Transition of asthmatic bronchial fibroblasts to myofibroblasts is inhibited by cell—cell contacts

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Summary
The role of airway wall remodelling in bronchial asthma is well established. Myofibroblasts, the cells displaying features intermediate between fibroblasts and smooth muscle cells, are involved in this process but the mechanism of myofibroblasts activation in the onset of the disease remains obscure. Myofibroblasts can differentiate from various cell types, including resident fibroblasts, and the fibroblasts to myofibroblasts transition (FMT) can be reproduced *in vitro*. We aimed to investigate the process of FMT in human bronchial fibroblasts (HBF) derived from non-asthmatic ($n = 7$) and asthmatic ($n = 7$) subjects. We also tested whether cell—cell contacts affect FMT by using N-cadherin blocking antibody. HBF plated in low or high cell density were treated with TGF-β_1 up to one week to induce FMT. The percentage of myo-fibroblsts was counted and expression of $\alpha-$smooth muscle actin was evaluated by cytoimmu-nofluorescence, flow cytometry and immunobloting. We demonstrated that the intensity of FMT induced by TGF-β_1 *in vitro* was strongly enhanced in asthmatic as compared to non-asthmatic HBF populations. This process was facilitated by low cell plating density in both groups of cultures. Furthermore, we proved that neither HBF-conditioned medium nor growth arrest in G_0/G_1 phase of cell cycle could stop the TGF-β_1-induced FMT in asthmatic cell popu-lations. However, even in sparse asthmatic HBF, the blocking of N-cadherin resulted in the inhi-bition of FMT. Our findings show for the first time that the initial absence or an induced loss of cell—cell adhesions in asthmatic HBF populations is important for the completion of FMT.
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Background

Myofibroblasts represent a heterogeneous group of cells displaying a phenotype intermediate between fibroblasts and smooth muscle cells. Myofibroblasts predominantly differentiate from tissue-specific fibroblasts, but can also originate from bone marrow-derived fibrocytes, tissue-derived stem cells, epithelial and smooth muscle cells.[1–3] In particular, wound healing depends on myofibroblast activity.[4,5] However, these cells - armed with the capacity of soft tissue remodelling - are also able to determine the incitement and maintenance of chronic fibrocontractive diseases.[5–7]

The tissue remodelling seems to be accomplished by myofibroblasts in both a mechanical and biochemical manner. Incorporation of de novo expressed α-smooth muscle actin (α-SMA) into stress fibres enables the formation of "supermature focal adhesions" (FAs) as well as specific cell—cell adherents junctions (AJs)[8,9] which help myofibroblasts to develop a high contractile activity.[10,11] Furthermore, the special pattern of synthesis and secretion of ECM components and growth factors, matrix metalloproteinases and their inhibitors participates in the remodelling of relevant tissues.[12,13]

Significantly, both systems shown to be responsible for fibroblasts to myofibroblasts transition (FMT) were also pinpointed as crucial for the development of bronchial asthma. Phenotypic FMT requires local accumulation of biologically active TGF-β[14,15] as well as the presence of specific ECM proteins.[16] Furthermore, the mechanical tension generated by myofibroblasts, and dependent on cell-ECM and cell—cell contacts, seems to play a role in this process.[10,17] In fact, the structural changes of the bronchial wall resulting from the myofibroblast activity, together with chronic inflammation, are fundamental factors in the process of the remodelling of the airways.[3,12,18] It was recently demonstrated that bronchial fibroblasts derived from asthmatic patients display in vitro a high potential to differentiate into myofibroblasts.[19,20] Moreover the capacity of fibroblast differentiation towards myofibroblastic phenotype is enhanced at low cell density.[21,22] In this study we aimed to elucidate the effect of cell density and intercellular contacts in the cultures of non-asthmatic and asthmatic bronchial fibroblast populations on the process of FMT.

Material and methods

Isolation and culture of human bronchial fibroblasts (HBF)

Primary HBF were isolated from bronchial biopsies as described previously.[23] Fibroblasts were derived from two groups comprising in a total number of 14 individuals. The first group consisted of 7 nonasthmatics in whom diagnostic bronchoscopy ruled out any serious airway pathology, including asthma, fibrotic lung disease, sarcoidosis, and cancer (group NA: 2 males and 5 females; average age: 46 years; mean forced expiratory volume in the first second (FEV_1): 112% predicted, range: 97—138%). The second group consisted of 7 patients diagnosed with asthma (group AS: 2

males, 5 females; average age: 41 years; mean duration of asthma: 7 years; mean FEV1%: 77.2% of predicted, range: 40—109%). All patients were treated in the Department of Medicine of Jagiellonian University and were in stable clinical condition. The study was approved by the University's Ethics Committee (KBET/362/B/2003) and all the patients provided informed consent to participate.

Cells were cultured in DMEM with 10% foetal bovine serum (FBS) at 37 °C in a humidified atmosphere with 5% CO_2 and used between 5 and 15 passages. For experiments, cells were plated at low (5000 cells/cm^2) or high (50,000 cells/cm^2) density and cultured in serum-free DMEM supplemented with 0.1% bovine serum albumin (BSA) (Sigma St. Louis, USA) with or without human recombinant TGF-$β_1$ (PeproTech EC, London, UK).

Generation of fibroblast-conditioned medium

HBF from AS group were plated at high density (50,000 cells/cm^2) in 10 cm diameter dishes in DMEM supplemented with 10% FBS for 24 h and then the medium was aspirated and cells were incubated in DMEM supplemented with 0.1% BSA and with 5 ng/ml TGF-$β_1$ for 3 or 5 days. This conditioned medium was then collected, filtered through a 0.4 μm filtration unit, and used for the experiments.

Immunofluorescence

The myofibroblasts were identified by immunodetection of α-SMA. The cells growing on glass coverslips were washed with PBS, fixed in 3.7% paraformaldehyde for 15 min, and permeabilized in 0.1% Triton X-100 for 10 min at room temperature. Following fixation, slides were blocked with 3% BSA for 30 min. Subsequently, slides were incubated with the mouse monoclonal antibody against human α-SMA (clone 1A4, Sigma—Aldrich, 1:400) for 1 h at room temperature. Cells were washed 5 times with PBS and incubated with Alexa Fluor 488 goat anti-mouse IgG (clone, Sigma—Aldrich, 1:200) for 45 min at room temperature. For staining of actin filaments TRITC-phalloidin (500 ng/ml, Sigma—Aldrich) was used. Fluorescent images were taken with a Leica DMIRE2 microscope.

Flow cytometry

Primary bronchial fibroblasts were seeded at the concentration of 5000 cells/cm^2 (low density) or 50,000 cells/cm^2 (high density) into 6-well plates and cultured overnight in DMEM supplemented with 10% FBS. Then the culture medium was changed for serum-reduced medium with or without TGF-$β_1$ (5 ng/ml). On day 7, fibroblasts were trypsinized and prepared for immunostaining. Detached cells were washed, fixed in 1% paraformaldehyde and permeabilised with 0.1% Triton X-100. To minimise non-specific binding, cells were blocked by incubation in 10% FBS in PBS. Expression of α-SMA was assessed with anti-α-SMA antibody and the specificity of staining was confirmed with mouse-IgG2a isotype control used at the same concentration. The cell suspensions were kept overnight at 4 °C, washed in 0.01% Triton X-100 PBS buffer, stained for 45 min with secondary FITC-conjugated goat anti-mouse IgG (Sigma—Aldrich), and analysed by flow

cytometry (Coulter EPICS XL, Beckman Coulter, Fullerton, CA). Results were expressed as a ratio of mean fluorescent intensity (MFI) of α-SMA labelled sample to MFI of corresponding isotype control sample.

Preparation of cell extracts and immunoblotting

Cells were cultured in cell culture Petri dishes until confluency. The cells were stimulated with TGF-β_1 (5 ng/ml) for 7 days. Fibroblasts were lysed with the lysis buffer (0.1 mol/L Tris-HC1, 15% glycerol, 2 mM EDTA, 2% SDS,

10 mM phenylmethytsulfonyl fluoride, and 10 μg/mL aprotinin, pH 7.4), and 10 μg of total cellular protein was subjected to SDS-PAGE in 15% acrylamide gels under reducing conditions and transferred into polyvinylidene difluoride membrane (Hybond-P, Amersham Pharmacia Biotech). After blocking in PBS-T (0.1% Tween 80 in PBS) containing 5% skimmed milk at room temperature, the membrane was incubated for 1 h with mouse monoclonal anti-α-SMA antibody (1:1000 in 3% BSA in PBS). After washing 3 times, the membrane was incubated for 1 h with anti-mouse IgG horseradish peroxidase-conjugated antibody (1:3000 in
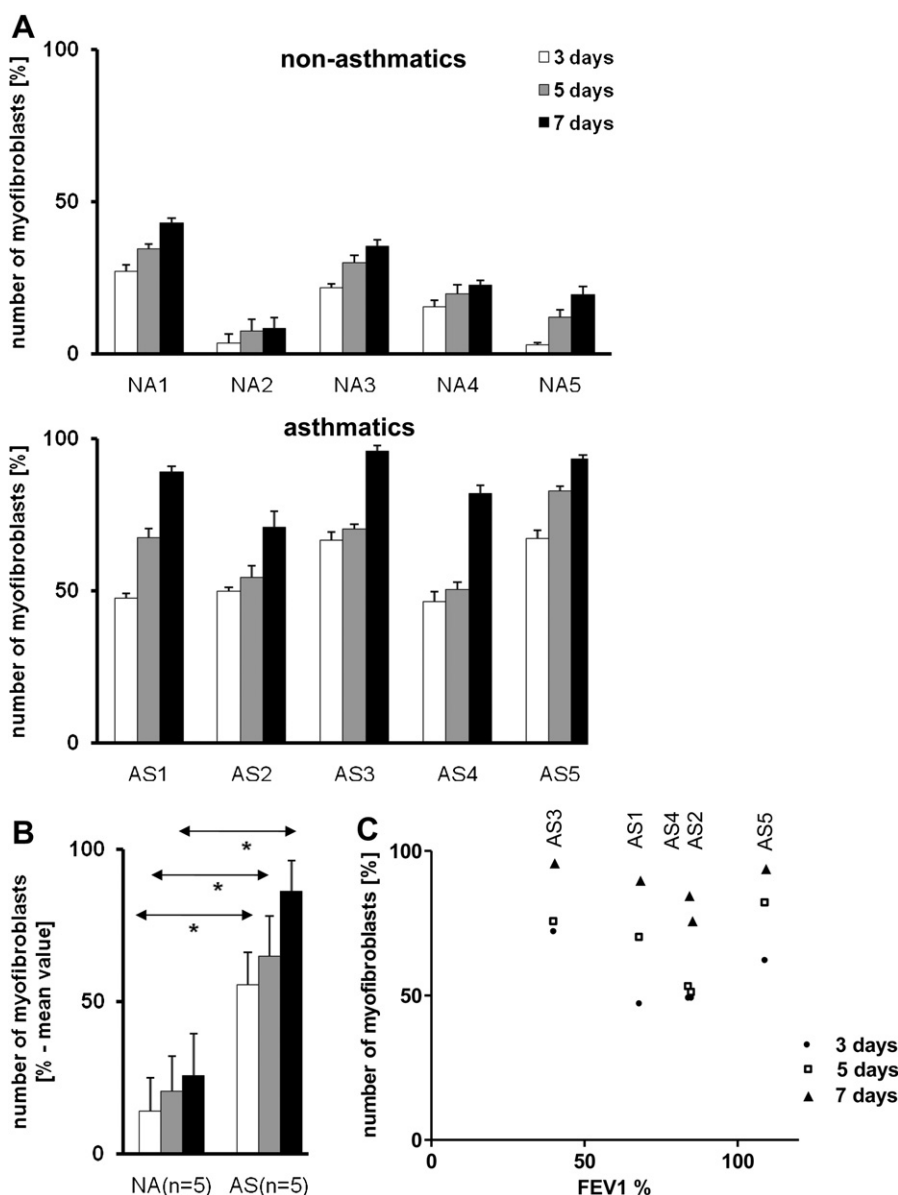
Figure 1 TGF-β_1 induced differentiation of HBF into myofibroblasts depends on the duration of the factor-treatment. HBF from non-asthmatics (NA: n = 5) and asthmatics (AS: n = 5) were cultured in DMEM with 10% FCS for 24 h, then with DMEM supplemented 0.1% BSA with 5 ng/ml TGF-β_1 for 3, 5 and 7 days. The cells were fixed and stained for α-SMA and the number of α-SMA positive cells (myofibroblasts) was counted. Each point represents the result obtained from particular cultures (A) or the mean values of both groups (NA and AS cultures), respectively (B). The results presented are the means of at least three separated experiments for each culture. The statistical analysis was performed using Mann—Whitney U-test, $*$ - $p < 0.01$ between groups of subjects (AS versus NA). (C) The graph illustrates the lack of correlation between the number of TGF-β_1-induced α-SMA positive cells in HBF derived from asthmatic patients and the asthma severity (FEV1%) of the donors (AS: n = 5).

PBS-T containing 5% skimmed milk; Santa Cruz Biotechnology). The extensively washed membranes were incubated with chemiluminescent reagent (ECL-Western blotting analysis system, Amersham) for 5 min and exposed to Kodak X-Omat film.

Results

"Asthmatic" HBF populations are more prone to FMT

First experiments were aimed to compare a time-dependence of FMT in NA and AS HBF cell populations undergoing a long-lasting TGF-β_1 treatment. Analyses of α-SMA-positive cells in NA and AS cell populations performed on day 3, 5 and 7 after TGF-β_1 stimulation revealed an increase in the percentage of myofibroblasts in both analysed groups (Fig. 1). However, FMT was much more frequent in asthmatic HBF cell populations (NA, between 9

and 40%; AS, between 70 and 95% on day 7). These data demonstrate differences in the potential of non-asthmatic and asthmatic fibroblasts to differentiate into myofibroblasts in response to TGF-β_1, and show that this process is time-dependent. It is striking that the lowest FMT level in AS group is much higher than the highest value in NA group. However, no significant correlation between TGF-β_1—induced FMT potential and the asthma severity (FEV1%) of the donors could be observed (Fig. 1C). Although the study group of asthmatics is too small to drive the final conclusion it can be assumed that the enhanced potential of FMT occurs throughout the spectrum of asthma severity.

FMT level is dependent on HBF culture density

We further compared TGF-β_1-reactiveness of NA and AS bronchial fibroblasts plated at 5000 cells/cm^2 (low density) and 50000 cells/cm^2 (high density cells) to elucidate density-dependence of FMT. When AS HBF were incubated in the presence of TGF-β_1 for 7 days, a decreased level of FMT was
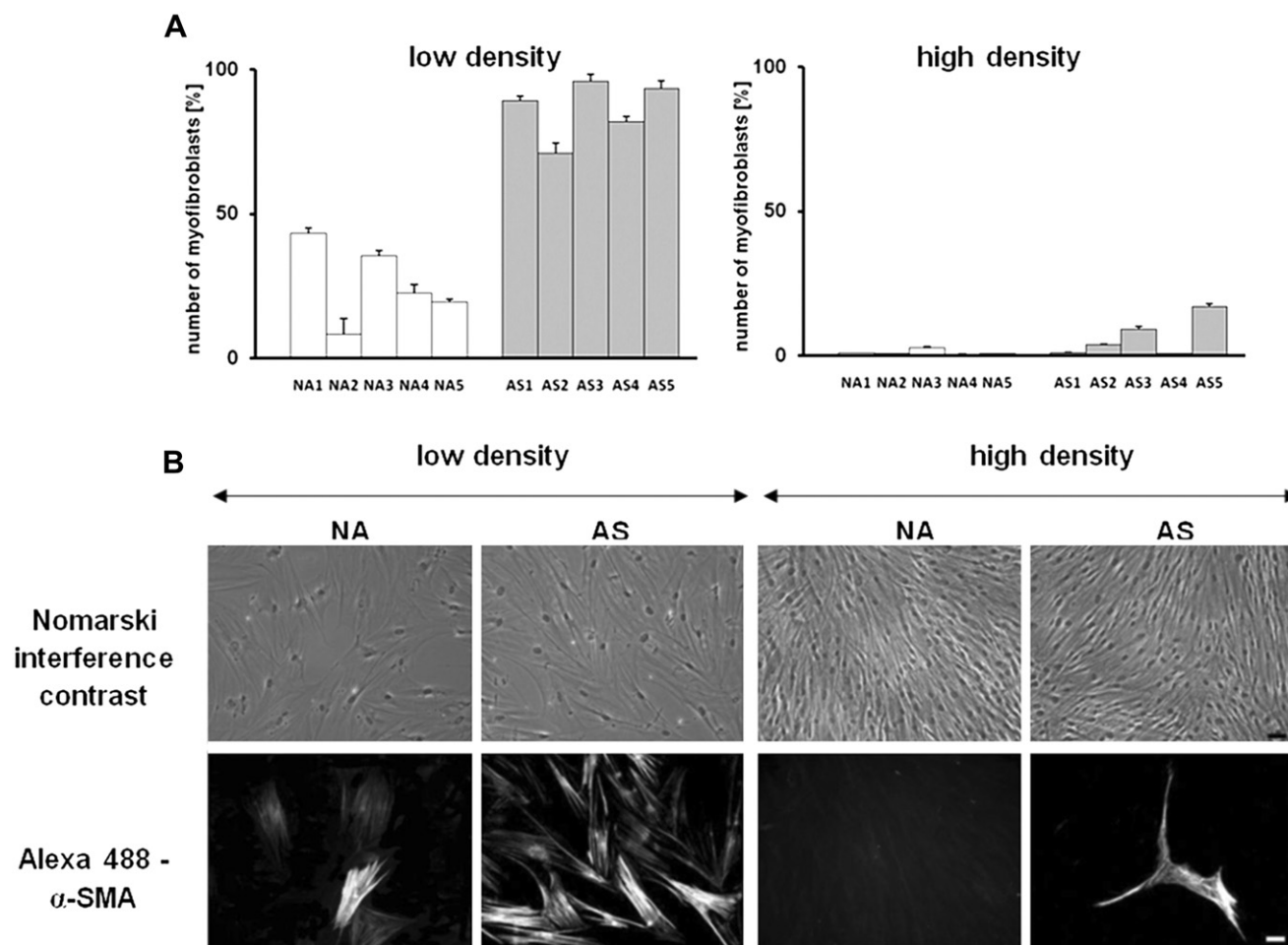

Figure 2 HBF differentiation into myofibroblasts is cell density-dependent. (A) HBF from non-asthmatics (NA: n = 5) and asthmatics (AS: n=5) were plating at low (5000 cells/cm^2) and high (50,000 cells/cm^2) density and cultured in DMEM with 10% FCS for 24 h, then with DMEM supplemented with 0.1% BSA and 5 ng/ml TGF-β_1 for 7 days. Cells were stained for α-SMA and the number of positive cells was counted. (B) Cells from representative non-asthmatic (NA) and asthmatic (AS) culture were photographed with Nomarski interference contrast optics and with epifluorescence mode. Note that majority of cells in low-density AS culture were myofibroblasts whereas in identical high-density culture only few α-SMA-staining cells appear to be growing surprisingly on top of the confluent fibroblasts. Bar = 50 μm.
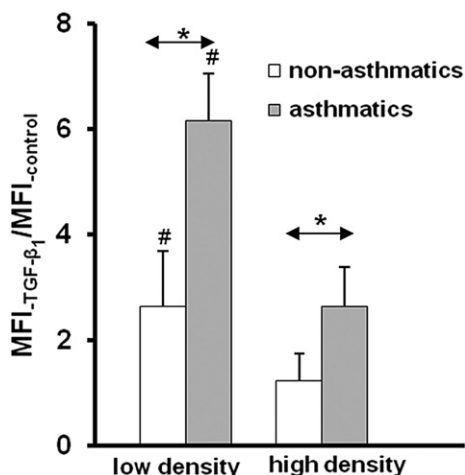
Figure 3 The cytometric analysis of α-SMA expression in HBF plated at low or high density. HBF were cultured in DMEM supplemented with 0.1% BSA and with or without TGF-β_1 (5 ng/ml) for 7 days. Cells were stained with anti α-SMA antibody. Mean fluorescence intensity of cells population (MFI) was measured using cell cytometry. Results are calculated as a ratio of MFI of TGF-β_1 treated to MFI of untreated culture (control) and presented as medians $\pm$quartiles (n = 7). The statistical analysis was performed using two ways ANOVA, $*$- $p < 0.05$ between groups of subjects (AS versus NA); # - $p < 0.01$ between low density and high density cultures.

observed with the higher cell density. In parallel, NA fibroblasts also reacted to the increased cell-seeding density with a decreased level of fibroblast-to-myofibroblast differentiation. However, in these cells, the effect was less pronounced (Fig. 2A and B). These data were also confirmed by FACS analyses. They revealed that asthmatic cells at the initial high density displayed a significantly lower increase of α-SMA MFI upon TGF-β_1 stimulation, as compared with their counterparts seeded at the low density. It is worth noting that in cells of AS group α-SMA content increased upon TGF-β_1 stimulation even in high density cultures, in contrast to cells of NA group (Fig. 3). The same conclusions could be drawn from α-SMA expression measured by immunoblotting (Fig. 4), which was
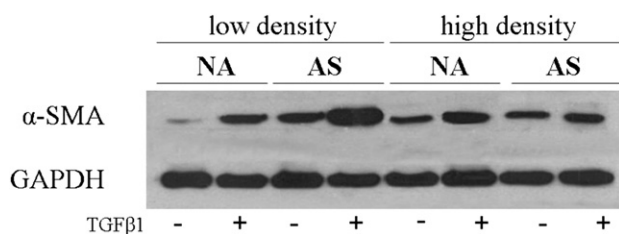
Figure 4 The expression of α-SMA in control and TGF-β_1-treated HBF of the NA and AS groups at low and high density. Cells were cultured as described in Fig. 2. Protein extracts (10 μg of protein per lane) were subjected to SDS-PAGE electrophoresis and α-SMA protein/GAPDH was detected by immunoblotting. Note the increased amount of α-SMA in TGF-β_1- treated cells in comparison to non-stimulated cells (derived from both AS and NA group) and that immunodetectable α-SMA was decreased considerably after high-density passage.

the highest in TGF-β_1-treated cells derived from AS group and cultured at low cell density. All these results demonstrate that the expression of α-SMA depends both on cell density and on cell origin and indicate that intercellular communication affects cellular susceptibility to TGF-β_1-induced FMT.

FMT in low density HBF cultures is not dependent on the humoral factors

In search of the factors responsible for the increased reactivity of bronchial fibroblasts to TGF-β_1 in low-density cultures, we focused on the possible inhibitory role of autocrine factors secreted by confluent HBF. The sparse HBF cultures, randomly chosen from the AS group, were subjected to TGF-β_1-stimulation in a medium conditioned by dense HBF cultures derived from the same subject. As shown in Fig. 5, no inhibitory effect of such a medium on the induction of FMT was observed. This is illustrated by similar percentages of α-SMA positive cells observed in TGF-β_1-supplemented with conditioned or fresh medium, respectively. Similarly, no statistically significant differences in the readiness of representative AS cells to differentiate into myofibroblasts between control and serum-starved cells were revealed (Fig. 6), indicating that contact-mediated HBF quiescence is not involved in the observed desensitisation of dense fibroblasts to TGF-β_1.

FMT is affected by N-cadherin—dependent intercellular adhesion

Thus, neither paracrine regulatory systems, nor the arrest of the cell cycle, affected the efficacy of density-dependent FMT of asthmatic HBF. Instead, these data
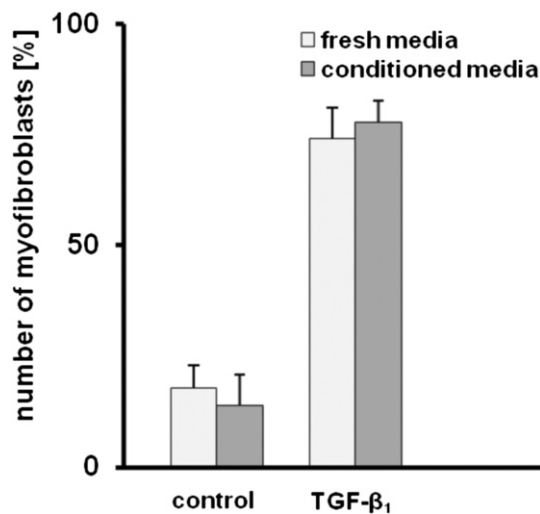
Figure 5 Asthmatics HBF grown at high density do not secrete factors which inhibit the process of myofibroblast differentiation. HBF from asthmatics were plated at low density (5000 cells/cm^2) and cultured in DMEM supplemented with 0.1% BSA (fresh media) or in the fibroblast-conditional medium (see Material and methods) derived from the same subject (conditioned media) without or with 5 ng/ml TGF-β_1. After 7 days cells were fixed and stained for α-SMA. The number of α -SMA positive cells was counted.
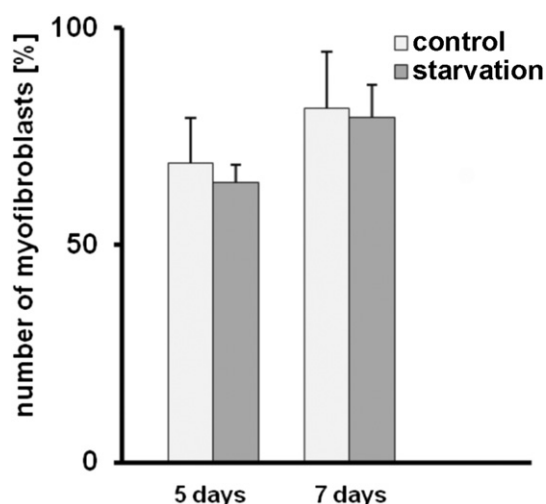
Figure 6 Fibroblast-to myofibroblast transition in low density HBF cultures is not affected by G0/G1 phase arrest of the cell cycle. HBF from asthmatics (AS: n = 5) were plated at density 3000 cells/cm^2 and cultured in DMEM with 10% FCS for 24 h, then with serum—free DMEM supplemented with 0.1% BSA for 5 days (starvation) or cells were plated at density 5000 cells/cm^2 and cultured in DMEM with 10% FCS for 24 h (control). After that as control cells as cells after serum starvation were culture in DMEM supplemented with 0.1% BSA and 5 ng/ml TGF-β_1 for 5 or 7 days, respectively. The cells were fixed and stained for α-SMA and the number of α-SMA positive cells was counted.

suggest an involvement of cell—cell contacts in the process of FMT. As intercellular contacts between fibroblasts are, among others, mediated by cell-surface, calcium-dependent N-cadherin adhesion molecules, in subsequent experiments we tested whether N-cadherin blocking antibody affected FMT. Cadherin-mediated intercellular contacts are abundant upon confluence in HBF cultures.[24] Unexpectedly, when N-cadherin blocking antibody was added to the dense AS cell cultures it further inhibited the process of density-dependent FMT (Fig. 7A). This observation suggests that binding of specific antibodies to N-cadherin molecules inhibits myofibroblasts formation in a manner similar to that caused by cell-to-cell contacts. In order to verify this hypothesis, we studied the effects of N-cadherin specific blocking on FMT in sparse culture to demonstrate that anti-N-cadherin antibody also inhibited the process of FMT to the level characteristic for unstimulated cultures (Fig. 7A). Moreover, N-cadherin blocking affected actin cytoskeleton organisation, as revealed by phalloidin staining. Cells grown in the N-cadherin blocked conditions demonstrated less numerous and thinner stress fibres (Fig. 7B). Altogether these observations indicate that N-cadherin-mediated events are crucial for HBF transdifferentiation.

Discussion

Although the role of myofibroblasts during the development of asthma is well established,[12,25] the process of bronchial wall remodelling in which these cells are involved remains largely obscure, and the specific mechanisms determining FMT in the asthma progression remain a matter of debate. It is known that increased secretion of factors belonging to TGF-β family by fibroblasts and infiltrating blood cells accompanies the development of asthmatic chronic inflammation.[18,26] Therefore, it can be assumed that stimulation of primary HBF in culture by TGF-β_1 to some extend mimics the milieu of fibroblasts in inflamed bronchial wall. Here, we demonstrate that the intensity of FMT induced by TGF-β_1 in vitro not only depends on the origin of analysed fibroblasts (derived from non-asthmatic vs. asthmatic patients) but also on the duration of TGF-β_1 treatment. Moreover, this process is positively affected by initial low cell densities, at the early stages of HBF differentiation.

The striking difference observed between the susceptibility of AS and NA fibroblasts to adopt myofibroblast phenotype in response to TGF-β_1 indicates that HBF derived from asthmatic patients bear some predisposition, which facilitates TGF-β_1-induced FMT. We suggest that a preselection might have occurred prior to the establishment of HBF primary cultures which resulted in the formation of subpopulation hyper-responsive to TGF-β signals. On the other hand, there was no difference in HBF proliferation rate in vitro between AS and NA groups (data not shown). Similar results were obtained by Ward et al., who studied the effect in larger groups of subjects.[27] Thus, some factors secreted in the early inflammatory foci might promote the proliferation of specific fibroblast subpopulations in asthmatic airways.[13,28]

Both AS and NA HBF were more apt to differentiate into myofibroblasts in the prolonged exposure to TGF-β_1 compared to short-term TGF-β_1 treatment.[20] This is consistent with the role of TGF-β_1 in the aetiology of asthma.[15,26] These data show the necessity of developing experimental models which maximally reflect the local HBF environment in vivo and enable the precise control of FMT efficacy. We claim that our experimental approach, based on the modulation of initial cell density of AS and NA HBF cultured in the presence of TGF-β_1 and the duration of the experiment at least partially reconstitutes the in vivo "asthmatic niche". It mimics early differentiation events crucial for the bronchial wall cell FMT. Although it is difficult directly to compare the spatial 3-D microenvironment of fibroblast in bronchial tissue to the planar model of cells cultured in a plastic vessel, it is evident that, at least in the initial stage of remodelling, the fibroblasts are sparse in the healthy bronchial wall and do not form many cell—cell contacts. When fibroblasts become more abundant due to their enhanced proliferation and recruitment of fibrocytes from the bloodstream or EMT,[3,29] the homophilic contacts between them are more likely. It is worth noting, that the main physiological role of fibroblasts adherence junctions in vivo might be the transmission of a force to ECM leading to tissue contraction — the process which may contribute to the asthmatic attack or bronchi remodelling.

Local cell density has long been regarded as a factor affecting the fate of normal cells.[30] We show that both AS and NA HBF reacted to the initial low densities in vitro with the increase of TGF-β_1 induced FMT. However this effect was much more pronounced in AS HBF populations than in their NA counterparts. This observation is in accordance with the previous data on the direction of fibroblast towards the myofibroblastic phenotype by the density at
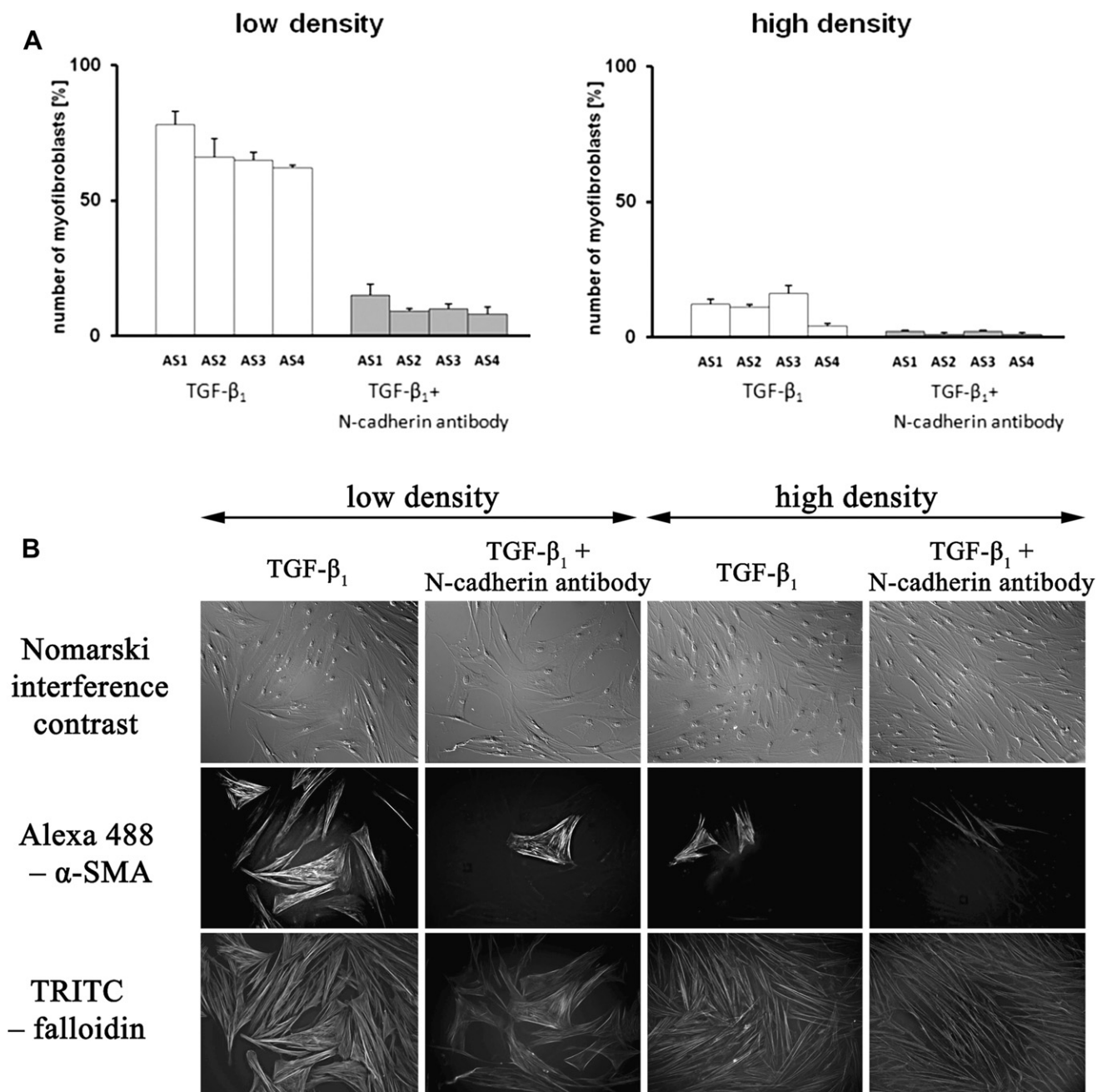
Figure 7 Fibroblast-to myofibroblast transition in HBF cultures is affected by N-cadherin—dependent intercellular adhesion. (A) HBF from asthmatics were plated at low (5000 cells/cm^2) and high (50,000 cells/cm^2) density in DMEM with 10% FCS or in the same medium containing the N-cadherin blocking antibody (dilution 1:100) for 24 h, and then with serum-free DMEM supplemented with 0.1% BSA and 5 ng/ml TGF-β1 without or with N-cadherin blocking antibody, respectively. After 7 days, the cells were fixed and stained for α-SMA and the number of α-SMA positive cells was calculated. (B) Cells from representative asthmatic cultures were photographed with Nomarski interference contrast optics and with epifluorescence mode. Bar = 50 μm.

which cells are plated.[21,22,31] Furthermore, we proved using our model system that neither HBF-conditioned medium nor growth arrest in G_0/G_1 phase of cell cycle could stop the TGF-β_1-induced FMT in asthmatic cell populations. Instead, the blocking of N-cadherin (a protein responsible for formation and maturation of intercellular junctions and creation of tension between cells[24]) in sparse asthmatic HBF resulted in the inhibition of FMT. The question arises about the mechanism of this phenomenon.

Mechanical stress has long been described as a prerequisite for the formation and the maintenance of stress fibres and the incorporation of α-SMA into microfilament bundles and subsequent development of differentiated myofibroblast.[10,14,32] Furthermore, focal adhesions were implicated in the development of mechanical tension in HBF cell populations.[8,33,34] It was suggested that the organisation of N-cadherin based cell—cell adhesions activated Rho GTP-ase activity in many types of cells[24,35] and that

Rho activation is crucial for FMT in lung fibroblasts.[36] It is thus possible that a low level of Rho activation in the absence of N-cadherin cell—cell contacts is insufficient for development of a myofibroblastic phenotype. In general, activation of small G protein Rho and its downstream targets leads to activation of an actin-myosin system (smooth muscle cell contraction) and development of tension (fibroblasts stress fibres formation).[10] Furthermore, Rho proteins have recently been recognised as a possible target for the treatment of airway hyper-responsiveness in bronchial asthma.[37]

It should be stressed that the investigation of cell behaviour for the elucidation of basic mechanisms responsible for the arousal of such complex diseases as asthma can only be reliably performed by employing model experimental systems. The experimental model used in this study mimics basic properties of bronchial fibroblasts during asthma and the chronic inflammation crucial for this disease. For example, typical Th2 cytokines produced within asthmatic bronchial wall by infiltrating cells induce prolonged production of TGF-β by structural cells *in vivo*.[38] It is a hallmark of persistent asthmatic inflammation which stimulates phenotypic changes of bronchial fibroblasts. In our hands, HBF maintain their original phenotype and are prone to TGF-β- induced FMT even following 5-15 divisions *in vitro*. Such a reductionist approach enabled us to test for between the groups differences in HBF susceptibility to TGF-β_1 - induced FMT, a topic very difficult to address by studies using *in vivo* models. Whereas these data should be verified using clinical approach, its is hard to expect simple correlations due to the complexity of the disease.

Our results show for the first time that FMT in asthmatic HBF populations is N-cadherin dependent, and can be effectively decreased by inhibition of intercellular adhesion junctions. The initial absence or an induced loss of cell—cell adhesions might be of special importance for an inhibition of FMT, similarly to the situation observed in epithelium.[39,40] It was shown recently that, upon induction by TGF-β, intestine fibroblasts overexpressed N-cadherin and increased basal cell migration, which also was prevented by N-cadherin inhibition.[41] Even if this example lacks its relevancy to the chronic bronchial disease, it points to a general mechanism of wound healing as a contributor to bronchial remodelling. Our findings strongly suggest, that inhibition of N-cadherin or its downstream targets, could be useful for anti-asthma therapy not only because of reduction of bronchial contraction driven by smooth muscle cells, but also by diminishing FMT-dependent remodelling of the airway wall.

Acknowledgements

Conflict of interest

None of the authors have any conflicts to disclose.

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