N-Acetyl-L-Cysteine abrogates fibrogenic properties of fibroblasts isolated from Dupuytren's disease by blunting TGF-β signalling

Jürgen Kopp [a] *, Harun Seyhan [a], Bastian Müller [a], Johanna Lanczak [a], Elke Pausch [a], Axel M. Gressner [b], Steven Dooley [c], Raymund E. Horch [a]

[a] Department of Plastic and Hand Surgery, University Medical Center, Erlangen, Germany
[b] Institute of Clinical Chemistry and Pathobiochemistry, RWTH University Hospital, Aachen, Germany
[c] Center of Molecular Alcohol Research, II. Medical Clinic, Faculty of Medicine Mannheim, University of Heidelberg

Abstract

Dupuytren's disease, a benign fibroproliferative disorder of the palmar fascia, represents an ideal model to study tissue fibrosis. Transforming growth factor-β_1 (TGF-β_1) and its downstream Smad signalling system is well established as a key player during fibrogenesis. Thus, targeting this basic pathomechanism seems suitable to establish new treatment strategies. One such promising treatment involves the substance N-acetyl-L-cysteine (NAC), shown to have antifibrotic properties in hepatic stellate cells and rat fibroblasts. In order to investigate antifibrotic effects of N-acetyl-L-cysteine (NAC), fibroblasts were isolated from surgically resected fibrotic palmar tissues (Dupuytren fibroblasts, DF) and exposed to different concentrations of NAC and recombinant TGF-β_1. Fibroblasts isolated from tendon pulleys served as controls (control fibroblasts, CF). Smad signalling was investigated by a Smad binding element driven reporter gene analysis. Both cell types express TGF-β_1, indicating autocrine signalling in DF and CF. This was confirmed by comparing reporter gene activity from LacZ and Smad7 adenovirus infected cells. NAC treatment resulted in abrogation of Smad mediated signalling comparable to ectopically overexpressed Smad7, even when the cells were stimulated with recombinant TGF-β_1 or ectopically expressed a constitutively active TGF-β receptor type I. Additionally, NAC dose-dependently decreased expression of three major indicators of impaired fibrotic matrix turnover, namely alpha-smooth muscle actin (α-SMA), α 1 type I procollagen (Col1A1), and plasminogen activator inhibitor-type I (PAI-1). Our results suggest that TGF-β signalling and subsequent expression of fibrogenesis related proteins in Dupuytren's disease is abrogated by NAC thus providing a basis for a therapeutic strategy in Dupuytren's disease and other fibroproliferative disorders.

Keywords: signal transduction • fibrogenesis • adenoviral gene transfer • Dupuytren's disease

Introduction

* Correspondence to: Dr. med. Jürgen KOPP
Abteilung Plastische und Handchirurgie, Chirurgische Universitätsklinik Erlangen-Nürnberg, Krankenhausstrasse 12
91054 Erlangen, Germany
Tel.:+49 9131 8533277
Fax:+49 9131 8539327
E-mail:juergen.kopp@chir.imed.uni-erlangen.de

Dupuytren's disease (DD) is a widespread benign fibroproliferative disorder of the palmar fascia that often results in severe disfigurement of hands and fingers with enormous functional impact. Most patients suffering from DD are native to or descendants from northern Europe, where approximately 10% of the male population older than 65 years is

affected [1]. Many etiological factors were suggested as candidates responsible for individual development of Dupuytren's disease, *e.g.* alcoholism [2], smoking [3], diabetes mellitus [4], trauma [5] and genetic predisposition [6]. The diseased aponeurotic tissue possesses biological features of benign neoplastic fibromatosis and was recently characterized as a useful model of fibrosis because it displays the entire temporal and histological architecture of cells, cytokines and extracellular matrix involved in fibroproliferative processes [7]. Until now, only surgical therapy, namely partial and total aponeurectomy, is established in patients suffering from palmar fibrosis, however, recurrence rates in individuals undergoing surgery are still high [8, 9]. Thus, new therapeutic approaches are required for more effective treatment.

Similar to other fibroproliferative disorders, TGF-β is a pivotal factor during pathogenesis of palmar fibrosis by initiating a specific signalling pathway mediated by Smad proteins [10]. Given the critical role of TGF-β in fibrogenesis, promising new therapeutic strategies exist that target its profibrogenic signalling route in order to decrease cytokine dependent overexpression of extracellular matrix (ECM) proteins in Dupuytren's disease. Recently, N-acetyl-L-cysteine (NAC) was identified as a ubiquitously available drug capable of blocking TGF-β-dependent Smad pathway signalling in hepatic stellate cells and rat fibroblasts [11, 12], thereby attenuating profibrotic capabilities of the cells.

This study addressed the question whether application of NAC can downregulate TGF-β dependent gene responses and protein expression in fibroblasts isolated from Dupuytren's disease.

Materials and methods

Materials

All reagents for cell culture (Dulbecco's modified Eagle medium (DMEM), fetal calf serum (FCS), Trypsin-EDTA and phosphate-buffered saline (PBS)) were from Gibco-BRL (Life Technologies, Rockville, USA) or BIOCHROM (Berlin, Germany). The monoclonal anti β-actin antibody was from Sigma (Munich, Germany). Polyclonal antibodies against PAI-1 and Col1A1 were from Santa Cruz Biotechnologies Inc., CA, USA. The monoclonal α-SMA antibody was from DAKO, Hamburg, Germany. The luciferase assay system was from Promega (Mannheim, Germany). All other chemicals were obtained from commercial sources at the purest grade available.

Cell culture

Tissues from Dupuytren's disease were obtained either by partial or total aponeurectomies of affected palmar fascias of 12 male (61 y ± 8.9) and 5 female (67 ± 9.3) northern European patients. All individuals suffered from contractures of the fingers rated as third degree deformities according to the Tubiana score [13]. Obtained tissues were separated by macroscopic appearance into cord and nodule origin and cultured separately. Normal tendon pulley fibroblasts were obtained from patients undergoing incision of tendon pulleys to treat tendovaginitis stenosans and served as controls. Written informed consent to the research use of material was obtained from all patients. The research protocol applied during the experiments was approved by the Ethics Committee of the Medical Faculty of the University of Erlangen.

Fibroblasts, either derived from cords of Dupuytren's disease (DF) or control tissues (CF), were isolated by using the explant culture technique as described [14] and grown in DMEM (Glucose 1.0 g/l, $NaHCO_3$ 3.7 g/l, N-Acetyl-L-alanyl-L-glutamine 1.0289 g/l, BIOCHROM) supplemented with 10% FCS, 40 µg/ml Gentamycin (GIBCO/BRL) without additional growth factors in 75 cm^2 culture flasks. All cultures were maintained at 37°C, 5% CO_2 in a humidified atmosphere. When cultures reached 70–80 % confluence, cells were subcultured or cryopreserved using 10% DMSO. For adenoviral infection, fibroblasts were seeded in 58 cm^2 Petri culture dishes (Greiner, Frickenhausen, Germany).

Vectors and gene transfer

Adenoviral stocks for $(CAGA)_9$-MLP-Luc were created using the Adeasy cloning and recombination procedure (Quantum, Appligene, www.quantum-appligene.com). Infections and coinfections of fibroblasts isolated from Dupuytren's disease and healthy tendon pulleys were performed according to other reports [15]. Routine infections were accomplished with 2 x 10^5 Dupuytren fibroblasts (DF) or control fibroblasts (CF) following second subculture at 50 m.o.i. with

single virus clones of the same virus stock preparation. Infection efficiency was proven by adenoviral constructs expressing β-galactosidase and, each time, about 90% of the cells were infected. Staining of infected cells was performed using an X-Gal staining kit (Roche, Mannheim, Germany). To perform luciferase assays, cells were cultured for 2 days in DMEM with 10 % FCS, and then infected with adenoviruses as indicated. Lysates for luciferase detection were prepared 24 hours after adenoviral infections. Adenoviral stocks for constitutively active TGF-β receptor I (AdcaTβRI) and Smad7 (AdSmad7) were described previously [16] and kindly provided by A. Moustakas, Ludwig Institute for Cancer Resarch, Uppsala, Sweden.

In vitro application of NAC and TGF-β₁

Following second subculture, CF and DF were adenovirally infected with Ad-(CAGA)$_9$-MLP-Luc [17]. Infected fibroblasts were treated with 5 or 10 mM NAC and 5 ng/ml TGF-β₁ (R&D Systems, Wiesbaden, Germany) separately or in combination as indicated. Untreated cells served as controls. Luciferase activity was determined 6h following NAC exposition and determined as light cycles per second and well (LCPS/well).

Preparation of total cell lysates, nuclear extracts and Western blot analysis

Total lysates from CF and DF, treated with 5 mM or 10 mM NAC and untreated were prepared with 0.9 ml RIPA buffer [1U Tris buffered saline (TBS), 1% Nonidet P-40 (Amresco, Solon, USA), 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS)]; protease inhibitors were provided as Protease Inhibitor Mix (Roche) just before use according to manufacturer's instructions. To inhibit protein dephosphorylation, Phosphatase Inhibitor Mix (Sigma) was added. Using a syringe fitted with a 21 gauge needle to shear DNA, lysates were transferred to a microcentrifuge tube. 10 µl of a 10 mg/ml PMSF stock was added, followed by a 30 to 60 min incubation on ice. Subsequently, cell lysates were cleared by centrifugation at 15.000 x g for 20 min at 4°C. 40 µl of lysates were separated by SDS-8% polyacrylamide gel electrophoresis (PAGE) and transferred to a 0.45

µm nitrocellulose membrane (Protran BA 85; Schleicher&Schüll, Dassel, Germany) for Western blot analysis to determine expression of PAI-1, COL1A1 and α-SMA as previously described [18]. Individual band intensities were analyzed using Scion Image for Windows (Release Beta 4.0.2, Scion Corporation, Frederick, MA, USA) and calculated in relation to obtained β-actin blots.

Statistical analysis

For statistical analysis of results obtained from Ad-(CAGA)$_9$-MLP-Luc experiments, GraphPad Prism (GraphPad Software for Science Inc., San Diego, California, USA) version 3.02 was used by applying the two tailed unpaired Student t test with a P value for significance set at least at 0.05.

Results

Reporter gene-infected DF and CF cells demonstrate functional Smad activity that can be inhibited by ectopic expression of Smad7

To evaluate intrinsic TGF-β downstream signal transduction, DF and CF were infected with an adenovirus containing a strongly responsive (CAGA)$_9$-MLP-luc sequence, including 9 copies of a PAI-1 specific TGF-β response element [17]. Infection rates were about 90%, as examined by microscopical galactosidase activity after Ad-LacZ infection (Fig. 1A). Both cell populations showed intrinsic stimulation of reporter gene activity with total counts of luciferase activity being higher in DF compared to CF in all experiments, indicating enhanced autocrine stimulation (Fig. 1B). Overexpression of a constitutively active TGF-β type I receptor (AdcaTβRI) resulted in a 38-fold increase of reporter activity in CF [(7.767 ± 2.003), $n = 12$ *vs.* (297.267 ± 181.392) $n = 12$, $P < 0.0001$] and a 20-fold upregulation in DF [(29.1 ± 1.664), $n = 12$ *vs.* (604.667 ± 187.763) $n = 12$, $P < 0.0001$]. Co-infection with AdSmad7 reduced autocrine stimulated luciferase activity significantly below basal levels in both cell populations [CF: (7.767 ± 2.003),
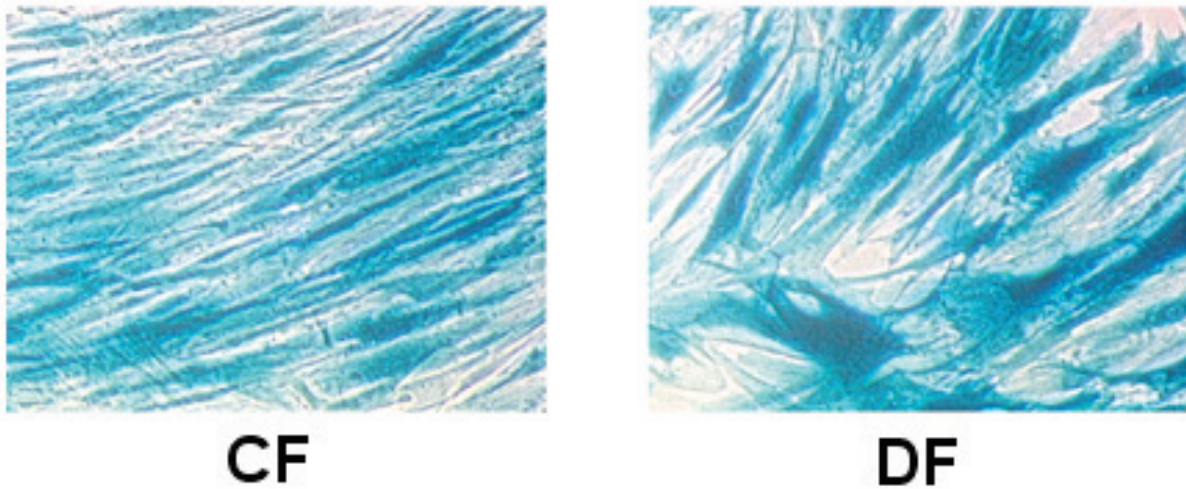
Fig. 1 Smad dependent TGF-β signalling in control (CF) and Dupuytren derived fibroblasts (DF) - Inhibition of autocrine stimulation by Smad7. 12 individual preparations of both cell populations were infected with adenovirus recombinants at 50 m.o.i. as indicated. (A) Infection rate was generally higher than 90%, as measured by positive β-Gal staining after AdLacZ infection.
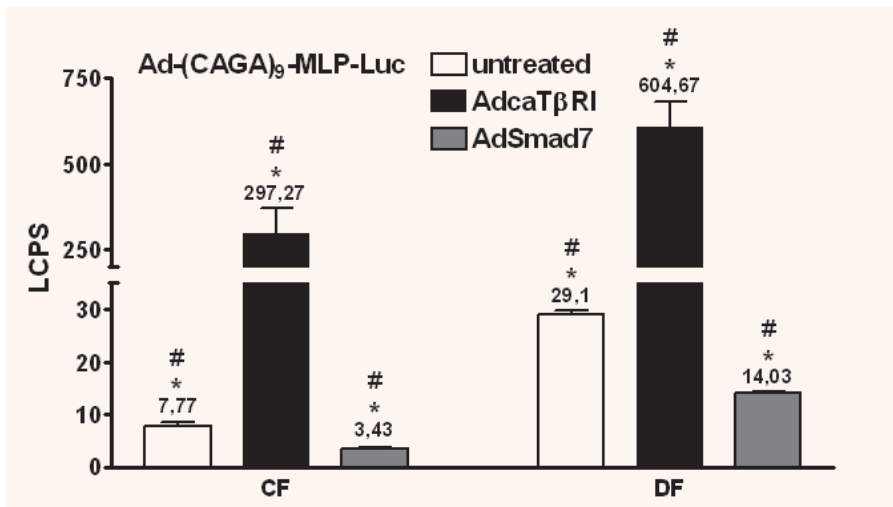

Fig. 1 (B) (CAGA)₉-MLP-luc dependent luciferase activity was measured 2 days after infection. The presented data are based on at least three independent experiments. LCPS, luciferase counts per second from collected data were measured ± SD and are indicated on top of each bar. Statistically significant differences (p < 0.05; Students t-Test) between CF and DF are indicated by #, and between various treatments within each cell type by *.

$n = 12$ *vs.* (3.433 ± 0.929) $n = 12$, $P < 0.0001$; DF: (29.1 ± 1.664), $n = 12$ *vs.* (14.033 ± 0.961) $n = 12$, $P < 0.0001$]. Throughout all experiments, data obtained from DF cells revealed significantly higher LCPS counts than CF when directly compared [CF *vs.* DF: (7.767 ± 2.003), $n = 12$ *vs.* (29.1 ± 1.664) $n = 12$, $P < 0.0001$; AdcaTβRI CF *vs.* AdcaTβRI DF: (297.267 ± 181.392), $n = 12$ *vs.* (604.667 ± 187.763) $n = 12$, $P < 0.0004$; AdSmad7 CF *vs.* AdSmad7 DF: (3.433 ± 0.929), $n = 12$ *vs.* (14.033 ± 0.961) $n = 12$, $P < 0.0001$]

TGFβ₁ dependent activation of (CAGA)₉-MLP-luc is downregulated by NAC

To evaluate the impact of NAC on TGF-β downstream signal transduction, CF and DF were infected with Ad(CAGA)₉-MLP-luc and exposed to 5 and 10 mM NAC (Fig. 2A). Both doses significantly reduced autocrine-stimulated luciferase activity in CF and DF, proving 10mM NAC more effective than 5 mM [CF *vs.* CF + 5 mM NAC: (233.667 ± 12.472), $n = 12$ *vs.* (136.333 ± 18.856)

Fig. 2 Inhibition of Smad dependent TGF-β signalling in DF and CF by N-acetyl-L-cysteine. 12 individual preparations of both cell populations were infected at 50 m.o.i. with adenoviral constructs as indicated. (A) Dose-dependent inhibition of autocrine TGF-β stimulation with NAC. Cells were infected with $(CAGA)_9$-MLP-luc. 24h later, 5 and 10 mM NAC were applied and reporter activity was evaluated by measuring luciferase activity as LCPS (luciferase counts per second) after 1.5h.
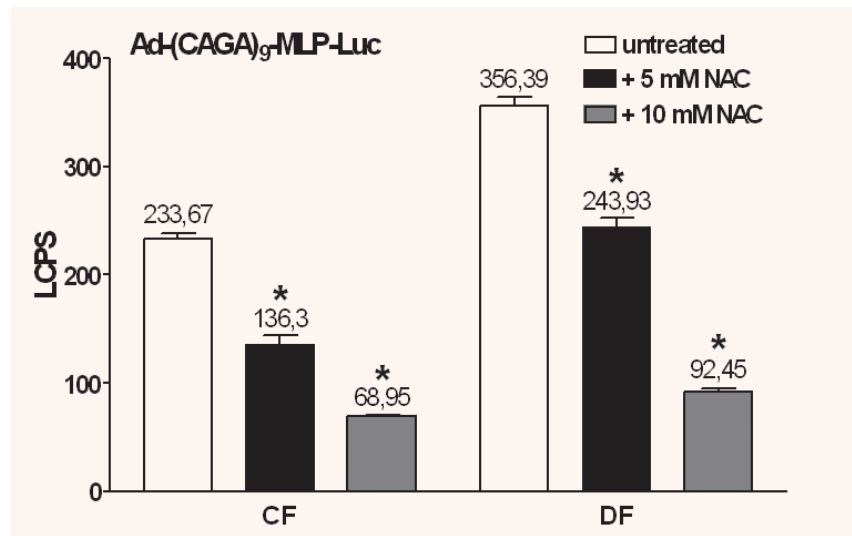

Fig. 2 (B) To further substantiate dose-dependent inhibitory capabilities of NAC, $(CAGA)_9$-MLP-luc infected CF and DF were stimulated with recombinant TGF-β_1 24h following adenovirus infection. NAC was applied 30 min before TGF-β stimulation and $(CAGA)_9$-MLP-luc dependent luciferase activity was measured 1.5h following TGF-β treatment. The presented collected data represent three independent experiments, each performed in quadruplicate (* indicates $p \leq 0.05$, Students t-Test).
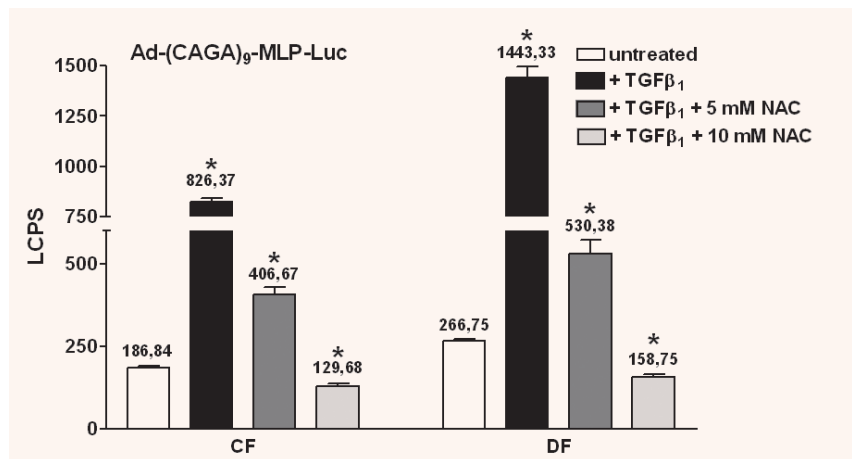
$n = 12$, $P < 0.0001$; CF *vs*. CF + 10 mM NAC: (233.667 ± 12.472), $n = 12$ *vs*. (68.949 ± 4.231) $n = 12$, $P < 0.0001$; DF *vs*. DF + 5 mM NAC: (356.387 ± 18.856), $n = 12$ *vs*. (243.927 ± 20.549) $n = 12$, $P < 0.0001$; DF *vs*. DF + 10 mM NAC: (356.387 ± 18.856), $n = 12$ *vs*. (92.454 ± 6.253) $n = 12$, $P < 0.0001$].

To further substantiate the suppressive effect of NAC, cells were additionally stimulated with 5 ng/ml TGF-β_1 1h following exposition to 5 and 10 mM NAC and reporter activity was evaluated 3h later (Fig. 2B). While stimulation with TGF-β_1 resulted in significant 4.4-fold stimulation in CF [(186.842 ± 10.473), $n = 12$ *vs*. (826.374 ± 44.969) $n = 12$, $P < 0.0001$] and 5.4-fold increase of reporter activity in DF [(266.749 ± 14.856), $n = 12$ *vs*. (1443.333 ± 134.247) $n = 12$, $P < 0.0001$], 5 as well as 10 mM NAC decreased $(CAGA)_9$-MLP-luc reporter gene activity. Treatment with 10 mM NAC was sufficient

to reduce gene response levels significantly below intrinsic basal levels in TGFβ_1 treated CF and DF [CF *vs*. CF + TGF-β_1 + 10 mM NAC: (186.842 ± 10.472), $n = 12$ *vs*. (129.679 ± 17.804) $n = 12$, $P < 0.0001$; DF *vs*. DF + TGF-β_1 + 10 mM NAC: (266.749 ± 14.856), $n = 12$ *vs*. (158.753 ± 21.454) $n = 12$, $P < 0.0001$].

Application of NAC downregulates protein expression in CF and DF

Cell lysates of CF and DF were screened for expression of α-SMA, Col1A1 and PAI-1 as major indicators for impaired fibrotic matrix turnover under standard culture conditions and following exposure to NAC (Fig. 3). 5 as well as 10 mM N-acetyl-L-cysteine distinctly reduced expression of α-SMA [CF *vs*. CF + 5 mM NAC = 86.43 *vs*. 60.49 = -30.01%; CF *vs*. CF + 10 mM NAC = 86.43 *vs*.
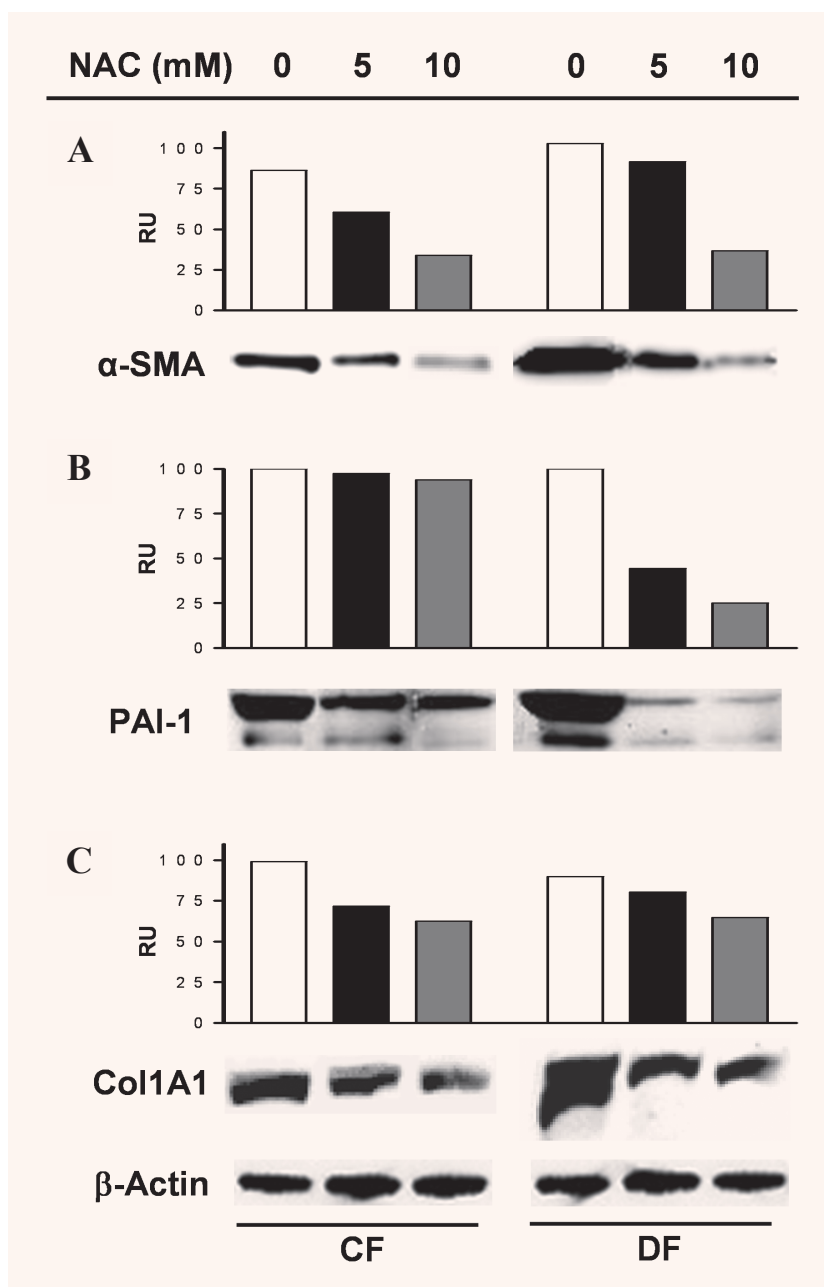
Fig. 3 Downregulation of myofibroblast marker protein expression in CF and DF by N-acetyl-L-cysteine treatment. Protein extracts were isolated from CF and DF cells cultured with or without 5 and/or 10 mM NAC. 40 µg cell lysates were analyzed by SDS-PAGE followed by Western blotting and immunodetection, using specific antisera against α-SMA (A), PAI-1 (B) and Col1A1 (C). Three representative blots are shown. CF, control fibroblasts; DF, fibroblasts derived from Dupuytren's disease. Experiments with the same samples were performed in duplicate. Band intensities were normalized to β-actin and measured in relative units [RU].

33.93 = -60.74%; DF *vs.* DF + 5 mM NAC = 100,01 *vs.* 91.55 = -8.46%; DF *vs.* DF + 10 mM NAC = 100,01 *vs.* 36.62 = -63.38%] (Fig. 3A) and Col1A1 [CF *vs.* CF + 5 mM NAC = 98.95 *vs.* 71.64 = -27.6%; CF *vs.* CF + 10 mM NAC = 98.95 *vs.* 62.46 = -36.87%; DF *vs.* DF + 5 mM NAC = 89.83 *vs.* 80.25 = -10.66%; DF *vs.* DF + 10 mM NAC = 89.83 *vs.* 64.65 = -28.03%] (Fig. 3B) in both cell populations. Expression of PAI-1 however was clearly downregulated only in DF [DF *vs.* DF + 5 mM NAC = 102.81 *vs.* 44.33 = -56.88%; DF *vs.* DF + 10 mM NAC = 106.34 *vs.* 25.16 = -75.53%] whereas evaluated proteins in CF were just slightly reduced [CF *vs.* CF + 5 mM NAC = 106.34 *vs.* 97.33 = -8,47%; CF *vs.* CF + 10 mM NAC = 106.34 *vs.* 93.92 = -11.68%].

Discussion

On the molecular level, there is evidence that Dupuytren's disease is influenced by a variety of growth factors [19, 20]. However, the transforming

growth factor beta isoforms TGF-β_1 and TGF-β_2 have been demonstrated to be the most potent biochemical effectors during fibrogenesis of the palmar fascia [21]. Since TGF-β_2 has a strong effect on cell proliferation, TGF-β_1 causes cytokine depending expression of α-SMA that has been identified as a critical hallmark in many fibroproliferative disorders. Also in Dupuytren's disease, it represents the differentiation of fibroblasts to myofibroblasts, a crucial step during pathogenesis [22–24]. Further, fibromatosis of the palmar fascia is characterized by excessive accumulation of collagen organized as cords and nodules [25, 26]. In Dupuytren's disease, expression of both proteins is a prerequisite for contractile phenomena to occur on fingers as well as on the *palma manus* [22]. Whereas in healthy connective tissues protein turnover is balanced through a fine equilibrium of matrix deposition and degradation processes, fibrotic tissues show high levels of PAI-1 expression, thereby blocking catabolic processes in involved tissues and organs [27]. Like in Dupuytren's disease, TGF-β_1 has already been identified as crucial factor in other fibroproliferative diseases, *e.g.* in liver [8, 9, 15, 28, 29], lung [30, 31] and kidney fibrosis [32] as well as in hypertrophic scar formation [33, 34].

Our experiments demonstrate that fibroblasts isolated from Dupuytren's disease exhibit elevated endogenous levels of TGF-β_1, a phenomenon, we have already described in hypertrophic scar derived fibroblasts [33]. Considering these findings, we have to postulate that progression of fibroproliferative processes in inflicted palmar fascias might be based on a self-perpetuating mechanism.

TGF-β_1 signalling is initiated by binding of the cytokine to type I and II receptors [35]. The activated type I receptor phosphorylates a subset of Smads, known as receptor-regulated Smads (R-Smads), which associate with Smad4 and move into the nucleus [36, 37]. In the nucleus, this complex may associate with specific DNA-binding proteins that direct it to the regulatory region of target genes encoding for proteins like procollagen, α-SMA, fibronectin or PAI-1 [17]. Smad mediated signalling itself is regulating the expression of specific inhibitory Smads (I-Smads) capable of blocking the phosphorylation of Smad 2/3, thereby providing a regulatory system in healthy tissues. TGF-β_1 signalling is blocked by Smad7,

whereas Smad6 is specific for bone morphogenetic protein signalling.

In hypertrophic scar derived fibroblasts, we have already demonstrated that ectopic overexpression of Smad7 abrogates the TGF-β signalling, thereby providing a therapeutic option for the treatment of fibrotic disorders [33]. However, since long term effects of *in vivo* overexpression or application of Smad7 remains unclear, other options have to be identified in order to establish new clinical strategies.

Recently, Meurer *et al.* identified NAC as a dose-dependent antagonist of TGF-β signalling as measured by reporter gene activation, Smad2 and Smad3 phosphorylation, and regulation of Smad7 mRNA expression [11].

Our results show that the application of NAC has comparable blunting effects on TGF-β signalling as ectopically overexpressing the natural antagonist Smad7. Related to the applied concentration, (CAGA)$_9$-MLP-luc reporter activity was downregulated, thus indicating abrogation of cytokine specific signalling in a dose-dependent manner. Importantly, in-parallel expression of proteins relevant for matrix accumulation and tissue contraction is reduced below basal levels.

Details of how NAC provides anti-TGF-β action are still speculative. Meurer *et al.* have suggested that these effects are based on disintegration of TGF-β and TGF-β receptor endoglin complex formation, as well as a reduced ligand binding capacity to betaglycan [11].

On the other hand, NAC is an efficient antioxidant and decreases oxidative stress, which was previously determined as a favouring multiplier of profibrogenic TGF-β effects [38–40].

Since we applied NAC only for the short incubation period of 6h, its therapeutic use in Dupuytren's contracture would require longer administration. The effects of long-term administration of NAC will require further investigation, as its antioxidant properties might delay senescence or apoptosis in resident cells and might be influenced by the age of the recipient [41, 42].

Details regarding anti-TGF-β and the antifibrotic function of NAC need to be established by further research. Since NAC is a ubiquitously available drug with no considerable side effects during short application periods, we consider its use a possible option in future clinical approach, especially in patients suffering from relapses of Dupuytren's disease.

Acknowledgement

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