblasts. 15 μg of NF or SF cytoplasmic extracts incubated in the presence or absence of CS or CSf (1–25 μg/ml), were separated on a 8% polyacrylamide gel in denaturing conditions. Then, proteins were transferred to a phenylmethylsulfonylfluoride (PVDF) membrane, and reacted with polyclonal antibody against TβRI (53 kD) (A, D), TβRII (75 kD) (B, E), Smad7 (47 kD) (C, F), β-Tubulin (55 kD) (A, B, C), and monoclonal anti-actin (40 kD) (D, E, F). Protein bands were revealed with a peroxidase coupled secondary antibody using a Western blot kit detection. DNA-binding was analysed by EMSA. Labelled double-stranded SBE probe was incubated with 7.5 μg of nuclear extracts from NF (G) and SF (H), treated or not with CS or CSf (50 μg/ml). Competition experiments were performed using a 50-fold molar excess of cold wild-type probe (indicated as ×50). Protein-DNA complexes are indicated by an arrow.

required to determine whether CS can modulate some of these messengers in dermal fibroblasts.

Indeed, very few data are available on the interaction mechanisms of CS with the cell membrane and the subsequent intracellular signalling pathways which are elicited by the GAG. Annexin 6 has been shown to act as a cell surface receptor for CS chains in human fibroblast WI38 and chick embryonic fibroblasts, which could be responsible of the anti-adhesive properties of the molecule [15]. Since annexin 6 has been found to make a complex with p120GAP (Ras GTPase-activating protein), Fyn (Src family kinase) and Pyk2 (focal adhesion kinase family member), we may hypothesize that the interaction of CS and annexin 6 causes the state

change of these signalling molecules [17]. Current study has been undertaken to clarify this point and further elucidate its relationships with the transcriptional machinery of type I collagen promoter. It has been suggested that low-molecular form of CS could be more effective in improving the symptoms of OA, as judged by oral administration in a rat model of the disease [50], probably because of a better absorption of the GAG. Therefore, it was tempting to search if a difference could also exist between the low- and high-molecular form of CS in our *in vitro* system. From the present data, we may deduce that the effects observed were globally similar for both CS molecular sizes, suggesting that they are equally capable of binding to fibroblasts and elicit the same signalling messengers.

In conclusion, besides its anti-adhesive and anti-inflammatory properties, chondroitin sulphate appears as a GAG capable of exerting anti-fibrotic effects by down-regulating fibroblast collagen synthesis. The present findings suggest that it may be useful in preventing peritoneal adhesions as well as in long-term treatment of any fibrotic situation, including scleroderma and hypertrophic scars.

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