

ORIGINAL ARTICLE

Opposite effect of fluticasone and salmeterol on fibronectin and tenascin-C expression in primary human lung fibroblasts

M. Degen^{*1,2}, S. Goulet^{†2}, J. Ferralli^{*}, M. Roth[†], M. Tamm[†] and R. Chiquet-Ehrismann^{*}

^{*}Friedrich Miescher Institute for Biomedical Research, Novartis Research Foundation, Basel, Switzerland and [†]Department of Research, Pulmonary Cell Research, University Hospital, Basel, Switzerland

Clinical & Experimental Allergy

Summary

Background Airway remodelling is a key feature of asthma and chronic obstructive pulmonary disease (COPD). The remodelling process involves the deposition of extracellular matrix (ECM) proteins within the airways. Current therapies for asthma and COPD consist of inhaled corticosteroids and long-acting β_2 -agonists (LABA). However, their effect on airway remodelling is not well understood so far.

Objective In this study we investigated the effect of fluticasone and salmeterol, either alone or in combination, on fibronectin and tenascin-C protein, isoform, and mRNA levels in primary human lung fibroblasts.

Methods In our model, fibroblasts cultured in serum-free medium represented a non-inflammatory condition and stimulation with 5% fetal calf serum and/or TGF- β_1 mimicked a pro-fibrotic environment with activation of tissue repair. Using these two different conditions, the effects of fluticasone and salmeterol on fibronectin and tenascin-C protein and mRNA levels were analysed by immunoblotting and semi-quantitative RT-PCR.

Results In both conditions, fluticasone increased fibronectin transcript and protein levels, whereas it decreased those of tenascin-C. Salmeterol neither affected fibronectin and tenascin-C synthesis significantly nor did it influence the effect of fluticasone when applied in combination. Furthermore, we found that treatment with fluticasone had an opposite effect on extra domain A and B containing fibronectin isoforms generated by alternative splicing compared with total fibronectin transcript levels, whereas tenascin-C isoforms were not differently modulated by fluticasone.

Conclusions Our results indicate that standard therapies for inflammatory lung disorders influence ECM protein composition and relative expression levels. In contrast to corticosteroids, LABA did not significantly alter the expression of tenascin-C and fibronectin in cultures of primary human lung fibroblasts.

Keywords alternative splicing, asthma therapy, fibronectin, human lung fibroblasts, tenascin-C

Submitted 10 June 2008; revised 11 December 2008; accepted 15 December 2008

Correspondence:

Ruth Chiquet-Ehrismann, Friedrich Miescher Institute for Biomedical Research, Novartis Research Foundation, Maulbeerstrasse 66, CH-4058 Basel, Switzerland. E-mail: chiquet@fmi.ch

Introduction

The extracellular matrix (ECM) consists of a complex network of proteins which cells secrete and organize around themselves and affects many aspects of cellular

behaviour. These include proliferation, differentiation, survival, adhesion, and migration of cells [1]. It is well established that the ECM profile in the airways of healthy individuals differs both in quantity and composition from that of asthmatic and chronic obstructive pulmonary disease (COPD) subjects. Whereas deposition of collagen IV and elastin is decreased in asthmatic patients compared with healthy individuals, deposition of other specific ECM proteins, including collagen I, III, and V, fibronectin, tenascin, versican, and laminin is increased [2–6].

¹Present address: Department of Dermatology, Brigham and Women's Hospital, Harvard Skin Disease Research Center, Harvard Medical School, Boston, MA, USA.

²Equal contribution.

Asthma and COPD are characterized by chronic inflammation and tissue remodelling resulting in irreversible structural changes in the lung. Lung fibroblasts are important mediators of the lung tissue repair process as they are involved in cell proliferation and synthesis of ECM components. Among the various ECM proteins, lung fibroblasts secrete tenascin-C and fibronectin, two large multifunctional and multidomain ECM glycoproteins highly expressed during morphogenesis and in tissue remodelling processes [7]. Both proteins are subject to alternative splicing resulting in various numbers of isoforms which are known to have specific functions and expression patterns during development and disease. Furthermore, both proteins are implicated, or at least highly expressed, in various lung diseases including asthma and COPD [7–10].

Current asthma and COPD therapies include inhaled corticosteroids and long-acting β_2 -agonists (LABA) applied either alone or in combination. Corticosteroids have a well-described anti-inflammatory effect. However, their influence on airway remodelling remains controversial [11–18]. More than a decade ago, Pauwels *et al.* [18] have shown that the addition of inhaled formoterol to budesonide resulted in improved clinical outcome in asthma patients than higher dose of budesonide. However, the effect of combined corticosteroids and LABA on lung ECM synthesis and deposition remains to be fully elucidated.

In the present study, we investigated the effect of corticosteroids and LABA, alone or in combination, on tenascin-C and fibronectin expression in primary human lung fibroblasts. As previously described, we used two experimental conditions in our culture model: a serum-deprived medium representing a non-inflammatory environment, and stimulation with 5% fetal calf serum (FCS), the common pro-inflammatory factor related to inflammation, and/or TGF- β_1 , the component in the serum triggering ECM remodelling. The latter culture model mimicked an early stage of a pro-fibrotic environment with vessel leakage and activated tissue repair [17].

Materials and methods

Cell culture

Primary human lung fibroblasts were established from non-diseased peripheral lung tissue samples obtained from patients undergoing lung resection following approval by the ethics committee (Faculty of Medicine, University Hospital, Basel, Switzerland). In this study we analysed cells isolated from three different patients. Cells were grown in RPMI 1640 (Cambrex Bio Science, Verviers, Belgium) supplemented with 10% FCS (Gibco BRL Invitrogen, Basel, Switzerland) and 1% MEM-vitamins (Cambrex Bio Science). Fibroblasts were stimulated with recombinant human TGF- β_1 (2.5 ng/mL; Sigma, Schnell-

dorf, Germany) and treated with fluticasone and salmeterol (10^{-7} M; GlaxoSmithKline, London, UK), formoterol (AstraZeneca, Lund, Sweden) or dexamethasone (10^{-6} M; Calbiochem, Lucerne, Switzerland). Culture conditions and optimal drug doses have been determined previously [17]. Before any treatment, cells were serum-deprived for 24 h in 0.3% human albumin. Fibroblasts between passages 3 and 6 were used for all experiments. HT1080 fibrosarcoma cells (ATCC # CCL-121) were cultured in DMEM (Invitrogen) supplemented with 10% FCS.

Immunofluorescence

For immunofluorescence staining of primary human lung fibroblasts, cells were plated on coverslips inside 3.5 cm dishes at a concentration of 10^5 cells/dish for 72 h. Cells were then fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 30 min followed by a PBS rinse. After permeabilization with 0.1% Triton in PBS for 5 min, cells were washed twice with PBS and incubated with the primary antibodies for 1 h. For the fibronectin and tenascin-C double-labelling, primary antibodies included the monoclonal anti-human tenascin-C B28-C13 [19] at 1 : 250 and the polyclonal anti-human fibronectin antiserum (1 : 500 [20]). In the case of the anti-EDA (F8 [21]) and anti-EDB (L19 [21]) the primary antibody incubation (1.6 μ g/mL) was followed by an additional incubation for 1 h with rabbit-anti-human IgE (1 : 500; Dako, Lausen, Switzerland) together with the anti-human fibronectin antiserum as mentioned above. Following these primary antibody incubations, cells were washed three times with PBS. After incubation with fluorescently labelled secondary antibodies for 1 h in the dark (Alexa Fluor 594 goat anti-rabbit and Alexa Fluor 488 goat anti-mouse at 1 : 1000 each; Invitrogen, Carlsbad, CA, USA) cells were washed three times with PBS, then once with water before coverslips were mounted onto slides in Mowiol (Calbiochem, La Jolla, CA, USA). Cells were analysed with a Zeiss Axioskop equipped with a HXP120 lamp (Carl Zeiss, Feldbach, Switzerland) and pictures were taken with a Hamamatsu ORCA-ER (Hamamatsu, Solothurn, Switzerland) camera.

Promoter studies

The human tenascin-C and fibronectin promoter constructs were cloned from human genomic DNA by PCR amplification using the Expand High Fidelity PCR System (Roche Diagnostic, Mannheim, Germany). The following two sets of primers were used for the tenascin-C constructs: 5'TAGCTAGCCTGGGGAGAGGAGTTTCGAG3'/5'TACTCGAGCTGCCTTTGTGCCACGGAG3' for pTNC-2016 and 5'ATGCTAGCGCAGGCGGGAATTCC TACTTT3'/5'TACTCGAGCTGCCTTTGTGCCACGGAG3' for pTNC-220.

The primers included *NheI* and *XhoI* restriction sites, respectively, allowing directional cloning into the reporter vector pSEAP2-Basic (Clontech, Mountain View, CA, USA). The fibronectin promoter constructs were obtained with primer sets 5'ATGCTAGCCTCTAACCAGACAGGATTC3'/5'ATCTCGAGCCGGCGCGGGCGTCCGAG3' for pFN-1761 and 5'ATGCTAGCCCAGCCGCTTCCCATCCCTTC3'/5'ATCTCGAGCCGGCGCGGGCGTCCGAG3' for pFN-317. The promoter sequences correspond to nucleotides 25203660–25201566 of accession NT_008470 for pTNC-2016, 25201858–25201566 of accession NT_008470 for pTNC-220, 66511971–66510198 of accession NT_005403 for pFN-1761 and 66510527–66510198 for pFN-317. Furthermore, a plasmid containing a glucocorticoid responsive element, pGRE-SEAP from the Pathway Profiling SEAP System (Clontech) was used as positive control.

HT1080 cells were plated at 8×10^4 cells/well in six-well plates 18 h before transfection. Cells were transfected with Eugene 6 Transfection reagent (Roche) using 2 µg DNA per well either in 10% FCS or 0.3% FCS with or without the addition of hormones as indicated in 'Results'. Twenty-four hours after transfection the medium was collected and secreted alkaline phosphatase was measured using the chemiluminescent alkaline phosphatase detection kit Great EscApe™ SEAP (Clontech) according to the manufacturer's procedure. For normalization co-transfection with the firefly luciferase-containing pGL3 basic vector (Promega, Madison, WI, USA) was performed. Twenty-four hours after transfection, cells were lysed with Passive Lysis Buffer (Promega) and assayed with the LARII Luciferase Assay System (Promega). Secreted alkaline phosphatase and firefly luciferase activity, were measured in a Luminometer Mithras LB 940 (Berthold technologies, Regensburg, Switzerland).

Reverse transcriptase-polymerase chain reaction

Confluent serum-deprived fibroblasts were treated with fluticasone and/or salmeterol (10^{-7} M) in 0.3% albumin or

5% FCS for 48 h. Total RNA was extracted using the RNeasy Mini kit (Qiagen GmbH, Hilden, Germany) and reverse-transcribed using M-MLV Reverse Transcriptase (Promega). PCR was performed using the fibroblast cDNAs as templates and primers amplifying specific amplicons of GAPDH, fibronectin, tenascin-C, tenascin-C isoforms, and the fibronectin splicing isoforms containing EDA and EDB (Microsynth AG, Balgach, Switzerland; Table 1). PCR conditions were initial denaturation at 94 °C for 2 min then denaturation at 94 °C for 30 s primer annealing at 55 °C for 30 s and extension at 72 °C for 1 min. PCR cycle numbers were optimized for each primer pair. PCR amplicons were size fractionated by electrophoresis on 1.5% agarose gels and visualized by ethidium bromide stain under UV light. The intensity of each band was analysed by densitometry using the software Gene Tools (SynGene, Cambridge, UK) and the relative mRNA expression of target gene was normalized to the GAPDH control. Results are expressed as relative to controls (without drug treatment) which were defined as 1.

Western blotting and quantification

Thirty microlitres of collected conditioned medium of primary human lung fibroblasts were mixed with 10 µl of 4× reducing SDS-PAGE sample buffer, boiled for 5 min at 95 °C and loaded onto a 6% SDS-PAGE gel. After electrophoresis, proteins were electro-transferred onto a polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA) using a semi-dry blotting apparatus (Millipore). Afterwards, membranes were stained with amido black to control equal protein loading. After blocking for 1 h at room temperature in TBS containing 0.05% Tween and 5% skim milk powder, membranes were incubated overnight at 4 °C with either the monoclonal antibody B28-13 raised against human tenascin-C (1:100) [19] or the monoclonal anti-human fibronectin antibody (1:1000)

Table 1. Sequences of primers used for RT-PCR

Primers	Sequences	Amplicon size(s)
GAPDH forw	5'-GAAGGTGAAGTCTCGGAGTC-3'	229 bp
GAPDH rev	5'-GAAGATGGTGATGGGATTTC-3'	
FN forw	5'-CGAACAACATGAGAGCACACC-3'	401 bp
FN rev	5'-TCACTGTGACAGCAGGAGCATC-3'	
TN-C forw	5'-GACACAGATTCAGCCATCACCA-3'	152 bp
TN-C rev	5'-GAGTGTTTCGTGGCCCTTCCAGT-3'	
EDA forw	5'-TTCITTCATTGGTCCGGTCTT-3'	EDA+: 496 bp
EDA rev	5'-GCAGCCACAGTGGAGTATGT-3'	EDA-: 226 bp
EDB forw	5'-CCCCTACAAACGGCCAGCAGGG-3'	EDB+: 731 bp
EDB rev	5'-GCCAGTTGGGGAATCAAGACCTG-3'	EDB-: 458 bp
TN-C isoforms forw	5'-ACCGCTACCGCCTCAATTACA-3'	331 bp/607 bp/883 bp/1159 bp/
TN-C isoforms rev	5'-GGTTCGGTCCACAGTTACCA-3'	1435 bp/1711 bp/1969 bp*

*Seven TN-C isoforms are detectable which correspond to the inclusion of seven FNIII extra-domains, each consisting of 276 bp (see Fig. 6b).

The major isoforms present in the primary human lung fibroblasts are highlighted in bold.

(Sigma, Buchs, Switzerland). Membranes were washed three times with TBS containing 0.05% Tween and then incubated with goat anti-mouse IgG coupled to horseradish peroxidase (1 : 10 000) (MP Biomedicals, Illkirch, France) for 1 h at room temperature. Blots were developed using Super Signal (Pierce, Rockford, IL, USA) and finally exposed on Kodak BioMax MR Films (Kodak, Rochester, NY, USA).

For Western blot quantification, the software Gene Tools was used. Different exposure times of the same blots were quantified to check accuracy of the quantification method. Results represent the quantification of three different blots using three different primary human lung fibroblasts cell lines. Graphs are expressed as relative to controls (without drug treatment), which were defined as 100%.

Statistical analysis

Data are expressed as means $\pm$ SEM. Statistical significances were established with the Student's *t*-test (available at the web server hosted by St John's University, St Joseph, MN, USA: <http://www.physics.csbsju.edu/stats/t-test.html>). Differences were considered significant if $P < 0.05$ compared with control.

Results

5% fetal calf serum and tumour growth factor- β_1 increase the expression of fibronectin and tenascin-C

Confluent fibroblasts cultured in serum-free medium, mimicking a non-inflammatory environment secreted a moderate constitutive level of fibronectin and tenascin-C detectable after 48 h by immunoblotting (Fig. 1). These control values were defined as 100% for further comparisons. Since we have shown previously that the addition of 5% FCS initiated a pro-inflammatory response in primary human lung fibroblasts [17] we investigated the effect of FCS on fibronectin and tenascin-C synthesis. Stimulation with 5% FCS showed only a slight increase of fibronectin levels but did not reach significance compared with control ($P = 0.06$) (Fig. 1a). In contrast, 5% FCS significantly increased tenascin-C levels by more than 2.5-fold compared with 0.3% albumin ($P < 0.001$) (Fig. 1b). In order to investigate the effect of TGF- β_1 which can function either as a pro- or anti-inflammatory cytokine on inflammatory cells, we treated fibroblasts with TGF- β_1 under both experimental conditions. Addition of TGF- β_1 into serum-free medium significantly augmented fibronectin ($P < 0.01$) and tenascin-C levels ($P < 0.001$) compared with controls. TGF- β_1 in the presence of 5% FCS significantly

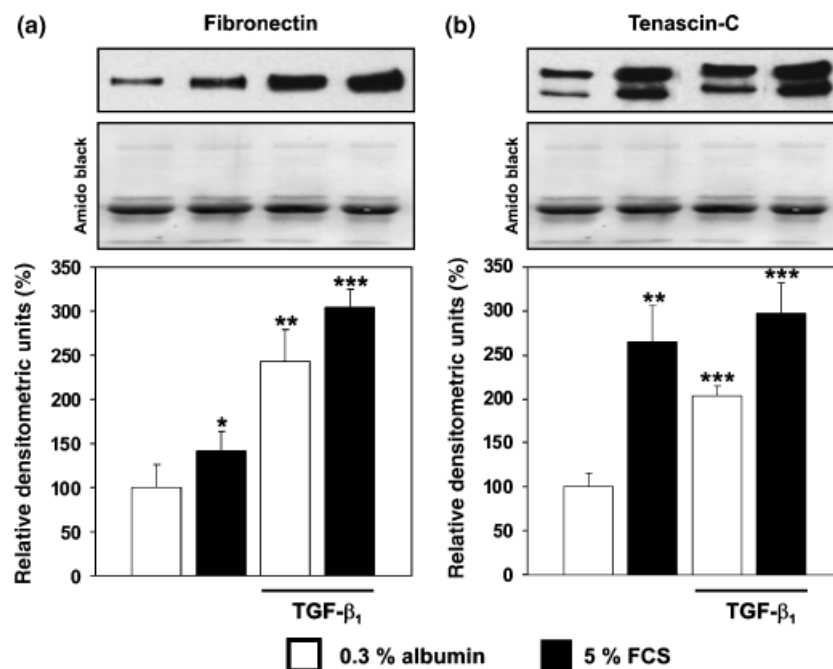


Fig. 1. Increased fibronectin and tenascin-C expression by 5% FCS and TGF- β_1 . Confluent serum-deprived primary human lung fibroblasts were stimulated with 5% FCS and/or TGF- β_1 (2.5 ng/mL) for 48 h. Supernatants were collected and fibronectin and tenascin-C were detected by immunoblotting. A representative immunoblot is shown for fibronectin (a, top) and for tenascin-C (b, top). Quantification of the immunoblots of three different primary cell lines is shown for fibronectin (a, bottom) and tenascin-C (b, bottom). Bars represent means $\pm$ SEM expressed as % of control, where 0.3% albumin was defined as 100%. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. The *P*-values correspond to the comparisons to 0.3% albumin. FCS, fetal calf serum.

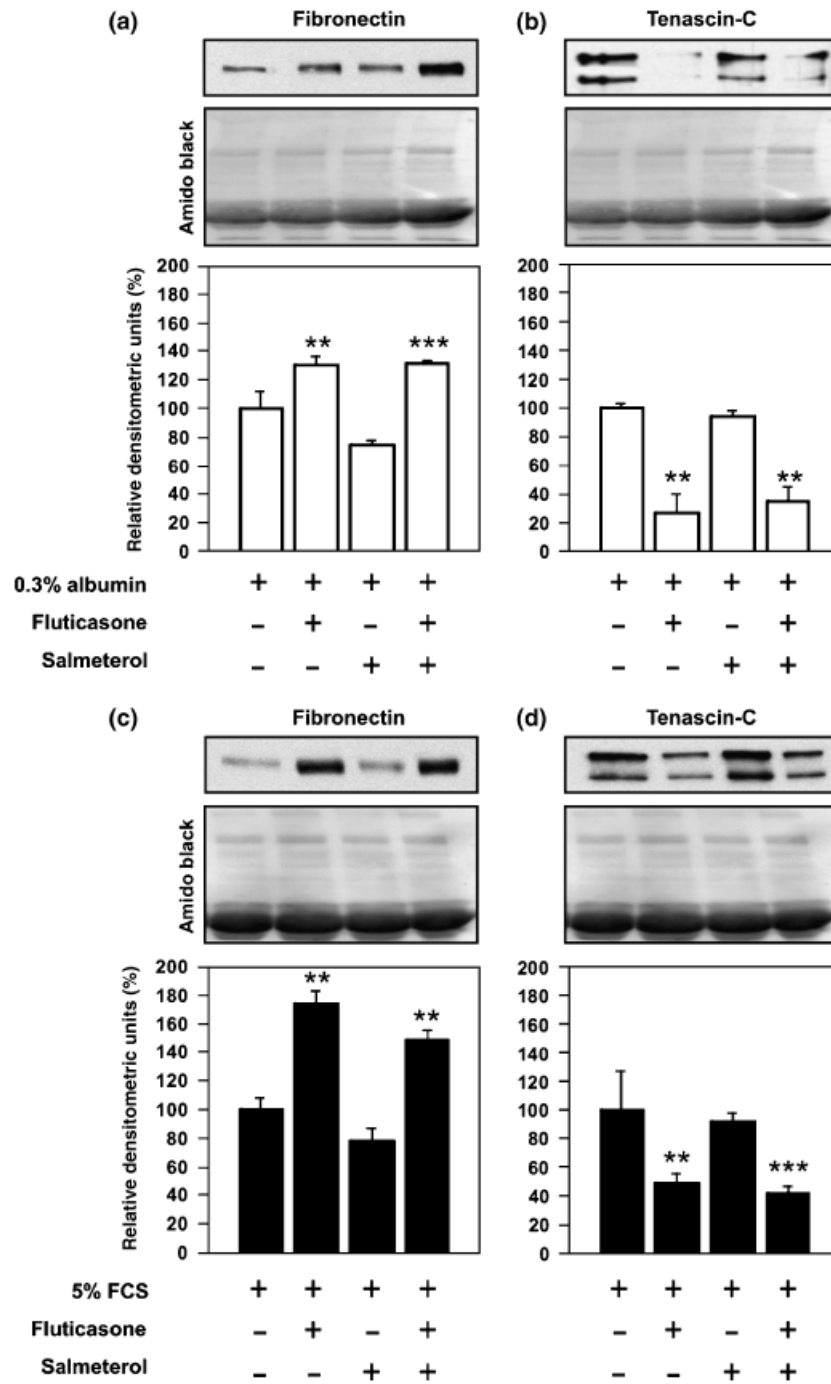


Fig. 2. Fibronectin and tenascin-C regulation by fluticasone and salmeterol. Confluent serum-deprived primary human lung fibroblasts were treated with fluticasone (10^{-7} M) and/or salmeterol (10^{-7} M) in 0.3% albumin and 5% FCS for 48 h. Supernatants were subjected to immunoblot analysis for fibronectin and tenascin-C. Amido black staining is included to show equal protein concentrations. A representative immunoblot is shown for fibronectin (a, c, top) and for tenascin-C (b, d, top). The same membrane was used for (a) and (b) as well as for (c) and (d). Quantification of the immunoblots of three different primary cell lines is shown for fibronectin (a, c, bottom) and tenascin-C (b, d, bottom). Bars represent means $\pm$ SEM expressed as % of control, where 0.3% albumin and 5% FCS were defined as 100%. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. The P -values correspond to the comparisons to control. FCS, fetal calf serum.

increased fibronectin levels by threefold compared with 0.3% albumin ($P < 0.001$) and more than twofold compared with the presence of 5% FCS alone ($P < 0.001$)

(Fig. 1a). Since tenascin-C levels were already very high in the presence of 5% FCS, we did not detect a further increase by treatment with TGF- β_1 (Fig. 1b).

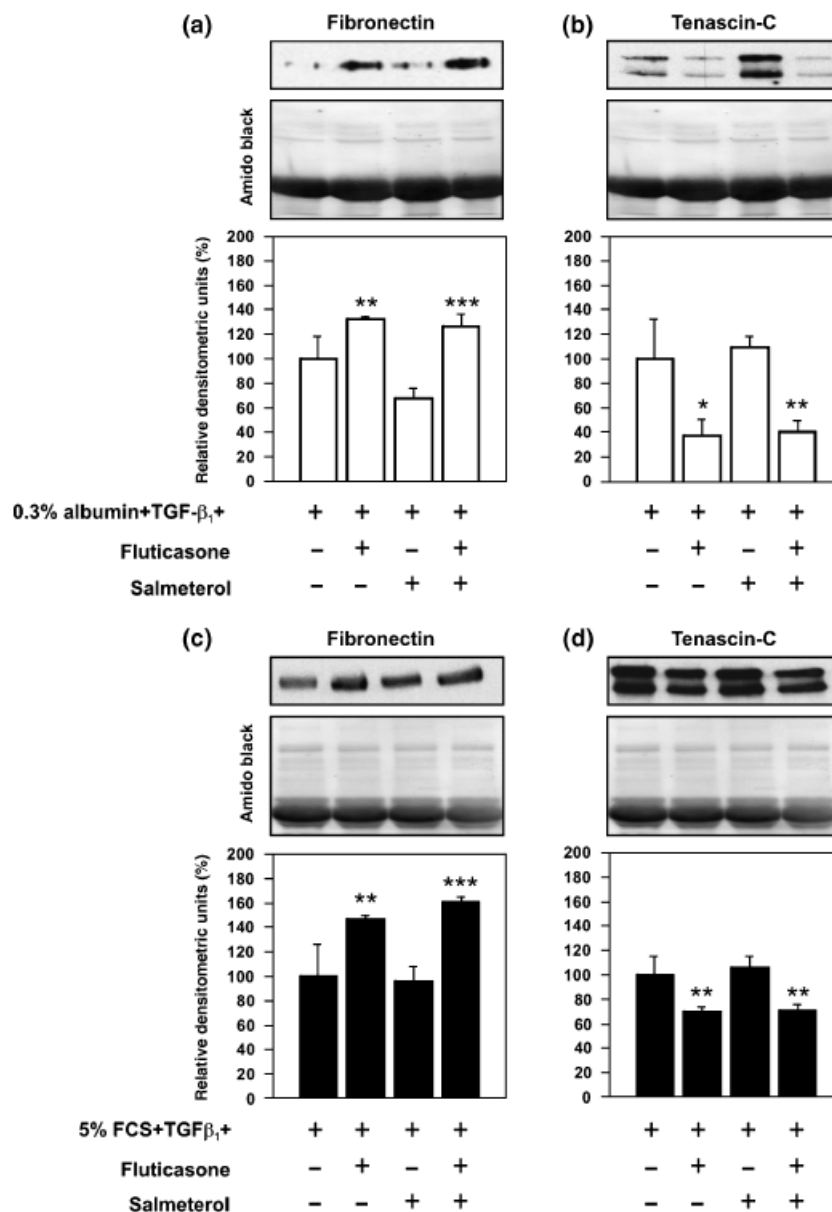


Fig. 3. Fibronectin and tenascin-C regulation by fluticasone and salmeterol in the presence of TGF- β_1 . Confluent serum-deprived primary human lung fibroblasts were treated with fluticasone (10^{-7} M) and/or salmeterol (10^{-7} M) in the presence of TGF- β_1 (2.5 ng/mL) in both 0.3% albumin and 5% FCS for 48 h. Supernatants were subjected to immunoblot analysis for fibronectin and tenascin-C. Amido black staining is included to show equal protein concentrations. A representative immunoblot is shown for fibronectin (a, c, top) and for tenascin-C (b, d, top). The same membrane was used for (a) and (b) as well as for (c) and (d). Quantification of the immunoblots of three different primary cell lines is shown for fibronectin (a, c, bottom) and tenascin-C (b, d, bottom). Bars represent means $\pm$ SEM expressed as % of control, where 0.3% albumin and 5% FCS were defined as 100%. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. The P -values correspond to the comparisons to control. FCS, fetal calf serum.

Fibronectin and tenascin-C are differently regulated by fluticasone and salmeterol in the presence or absence of 5% fetal calf serum and/or tumour growth factor- β_1

Next we evaluated by immunoblotting the effects of fluticasone and salmeterol, applied alone or in combination, in the presence or absence of 5% FCS on fibronectin and tenascin-C synthesis (Fig. 2). Under serum-free conditions, fluticasone led to an increase in fibronectin

expression compared to the control ($P < 0.01$), whereas salmeterol had no effect. Combined fluticasone and salmeterol increased fibronectin levels ($P < 0.001$) similarly to fluticasone administration alone (Fig. 2a). Fluticasone had an opposite effect on tenascin-C levels, which were significantly decreased by fivefold compared with the control ($P < 0.01$). Salmeterol alone did not modulate tenascin-C expression compared with controls nor did it affect the decrease observed with fluticasone in

combination (Fig. 2b). Inflammatory conditions with 5% FCS stimulation did not influence the effects of both drugs on fibronectin and tenascin-C expression in comparison to the one observed in serum-deprived fibroblasts. Indeed, fluticasone augmented fibronectin levels ($P < 0.01$), whereas it decreased tenascin-C levels secreted into the conditioned medium ($P < 0.001$). Again, salmeterol administration alone in the presence of 5% FCS did not show any effect on fibronectin and tenascin-C levels. However, combination with fluticasone significantly reduced fibronectin as well as tenascin-C expression in the presence of 5% FCS (Figs 2c and 2d). These effects of fluticasone and salmeterol on fibronectin and tenascin-C expression were not modulated by further stimulation with TGF- β_1 under both 0.3% albumin and 5% FCS (Fig. 3). We observed the same results when the primary human lung fibroblasts were treated with other corticosteroids and/or LABAs such as budesonide, formoterol, or dexamethasone (data not shown).

Response of tenascin-C and fibronectin promoter constructs to corticosteroids

It is known that fibronectin and tenascin-C protein expression in HT1080 cells shows a reverse response to dexamethasone [22]. Therefore, we used these cells to test whether glucocorticoids could regulate tenascin-C and fibronectin expression on the promoter level. We analysed the response of HT1080 cells transfected with promoter reporter constructs of tenascin-C, fibronectin, or a glucocorticoid responsive promoter plasmid pGRE as control (Fig. 4). Interestingly, while the activity of the pGRE plasmid was highly induced by the addition of dexamethasone or fluticasone, the promoter activities of both tenascin-C constructs were inhibited by more than 40% in the presence of glucocorticoids. In contrast, the promoter activity of the fibronectin constructs was not affected by the addition of either corticosteroid. Thus, transcription of tenascin-C seems to be directly repressed by corticosteroids and the responsive region is contained within the proximal promoter within 220 bp of the transcription start while the activities of fibronectin promoter constructs are not affected by corticosteroid treatments.

Modulation of fibronectin and tenascin-C mRNA expression by fluticasone and salmeterol

So far we investigated the effects of fluticasone and salmeterol alone or in combination on fibronectin and tenascin-C protein levels and on the level of direct activation of their promoters using promoter reporter constructs. In order to determine the drug's effect on fibronectin and tenascin-C transcript levels, we performed RT-PCR analysis on mRNA isolated from primary human

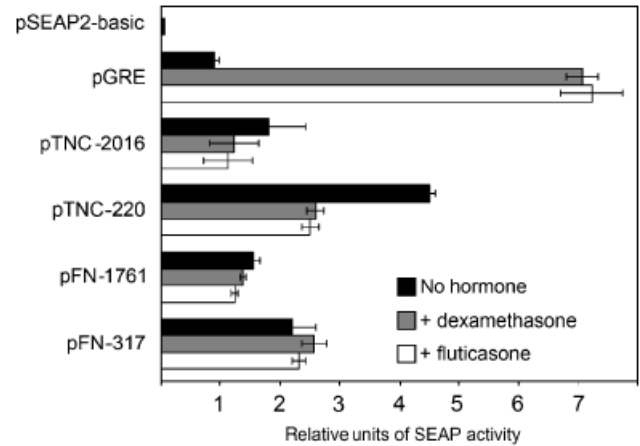


Fig. 4. Effect of corticosteroids on promoter reporter constructs of tenascin-C and fibronectin. Cells were transfected with either a promoter-free control plasmid pSEAP2-basic, a plasmid containing a glucocorticoid-responsive promoter pGRE, tenascin-C promoter plasmids including 2016 bp (pTNC -2016) or 220 bp (pTNC -220) upstream of the transcription start site or fibronectin promoter plasmids including 1761 bp (pFN -1761) or 317 bp (pFN -317) upstream of the transcription start site in the presence or absence of dexamethasone (10^{-6} M) or fluticasone (10^{-7} M). All transfections were done in 0.3% FCS except for the tenascin-C constructs which were transfected in 10% FCS. Tenascin-C promoter activity stimulated by serum was greatly reduced by the addition of dexamethasone and fluticasone. The plasmid pGRE was highly induced by the addition of the hormones while the fibronectin reporter plasmids remained unaffected. FCS, fetal calf serum.

lung fibroblasts cultured under both 0.3% albumin and 5% FCS. The agarose gels shown in Fig. 5a confirm our immunoblot results. Expression of the transcript levels was analysed by densitometry analysis and normalized to GAPDH and the relative gene expression from three cell lines is depicted in Fig. 5b. Treatment with fluticasone under any experimental condition is associated with a significant increase in fibronectin mRNA accumulation ($P < 0.05$), and with a significant reduction in tenascin-C transcript levels ($P < 0.05$). This indicates that glucocorticoids may regulate fibronectin transcript levels post-transcriptionally. Again, administration of salmeterol applied alone or in combination with fluticasone did neither modulate fibronectin nor tenascin-C mRNA levels compared with controls (Fig. 5b).

To test the modulatory effects of fluticasone and salmeterol on the major splice variants of fibronectin and tenascin-C, we specifically designed primers which flank the sequences that are subjected to alternative splicing in the human fibronectin and tenascin-C cDNAs (Table 1, Fig. 6b top). Treatment with fluticasone in either 0.3% albumin or 5% FCS, but in the absence of TGF- β_1 was associated with a down-regulation of the extra domain A containing isoform (EDA+) compared with non-treated cells, whereas salmeterol did not influence EDA-containing transcripts (Fig. 6a). This regulation of the EDA+

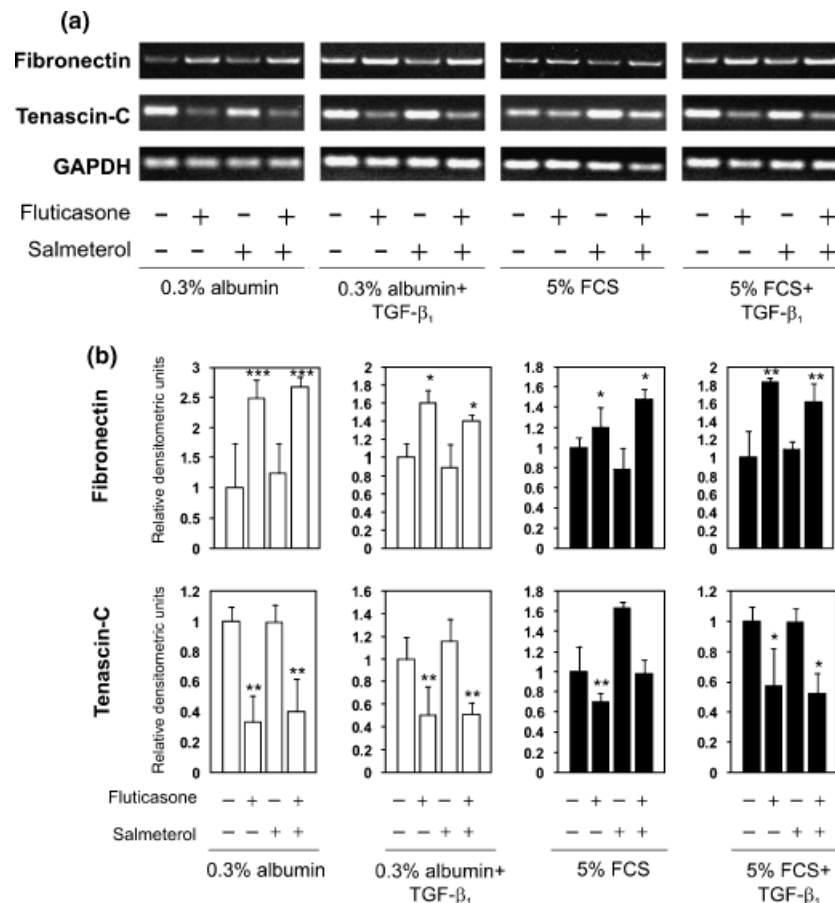


Fig. 5. Modulation of fibronectin and tenascin-C mRNA expression by fluticasone and salmeterol. Confluent serum-deprived primary human lung fibroblasts were treated with fluticasone and/or salmeterol (10^{-7} M) in the absence or presence of TGF- β_1 (2.5 ng/mL) under both 0.3% albumin and 5% FCS. Total RNA was extracted after 48 h and cDNA was synthesized. Representative PCR gels are shown in (a) and quantification of three cell lines is shown in (b). Bars represent means $\pm$ SEM expressed as fold induction compared with control and are normalized to GAPDH. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. The P -values correspond to the comparisons to 0.3% control. FCS, fetal calf serum.

isoform is the exact opposite to total levels of fibronectin transcripts. However, in the presence of TGF- β_1 , the changes in EDA+ mRNA expression are masked. The extra domain B containing isoform (EDB+) is regulated in the same manner as the EDA+ transcripts. However, the decrease of EDB+ by fluticasone was also observed in the presence of TGF- β_1 (Fig. 6a). In contrast to fibronectin, treatment with fluticasone and/or salmeterol did not differently modulate tenascin-C isoform expression generated by alternative splicing compared with total tenascin-C transcript levels (Fig. 6b, bottom).

Next we analysed the deposition of tenascin-C and fibronectin as well as the fibronectin splice variants in the ECM deposited by the lung fibroblasts. We found that fibronectin and tenascin-C were deposited independently in different fibrillar patterns, while the staining patterns for total fibronectin and EDA+ or EDB+ fibronectin were indistinguishable (Figs 7a–d). It appears that tenascin-C accumulates preferentially in areas where cells are particularly dense.

In summary, we show here that fluticasone regulates tenascin-C and fibronectin protein and mRNA levels in an opposite manner, and the localization of the two proteins in the ECM is different as well. In the case of fibronectin and its alternative splicing variants, transcripts accumulate differentially after fluticasone treatment but the protein variants are deposited in the same ECM fibres. In contrast to fluticasone, salmeterol has no significant effect on fibronectin, fibronectin variants or tenascin-C levels under any conditions tested.

Discussion

Asthma is more or less effectively controlled in most patients with maintenance treatment. Control may be achieved with an inhaled corticosteroid or with a more effective approach of a combination inhaler containing a corticosteroid and a LABA. However, their exact mechanism of action and direct influence on tissue remodelling are not well understood so far.

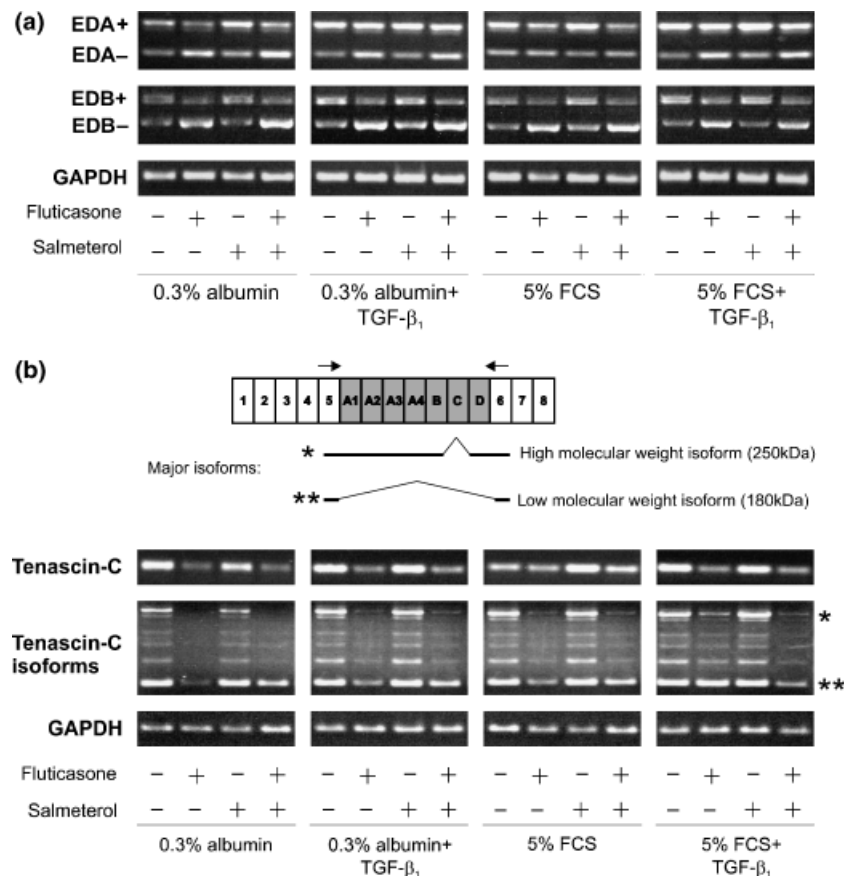


Fig. 6. Modulation of fibronectin and tenascin-C isoform mRNA expression by fluticasone and salmeterol. Confluent serum-deprived primary human lung fibroblasts were treated with fluticasone and/or salmeterol (10^{-7} M) in the absence or presence of TGF- β_1 (2.5 ng/mL) under both 0.3% albumin and 5% FCS. Total RNA was extracted after 48 h and cDNA was synthesized. (a) Representative PCR gels of the major fibronectin splice variants, where EDA+ and EDB+ indicate the EDA-containing fibronectin, or the EDB-containing fibronectin isoform respectively are shown. (b) Schematic representation of the 15 fibronectin type III repeats (white boxes: constant repeats; grey boxes: repeats subject to alternative splicing) present in tenascin-C and the primers used to detect the tenascin-C isoforms is shown (top). Representative PCR gels of the tenascin-C splice variants are shown (bottom). Seven tenascin-C isoforms are detectable corresponding to the inclusion of the seven fibronectin type III repeats (grey boxes, top), each containing 276 bp. The major isoforms present in the primary human lung fibroblasts are highlighted by asterisks (*, **) and they give rise to the 250 and 180 kDa protein respectively. FCS, fetal calf serum.

In our report, we studied the effect of fluticasone and salmeterol on tissue remodelling, a prominent characteristic of asthma and COPD. We specifically, analysed total fibronectin, tenascin-C, fibronectin-, and tenascin-C isoform accumulation in the medium by primary human lung fibroblasts in response to the drugs.

We observed an increase of fibronectin protein and mRNA levels by primary human lung fibroblasts upon treatment with budesonide as well as with dexamethasone (data not shown). This is in agreement with other reports showing that corticosteroid treatment is associated with fibronectin accumulation [23–26]. This was also the case in an *in vivo* model of asthma in rats [27]. Down-regulation of tenascin-C by corticosteroids seems to be common to many cell types as has been shown in several reports [6, 26, 28–30]. Interestingly, our results show that even the

presence of TGF- β_1 , an inducer of tenascin-C production [31, 32] did not counteract the reducing effect of fluticasone on tenascin-C levels. Thus, corticosteroids can down-regulate tenascin-C synthesis even in the presence of local stimulatory growth factors. The same has been observed before for collagen type I, III, and V [17]. Salmeterol did not affect fibronectin or tenascin-C levels compared with control, nor did it attenuate the effect of fluticasone when applied in combination. This stands in contrast to the effect of LABA on total ECM and collagen deposition by lung fibroblasts, which are reduced by them [17]. Furthermore, we show that fibronectin and tenascin-C are influenced in the opposite way by corticosteroids applied alone or in combination with LABA. This may have functional consequences, since it was shown that fibronectin and tenascin-C can directly interact with each other and that the presence of tenascin-C results in abolishment of

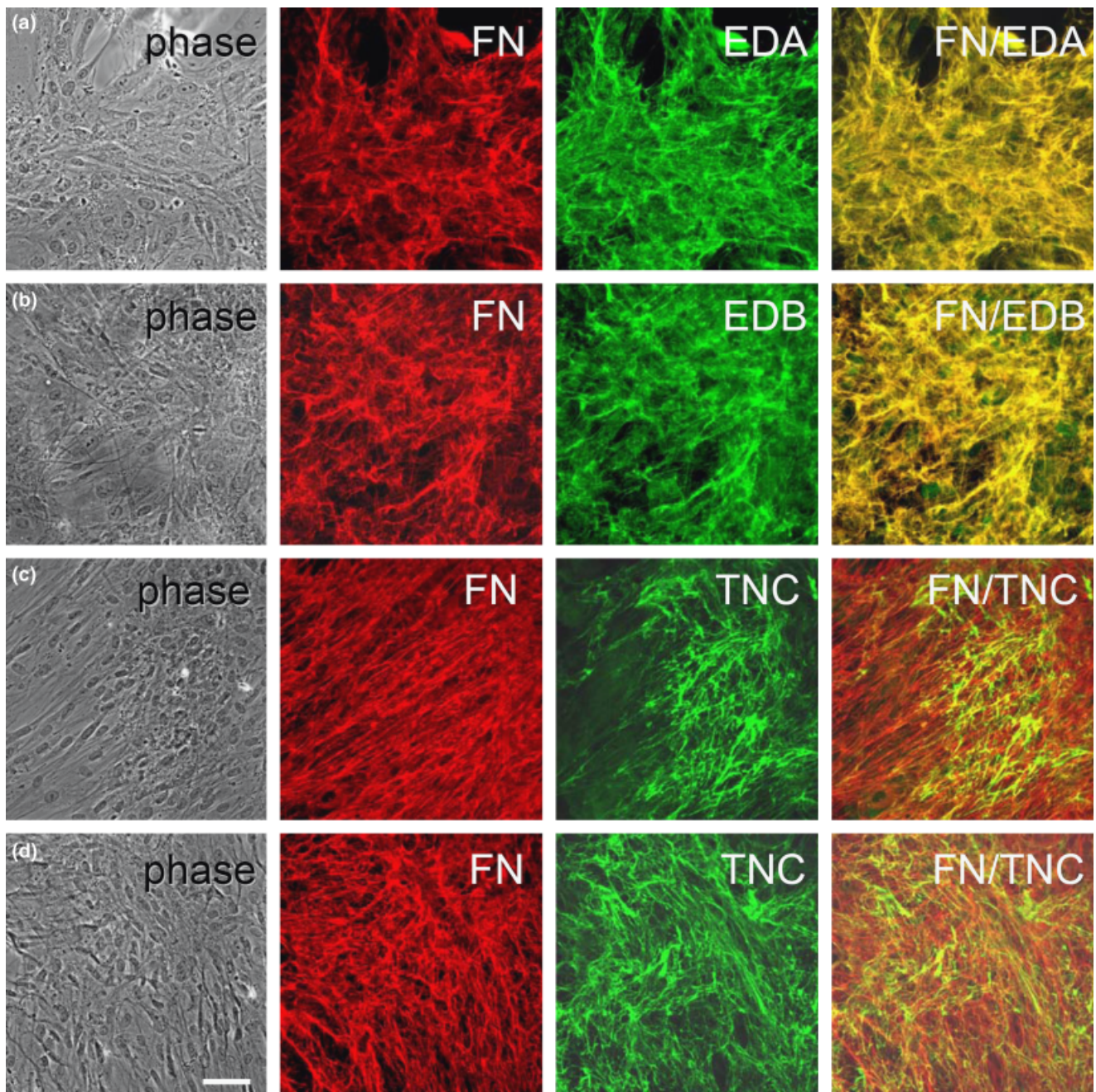


Fig. 7. Deposition of fibronectin variants and tenascin-C in the ECM synthesized by primary lung fibroblasts. Phase contrast pictures (phase) and double immunostainings of total fibronectin (FN) in red with EDA + fibronectin (EDA) in green panel (a), EDB + fibronectin (EDB) in green panel (b) or tenascin-C (TNC) in green panels (c and d) with the corresponding overlays are shown. Whereas all fibronectin antibodies stained the same ECM fibres, the immunostaining of fibronectin and tenascin-C was to a large extent non-overlapping. Panel (d) shows a different cell line than panels (a–c) with the same result. Bar, 100 μ m. ECM, extracellular matrix.

fibronectin-mediated cell spreading [33, 34]. At least in tumor cells, fibronectin and tenascin-C interaction is associated with reduced cell adhesion and enhanced cell proliferation [34, 35]. This might also be true in other pathological conditions such as asthma or COPD. Thus, indirect effects of corticosteroids may involve the modulation of cell–cell or cell–ECM interactions.

Alternative splicing in fibronectin, which is regulated both temporally and spatially, is a very prominent feature of this complex ECM protein [36]. Alternative splicing is clearly adding another level of complexity to the many functions ascribed to fibronectin. EDA+ and EDB+ fibronectin expressions are high during embryonic development and basically absent in the adult tissues. However,

these extra domains are included into fibronectin during pathological conditions such as tumorigenesis, inflammation, or tissue remodelling [37]. More recently, the discrimination between EDA+ and EDB+ fibronectin expression in pathological conditions and normal tissues was used to specifically target or image sites of tumor angiogenesis or chronic skin inflammation using EDA or EDB-specific human monoclonal antibodies [21, 38]. Therefore, EDA+ and EDB+ fibronectin have the potential to be used as marker for different pathological conditions such as a vascular marker of solid tumours and metastases, a marker for chronic skin inflammation or a marker for atherosclerotic plaques [21, 38, 39]. Interestingly, in the present study it seemed that treatment of primary human lung fibroblasts with fluticasone resulted in a differential effect on EDA+ and EDB+ mRNA as compared with fibronectin transcripts without these extra repeats. It has been shown previously that dexamethasone greatly increases fibronectin protein and transcript levels and that this effect is based on the stabilization of transcripts rather than on transcriptional regulation. Furthermore, Dean et al. showed that while fibronectin promoter reporter constructs could be activated by TGF- β , the same reporter constructs did not respond to dexamethasone treatment [24, 25]. These results were confirmed here in our study, and we found that in contrast to fibronectin, the activity of tenascin-C promoter reporters was repressed by corticosteroids. Thus, the differential effect of corticosteroids on tenascin-C vs. fibronectin expression could be explained by their different means of regulation. While tenascin-C appears to be regulated on a transcriptional level by corticosteroids, fibronectin transcripts are regulated post-transcriptionally through mRNA stabilization. The differential effect on EDA+ and EDB+ fibronectin could be based on factors involved in the stabilization of mRNA acting specifically on fibronectin transcripts devoid of extra repeats EDA and EDB. Tenascin-C is also subject to alternative splicing. However, we do not have any evidence that tenascin-C splice variants are regulated in an opposite manner by corticosteroids and LABA compared with total tenascin-C levels as observed for fibronectin splice variants.

In summary, we analysed the effect of corticosteroids and LABA on the expression levels of tenascin-C and fibronectin in primary human lung fibroblasts. Corticosteroid treatment negatively regulated expression of tenascin-C. The effect of corticosteroids on fibronectin was exactly the opposite: corticosteroids increased fibronectin deposition. LABA neither showed a prominent effect on tenascin-C or fibronectin expression nor modulated the effect of corticosteroids. However, since we have previously shown that total ECM and collagen deposition were decreased by LABA [17], it becomes clear that in relation to other ECM-proteins the relative amounts of tenascin-C, fibronectin, and its variants are shifting with

corticosteroids as well as with LABA. Our results strengthen the idea that drug treatment of asthma patients might have different effects on specific ECM proteins and hence on the remodelling process during pathogenesis.

Acknowledgements

We gratefully acknowledge the generous gift of antibodies F8 and L19 against EDA and EDB domains of fibronectin by Prof. Dario Neri (ETH Zuerich, Switzerland). This study was supported by the Novartis Research Foundation to MD and RC-E, by the Swiss National Science Foundation (SNF3100A0-1202235) to RC-E and by the Swiss National Science Foundation (SNF3200B0-105737/1) and GlaxoSmithKline to SG, MR and MT.

References

- 1 Bosman FT, Stamenkovic I. Functional structure and composition of the extracellular matrix. *J Pathol* 2003; **200**:423–8.
- 2 Roche WR, Beasley R, Williams JH, Holgate ST. Subepithelial fibrosis in the bronchi of asthmatics. *Lancet* 1989; **1**:520–4.
- 3 Roberts CR, Burke AK. Remodelling of the extracellular matrix in asthma: proteoglycan synthesis and degradation. *Can Respir J* 1998; **5**:48–50.
- 4 Bousquet J, Chanez P, Lacoste JY et al. Asthma: a disease remodeling the airways. *Allergy* 1992; **47**:3–11.
- 5 Altraja A, Laitinen A, Virtanen I et al. Expression of laminins in the airways in various types of asthmatic patients: a morphometric study. *Am J Respir Cell Mol Biol* 1996; **15**:482–8.
- 6 Laitinen A, Altraja A, Kampe M, Linden M, Virtanen I, Laitinen LA. Tenascin is increased in airway basement membrane of asthmatics and decreased by an inhaled steroid. *Am J Respir Crit Care Med* 1997; **156**:951–8.
- 7 Chiquet-Ehrismann R, Chiquet M. Tenascins: regulation and putative functions during pathological stress. *J Pathol* 2003; **200**:488–99.
- 8 Trifilieff A, El-Hashim A, Bertrand C. Time course of inflammatory and remodeling events in a murine model of asthma: effect of steroid treatment. *Am J Physiol Lung Cell Mol Physiol* 2000; **279**:L1120–8.
- 9 Karjalainen EM, Lindqvist A, Laitinen LA et al. Airway inflammation and basement membrane tenascin in newly diagnosed atopic and nonatopic asthma. *Respir Med* 2003; **97**:1045–51.
- 10 Nakahara H, Gabazza EC, Fujimoto H et al. Deficiency of tenascin C attenuates allergen-induced bronchial asthma in the mouse. *Eur J Immunol* 2006; **36**:3334–45.
- 11 Olivieri D, Chetta A, Del Donno M et al. Effect of short-term treatment with low-dose inhaled fluticasone propionate on airway inflammation and remodeling in mild asthma: a placebo-controlled study. *Am J Respir Crit Care Med* 1997; **155**:1864–71.
- 12 Hoshino M, Takahashi M, Takai Y, Sim J. Inhaled corticosteroids decrease subepithelial collagen deposition by modulation of the balance between matrix metalloproteinase-9 and tissue inhibitor of metalloproteinase-1 expression in asthma. *J Allergy Clin Immunol* 1999; **104**:356–63.
- 13 Boulet LP, Turcotte H, Laviolette M et al. Airway hyperresponsiveness, inflammation, and subepithelial collagen deposition in

- recently diagnosed versus long-standing mild asthma. Influence of inhaled corticosteroids. *Am J Respir Crit Care Med* 2000; **162**:1308–13.
- 14 Bergeron C, Hauber HP, Gotfried M *et al*. Evidence of remodeling in peripheral airways of patients with mild to moderate asthma: effect of hydrofluoroalkane-flunisolide. *J Allergy Clin Immunol* 2005; **116**:983–9.
 - 15 Ward C, Reid DW, Orsida BE *et al*. Inter-relationships between airway inflammation, reticular basement membrane thickening and bronchial hyper-reactivity to methacholine in asthma; a systematic bronchoalveolar lavage and airway biopsy analysis. *Clin Exp Allergy* 2005; **35**:1565–71.
 - 16 Tomic R, Lassiter CC, Ritzenthaler JD, Rivera HN, Roman J. Anti-tissue remodeling effects of corticosteroids: fluticasone propionate inhibits fibronectin expression in fibroblasts. *Chest* 2005; **127**:257–65.
 - 17 Goulet S, Bihl MP, Gambazzi F, Tamm M, Roth M. Opposite effect of corticosteroids and long-acting beta(2)-agonists on serum- and TGF-beta(1)-induced extracellular matrix deposition by primary human lung fibroblasts. *J Cell Physiol* 2007; **210**:167–76.
 - 18 Pauwels RA, Lofdahl CG, Postma DS *et al*. Effect of inhaled formoterol and budesonide on exacerbations of asthma. Formoterol and Corticosteroids Establishing Therapy (FACET) International Study Group. *N Engl J Med* 1997; **337**:1405–11.
 - 19 Schenk S, Muser J, Vollmer G, Chiquet-Ehrismann R. Tenascin-C in serum: a questionable tumor marker. *Int J Cancer* 1995; **61**:443–9.
 - 20 Ehrismann R, Chiquet M, Turner DC. Mode of action of fibronectin in promoting chicken myoblast attachment. Mr = 60,000 gelatin-binding fragment binds native fibronectin. *J Biol Chem* 1981; **256**:4056–62.
 - 21 Rybak JN, Roesli C, Kaspar M, Villa A, Neri D. The extra-domain A of fibronectin is a vascular marker of solid tumors and metastases. *Cancer Res* 2007; **67**:10948–57.
 - 22 Chiquet-Ehrismann R, Hagios C, Schenk S. The complexity in regulating the expression of tenascins. *BioEssays* 1995; **17**:17873–8.
 - 23 Furcht LT, Mosher DF, Wendelschafer-Crabb G, Woodbridge PA, Foidart JM. Dexamethasone-induced accumulation of a fibronectin and collagen extracellular matrix in transformed human cells. *Nature* 1979; **277**:393–5.
 - 24 Dean CD, Newby RF, Bourgeois S. Regulation of fibronectin biosynthesis by dexamethasone, transforming growth factor β , and cAMP in human cell lines. *J Cell Biol* 1988; **106**:2159–70.
 - 25 Pataki A, Madarasz E, Kurucz I. Fibronectin quantification without antibodies: a bioassay for the detection of the gelatin-captured macromolecule. *J Biochem Biophys Methods* 2006; **68**:113–26.
 - 26 Talts JF, Weller A, Timpl R, Ekblom M, Ekblom P. Regulation of mesenchymal extracellular matrix protein synthesis by transforming growth factor-beta and glucocorticoids in tumor stroma. *J Cell Sci* 1995; **108**:2153–62.
 - 27 Vanacker NJ, Palmans E, Pauwels RA, Kips JC. Effect of combining salmeterol and fluticasone on the progression of airway remodeling. *Am J Respir Crit Care Med* 2002; **166**:1128–34.
 - 28 Zhao Y. Tenascin is expressed in the mesenchyme of the embryonic lung and down-regulated by dexamethasone in early organogenesis. *Biochem Biophys Res Commun* 1999; **263**:597–602.
 - 29 Fassler R, Sasaki T, Timpl R, Chu ML, Werner S. Differential regulation of fibulin, tenascin-C, and nidogen expression during wound healing of normal and glucocorticoid-treated mice. *Exp Cell Res* 1996; **222**:111–6.
 - 30 Ekblom M, Fassler R, Tomasini-Johansson B, Nilsson K, Ekblom P. Downregulation of tenascin expression by glucocorticoids in bone marrow stromal cells and in fibroblasts. *J Cell Biol* 1993; **123**:1037–45.
 - 31 Pearson CA, Pearson D, Shibahara S, Hofsteenge J, Chiquet-Ehrismann R. Tenascin: cDNA cloning and induction by TGF-beta. *Embo J* 1988; **7**:2977–82.
 - 32 Sakai T, Kawakatsu H, Ohta M, Saito M. Tenascin induction in tenascin nonproducing carcinoma cell lines in vivo and by TGF-beta 1 in vitro. *J Cell Physiol* 1994; **159**:561–72.
 - 33 Chiquet-Ehrismann R, Kalla P, Pearson CA, Beck K, Chiquet M. Tenascin interferes with fibronectin action. *Cell* 1988; **53**:383–90.
 - 34 Huang W, Chiquet-Ehrismann R, Moyano JV, Garcia-Pardo A, Orend G. Interference of tenascin-C with syndecan-4 binding to fibronectin blocks cell adhesion and stimulates tumor cell proliferation. *Cancer Res* 2001; **61**:8586–94.
 - 35 Ruiz C, Huang W, Hegi ME *et al*. Growth promoting signaling by tenascin-C [corrected]. *Cancer Res* 2004; **64**:7377–85.
 - 36 Ffrench-Constant C, Hynes RO. Alternative splicing of fibronectin is temporally and spatially regulated in the chicken embryo. *Development* 1989; **106**:375–88.
 - 37 Ffrench-Constant C, Van de Water L, Dvorak HF, Hynes RO. Reappearance of an embryonic pattern of fibronectin splicing during wound healing in the adult rat. *J Cell Biol* 1989; **109**:903–14.
 - 38 Trachsel E, Kaspar M, Bootz F, Detmar M, Neri D. A human mAb specific to oncofetal fibronectin selectively targets chronic skin inflammation in vivo. *J Invest Dermatol* 2007; **127**:881–6.
 - 39 von Lukowicz T, Silacci M, Wyss MT *et al*. Human antibody against C domain of tenascin-C visualizes murine atherosclerotic plaques ex vivo. *J Nucl Med* 2007; **48**:582–7.