Invasive bronchial fibroblasts derived from asthmatic patients activate lung cancer A549 cells in vitro

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Abstract. Epidemiological data suggests that there are functional links between bronchial asthma and lung carcinogenesis. Bronchial fibroblasts serve a prominent role in the asthmatic process; however, their involvement in lung cancer progression remains unaddressed. To estimate the effect of the asthmatic microenvironment on the invasiveness of lung cancer cells, the present study compared the behavior of human non-small cell lung cancer A549 cells exposed to the signals from human bronchial fibroblasts (HBFs) derived from non-asthmatic donors (NA HBFs) and from asthmatic patients (AS HBFs). NA HBFs did not significantly affect A549 motility, whereas AS HBFs and the media conditioned with AS HBF/A549 co-cultures increased Snail-1/connexin43 expression and motility of A549 cells. In contrast to NA HBFs, which formed A549-impenetrable lateral barriers, α-SMA+ AS HBFs actively infiltrated A549 monolayers and secreted chemotactic factors that arrested A549 cells within AS HBF/A549 contact zone. However, small sub-populations of A549 cells could release from this arrest and colonize distant regions of AS HBF monolayers. These data indicated that the interactions between lung cancer cells and HBFs in asthmatic bronchi may facilitate the colonization of lung tumors by fibroblasts. It further stabilizes the tumor microenvironment and potentially facilitates collective colonization of novel bronchial loci by cancer cells.

Potential mechanistic links between the asthmatic process and lung cancer progression suggest that bronchial asthma should be included in the list of potential prognostic markers for lung cancer therapy.

Introduction

Bronchial asthma is one of the most common chronic diseases throughout the world and its rate of prevalence has continuously increased over the last decades (1). During the asthmatic process, epithelial damage is followed by the thickening of asthmatic bronchial walls, which is mechanistically linked to the local inflammation and the activation of bronchial fibroblasts (2-4). Local inflammation induces the differentiation of bronchial fibroblasts into α-smooth muscle actin (α-SMA)positive myofibroblasts and increases their longevity (5) through paracrine (for example TGFβ-dependent) and cell adhesion-dependent mechanisms (3,6,7). A high extracellular matrix-producing activity and contractility of myofibroblasts (8) participates in bronchial fibrosis, fibrotic remodeling of airways and lung parenchymal compartments, and a pulmonary dysfunction. The asthmatic process may also facilitate the progression of other chronic lung diseases. Importantly, epidemiologic data show the correlation between the incidence of asthma and lung cancer in developed countries (9).

Chronic inflammation and tissue fibrosis have long been considered to induce phenotypic shifts in cancer cells, such as epithelial-mesenchymal transition (EMT), which accounts for the formation of cancer invasive front. In particular, cancer-associated fibroblasts (CAFs) participate in cancer progression and metastasis (10,11). During lung cancer progression, the connective tissue of bronchial walls provides the scaffold for developing tumors, forms tissue barriers against immune responses of the host and/or constitutes the routes for the invasion of cancer cells (12). Accordingly, the activation of fibroblasts/myofibroblasts, which is characteristic for the asthmatic process, may facilitate lung cancer progression. However, the contribution of asthmatic bronchial fibroblasts in systemic dissemination of lung cancer cells remains unaddressed. We have previously shown that human bronchial fibroblasts (HBFs) derived from asthmatic patients (AS HBFs) display relatively high susceptibility to TGFβ-inducible fibroblast-myofibroblast

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Abbreviations: HBF, human bronchial fibroblasts; Cx43, connexin43; α-SMA, α-smooth muscle actin; EMT, epithelial-mesenchymal transition; FMT, fibroblast-myofibroblast transition; CAFs, cancer-associated fibroblasts; CM, conditional media; CME, coefficient of movement efficiency

Key words: lung cancer, bronchial asthma, invasion, CAFs, intercellular communication
transition [FMT; (13-15)]. These heritable pro-fibrotic properties of AS HBFs prompted us to hypothesize that AS HBFs may contribute to lung carcinogenesis. To elucidate the links between their function and the progression of lung cancer, we compared the behavior of human non-small cell lung cancer A549 cells exposed to the signals from monolayers of AS HBFs and from HBFs derived from non-asthmatic (NA) donors.

Materials and methods

Isolation and propagation of primary HBFs. Primary HBFs were isolated from bronchial biopsies derived from i) the individuals in whom diagnostic bronchoscopy ruled out any serious airway pathology, i.e., asthma, chronic inflammatory lung disease or cancer (NA group; N=5, averaged age, 57.4±9.9 years; FEV1(%)=101.83±14.7) and ii) from the patients with moderate asthma (AS group; N=5; age, 39±17.6 years; mean duration of asthma=16.7±12.2 years; FEV1(%)=48.75±0.86). Patients were treated in the Department of Medicine of Jagiellonian University (Kraków, Poland) and were in a stable clinical condition. AS HBF lineages were derived from a 38 years old man/66 years old woman (asthma duration=10/30 years; FEV1(%)=35/52.5). These lineages displayed a considerably higher pro-fibrotic potential than their non-asthmatic counterparts; however, no relationship between their phenotypic properties and the age/sex of the donors, or with the development of asthma severity was observed (6,13-15). The present study was approved by the Jagiellonian University’s Ethics Committee (decision no. 122.6120.69.2015) on the basis of written informed consent for the use of HBF cell lines in basic research, which had previously been obtained from all donors. For endpoint experiments, HBFs were harvested between the 5th and 15th passage after the establishment of primary cultures.

Co-cultures of A549 cells with HBFs. HBFs were propagated in DMEM supplemented with a 10% FCS and antibiotic-antimycotic cocktail (both Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) whereas human lung carcinoma A549 cells (ECACC 86012804) were cultured in RPMI-1640 medium (Sigma-Aldrich; Merck KGaA) with the same supplements. Cells were incubated in a humidified atmosphere with 5% CO₂ at 37°C to reach 80% confluence. For ‘open’ co-cultures with HBFs, A549 cells were seeded on coverslips at the density of 10⁵ cells/cm² together with HBFs (seeded at 10³ cells/cm²) and incubated for 48 h in DMEM supplemented with a 10% FCS. Alternatively, A549 and HBFs (NA or AS) were seeded into the chambers of Ibidi µ-Slide 2 Well Co-Culture dish (81806; Ibidi GmbH, Munich, Germany) for 48 h in 2 ml of the same medium (i.e., in the conditions allowing only for exchange of the medium between the chambers; ‘isolated’ co-cultures). Furthermore, A549 cells and HBFs were seeded in the chambers of 2 well silicone inserts with a defined cell free gap (81176; Ibidi GmbH) cultured for 6 h and the next 42 h after the removal of silicone insert (‘confronted’ co-cultures). Media conditioned by HBFs and 3 variants of HBF/A549 co-cultures were collected and kept in -80°C for endpoint experiments. For the estimation of the effect of A549/HBF ‘secretomes’ on A549 motility/transmigration, the cells were incubated in the presence of the mixture of relevant conditioned medium/fresh medium (1:1 v/v). For 2D invasion assays A549 cells and HBFs were seeded in the chambers of 2-well silicone inserts (81176; Ibidi GmbH). The cells were cultured to monolayers (48 h) and allowed to migrate for the next 48 h after removal of the silicone insert. Then, the cells were fixed and stained (see below) to estimate the effect of paracrine/juxtacrine signaling between A549 cells and HBFs on A549 invasive front formation.

Cell migration and transmigration tests. Movement of A549 cells in co-cultures with HBFs and in the conditioned media was registered using Leica DMI6000B time-lapse videomicroscopy system (Leica Microsystems GmbH, Wetzlar, Germany) equipped with a temperature/CO₂ chamber and Integrated Modulation Contrast (IMC) optics. Sequences of cell centroid positions were recorded at 300 s time intervals for 6 h using a dry 20x, NA-0.75 objective (16). The averaged total length of cell trajectory (i.e., the ‘Distance’ covered by the cells during the registration time; μm) and the total length of cell displacement (‘Displacement’, i.e., the distance from the starting point directly to the cell’s final position; μm) were quantified from cell trajectories with the purpose-designed Hiro program. The averaged velocity of cell motility (VCM) and velocity of cell displacement (VCD) were defined as the averaged ‘Distance/time of registration and averaged ‘Displacement/time of registration, respectively. Cell trajectories (N>50) from no less than three independent experiments (N>3) were taken for the estimation of statistical significance. For transmigration assays, A549 cells were seeded into chambers containing microporous membranes (pore diameter 8 μm; membrane diameter 6.5 mm; 2422; Corning, NY, USA) at the density of 300 cells/mm². The inserts were placed in the wells of 24-well plates filled with control, HBF and HBF/A549-conditioned media (1:1 v/v with a fresh medium) and the cells were allowed to transmigrate for 48 h. The numbers of the transmigrated cells were determined with Coulter counter after the next 48 h (17).

Immunofluorescence and cytofluorimetry. For the immunofluorescence analyses of Snail-1/α-SMA/Cx43, the cells were fixed in MetOH/Acetone (7:3) solution for 10 min. in -20°C. The fixed cells were incubated in 3% BSA solution and incubated with the primary antibodies (mouse anti-α-SMA IgG; Sigma-Aldrich; Merck KGaA; A2547; 1:300); rabbit anti-Snail-1 antibody (SAB 4504319; 1:100) and mouse anti-Cx43 IgM (both Sigma-Aldrich; Merck KGaA; C8093; 1:300) for 1 h. Then, the cells were incubated with the secondary antibody [Alexa Fluor® 647-conjugated goat anti-mouse IgG (A21235), Alexa Fluor® 488-conjugated goat anti-rabbit IgG (A11008) or Alexa Fluor® 546-conjugated goat anti-mouse IgM (A21045); all 1:500; Thermo Fisher Scientific, Inc., Waltham, MA, USA]. Image acquisition was performed with Leica DMI6000B microscope (DMI7000 version; Leica Microsystems GmbH) equipped with the Nomarski Interference Contrast (DIC) module. Cytofluorimetric analyses were performed with LasX software (Leica Microsystems GmbH) and ImageJ freeware (National Institutes of Health, Bethesda, MD, USA), respectively (17) to generate histograms that show the co-localization of immunofluorescence signals along the scan line.

Statistical analysis. Statistical analysis was performed using one-way analysis of variance followed by Tukey's honestly
significant difference post-hoc and Statistica Data Miner Software version 13 (StatSoft, Inc., Tulsa, OK, USA). Data are expressed as the mean ± standard error of the mean. *P<0.05 was considered to indicate a statistically significant difference.

**Results**

**AS HBFs stimulate the invasive behavior of A549 cells.** To estimate how asthmatic bronchial microenvironment participates in lung cancer progression, we used an experimental approach based on the ‘open’ co-cultures of non-small cell lung cancer (A549) cells with bronchial fibroblasts derived from 2 asthmatic patients (AS HBFs). An activation of A549 cells in the proximity of both AS HBF lineages was illustrated by the induction of their motility in the co-cultures (Fig. 1A). Interestingly, AS1 HBFs activated A549 cell locomotion without any considerable effect on its efficiency (the cell displacement), whereas the increased velocity of A549 displacement was observed in the proximity of AS2 HBFs (Fig. 1C; cf. Table I). Differences in A549 cell reactions to the signals from the analyzed AS HBF lineages were accompanied by different behavior of AS and NA HBFs in the proximity of A549 cells. AS HBFs of both lineages were dispersed among A549 cells, whereas NA HBFs formed compact multicellular clusters (Fig. 1B) and only slightly affected the motility of A549 cells (Fig. 1D). Pictures in Fig. 1B illustrate the morphology of NA and AS HBFs in confluent co-cultures with A549 cells. Some differences in the HBF density result from more efficient spreading of the fibroblasts from AS group. These observations suggest that HBFs from asthmatic bronchi can differentially activate lung cancer cells via paracrine and/or juxtacrine signaling pathways.

**ASHBFs induce the motility of A549 cells via contact-modulated paracrine signaling.** Numerous signaling systems have been implicated in the regulation of cancer cell motility by CAFs (18). In our hands, media conditioned by both analyzed AS HBF lineages augmented the motility of A549 cells (Fig. 2A). A549 displaced more efficiently in the presence of AS1 HBF-conditioned medium, whereas AS2 HBF secretome had no significant effects on the efficiency of A549 displacement, even though it induced the movement of A549 cells (Fig. 2C; Table I). An induction of A549 displacement was also observed in the presence of the media conditioned by AS HBF/AS459 cells co-cultured in the conditions allowing for their mutual paracrine (i.e., in ‘isolated’ co-cultures) and paracrine/juxtacrine interactions (i.e., in ‘confronted’ co-cultures). Generally, this effect was stronger than in the presence of the media conditioned by AS HBFs and ‘open’ AS HBF/AS459 co-cultures; however certain differences were seen in A549 responses to AS1 HBF/AS459 and AS2 HBF/AS459 secretome (Fig. 2C). We also observed nuclear accumulation of Snai1 and Cx43 up-regulation in A549 cells cultured in the media from the ‘isolated’ co-cultures (Fig. 2B). On the other hand, NA HBF/AS459 secretomes displayed a considerably lower pro-invasive activity regardless of the culture conditions (Fig. 2D; Table I). Collectively, these data show that paracrine/juxtacrine communication between A549 cells and AS HBFs affects their secretome, thus regulating A549 invasive behavior.

**AS HBFs selectively modulate the invasion of A549 cells.** To further estimate the biological significance of juxtacrine/paracrine loops between bronchial fibroblasts and lung cancer cells, we analyzed the behavior of HBFs and A549 cells at the confrontation front of their monolayers. When confronted with A549 cells, NA HBFs formed lateral barrier structures, which were similar to these seen in the ‘open’ co-cultures (see Fig. 1C) and remained impenetrable to A549 cells (Fig. 3A). In turn we observed α-SMA up-regulation in AS HBFs. Concomitantly, α-SMApositive AS HBFs efficiently...
infiltrated A549 monolayers, which was accompanied by the remodeling of interfaces between AS HBF and A549 monolayers. Noteworthy, α-SMA expression was also observed in A549 cells and in AS HBFs exposed to AS HBF/A549 conditioned media (Fig. 3B). On the other hand, we did not observe any collective infiltration of AS HBF continua by A549 cells, even though Snail-1 was accumulated in A549 nuclei throughout the contact zone with AS HBFs (Fig. 3C). Instead, small and scattered clusters of Cx43<sup>positive</sup> A549 cells were observed within AS HBF continua (Fig. 3A and B; inserts). This was accompanied by a relatively high chemotactic response of A549 cells to the media conditioned by ‘open’ A549/AS HBF co-cultures (Fig. 3D). These data indicate that juxtacrine/paracrine signaling in A549/HBF co-cultures facilitates the activation of AS HBFs and secretion of chemotactic factors, which arrest lung cancer cells in A549/HBF confrontation front. Small sub-populations of resistant cells can overcome this arrest and migrate through cell-penetrable interfaces between AS HBF and A549 monolayers.

**Discussion**

The contribution of the tissue microenvironment to the cancer disease is exemplified by the involvement of endothelial, immune and connective tissue cells in cancer promotion and progression. These processes are regulated by paracrine and juxtacrine loops that are locally established between cancer and stromal cells (19-21). In particular, the interactions between cancer cells and CAFs participate in the formation of the scaffolds that sustain the structure of tumor-protective tissue barriers (22,23). CAFs can also generate signals crucial for the microevolution and expansion of invasive cancer cell sub-populations. Our study is the first to suggest the functional links between lung cancer progression and pro-fibrotic properties of fibroblasts that reside in asthmatic bronchi.

We have previously shown a high pro-fibrotic activity of the fibroblasts derived from asthmatic bronchi. In response to TGFβ, AS HBFs undergo fibroblast-myofibroblast-transition (FMT), which facilitates bronchial remodeling and asthmatic process in vivo (13,15,24). Here we have shown that AS HBFs react to A549 cells and to AS HBF/A549 secreteme with α-SMA/Cx43 up-regulation, which is a sign of their myofibroblastic differentiation (15). Concomitantly, Snail-1/Cx43 activation and the induction of A549 cell motility was detected in A549 cells exposed to direct contacts with AS HBFs and to AS HBF/A549 secreteme. Snail-1/Cx43-dependent axis has been suggested to regulate the invasiveness of the prostate (17,25) and lung cancer cells (26). Therefore, these observations confirm that paracrine/juxtacrine interactions

| Variant                  | Distance ± SEM (µm) | Displacement ± SEM (µm/min) | CME ± SEM (%) |
|--------------------------|---------------------|-----------------------------|---------------|
| Control (A549)           | 268.797±9.291       | 18.611±1.376                | 6.880±0.723   |
| +CM HBFs                 |                     |                             |               |
| NA1                      | 447.073±13.836<sup>a</sup> | 28.226±2.796                | 7.582±0.900   |
| NA2                      | 417.337±13.776<sup>a</sup> | 20.420±3.197<sup>a</sup>    | 5.632±0.844   |
| AS1                      | 325.099±9.794       | 34.768±2.932<sup>a</sup>    | 13.805±1.539<sup>a</sup> |
| AS2                      | 584.146±17.543<sup>a</sup> | 21.884±2.074                | 5.010±0.768   |
| +CM HBFs isolated        |                     |                             |               |
| NA1                      | 316.663±11.869      | 27.537±2.269                | 11.206±1.205  |
| NA2                      | 380.407±14.506<sup>a</sup> | 24.401±2.031                | 8.418±0.947   |
| AS1                      | 416.148±12.914<sup>a</sup> | 36.541±2.694<sup>a</sup>    | 10.534±1.041  |
| AS2                      | 422.320±16.828<sup>a</sup> | 41.202±3.436<sup>a</sup>    | 12.924±1.446  |
| +CM A549/HBFs CONFRONTED |                     |                             |               |
| NA1                      | 325.500±11.423      | 22.790±2.187                | 7.905±0.851   |
| NA2                      | 463.809±15.997<sup>a</sup> | 25.114±2.320                | 6.997±0.853   |
| AS1                      | 387.730±14.364<sup>a</sup> | 44.422±3.688<sup>a</sup>    | 15.099±1.617<sup>a</sup> |
| AS2                      | 435.521±12.779<sup>a</sup> | 33.665±3.002<sup>a</sup>    | 9.635±1.177   |
| A549/HBFs CO-culture     |                     |                             |               |
| NA1                      | 234.485±6.225       | 19.744±1.888                | 9.666±1.117   |
| NA2                      | 239.516±8.294       | 15.692±1.574                | 7.505±0.767   |
| AS1                      | 383.326±10.824<sup>a</sup> | 19.198±1.381                | 5.556±0.528   |
| AS2                      | 265.195±9.993       | 32.397±2.353<sup>a</sup>    | 15.779±1.665<sup>a</sup> |

Distance is defined as the total length of cell trajectory (6 h); cell displacement is defined as the total length of cell displacement from the starting point to the final cell position/time of recording (6 h); CME is the ratio of cell displacement to cell trajectory length, which was calculated as: (Total length of cell displacement/total length of cell trajectory) x 100. CME would equal 100 for cells moving persistently along one straight line in one direction and 0 for a random movement. *P<0.05 vs. control. SEM, standard error of the mean; CME, Coefficient of Movement Efficiency; CM, conditional medium; HBFs, human bronchial fibroblasts; AS, asthmatic donors; NA, non-asthmatic donors.
between asthmatic CAFs and lung cancer cells contribute to the phenotypic dynamics at the interface between the cancerous tissue and bronchial stroma. The lack of the corresponding activation of NA HBFs and A549 cells in NA HBF/A549 co-cultures suggests the absence of the corresponding paracrine loops in non-asthmatic bronchi.

On the other hand, we noticed the differences in the quantity of motility-related A549 reactions to AS1 and AS2 HBFs. They can be ascribed to the apparent phenotypic differences between the discrete AS HBF lineages. In general, AS HBFs lineages derived from different patients display a very high pro-fibrotic potential in comparison to their counterparts from NA donors (6,13-15). However, they differ in morphology, a proliferation rate, susceptibility to TGFβ, and the efficiency of TGFβ-induced FMT. This is not surprising, since the phenotypic characteristics of HBF lineages can be interpreted as the snapshots of the resident cells' characteristics, which may differ between the patients. A certain diversity of A549 reactions to AS1 and AS2 HBFs may thus illustrate a differential contribution of HBF lineages to the lung cancer microenvironment in vivo. Collectively, these data indicate that the asthmatic process may constitute a local bronchial microenvironment that promotes lung cancer remodeling and progression.

The potential significance of AS HBFs for cancer development in vivo was also emphasized by their invasive behavior in the proximity of A549 cells. AS HBFs failed to form lateral barrier structures that are characteristic for their non-asthmatic counterparts; instead, they collectively infiltrated A549 monolayers (4). On the other hand, we observed a relatively low translocation of A549 in co-cultures with AS HBFs and the lack of collective infiltration of AS HBF continua by A549 cells. This somewhat unexpected observation can be interpreted in terms of a strong chemotactic activity of the factors preferentially secreted by AS HBFs/A549 cells within the contact zone. It suggests that combined juxtacrine/paracrine interactions between AS HBFs and A549 cells counteract their chemodynamic effect on A549 cells. These observations also confirm the modulating effect of juxtacrine signaling on the quality/quantity of integrated AS HBF/A549 secretome. Noteworthy, scattered A549 cells were seen within AS HBF monolayers beyond AS HBF/A549 confrontation zones. This is consistent with our previous report on the heterogeneity of A549 invasive potential (26). It shows that small sub-populations of chemotaxis-resistant A549 cells can still colonize more distant regions of asthmatic bronchi.

Epidemiologic association between asthma and the risk of lung cancer formation is a controversial matter (9,27). For the first time we have shown that the microenvironment of asthmatic airways promotes the establishment of signaling loops between bronchial fibroblasts and lung cancer cells. This observation remains in concordance with the reports on intercellular signaling between cancer cells and CAFs during cancer progression (18,28,29). Accordingly, the infiltration of lung tumors by CAFs, which is induced by paracrine loops between asthmatic CAFs and lung cancer cells, may stabilize the structure of lung tumors. Chemotactic arrest of lung cancer cells, enforced by the gradients of chemotactic signals generated at the contact zone between tumor cell mass and stroma, can further strengthen this effect. Concomitant activation of cancer cells may stimulate local remodeling of cancerous tissue. However, small sub-populations of invasive, chemotaxis-resistant lung cancer cells, which penetrate the
compromised stromal barriers, can form new chemotactic loci and prompt collective invasion of other cancer cells. Further research based on the more comprehensive spectrum of lung cancer cell lineages is necessary to verify this hypothesis, to elucidate a biological significance of the expression and nuclear accumulation of α-SMA in A549 cells and to identify the elements of AS HBF/A549 secretome, which are responsible for lung cancer cell activation. However, our data suggest the role of the asthmatic bronchial microenvironment and chronic inflammation in the formation and stabilization of the invasive front(s) of lung tumors. Accordingly, the potential mechanistic links between the asthmatic process and lung cancer progression justify the inclusion of asthma into the long list of potential prognostic markers in the lung cancer therapy.

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Availability of data and materials
All data generated or analyzed during this study are included in this published article.

Authors' contributions
DR designed the study, obtained the data, performed data analysis and prepared the manuscript. FR and KR obtained the data and performed data analysis. JCa conducted data analysis and prepared the manuscript. TW analyzed the data and prepared the manuscript. MM designed the study. JCz conceived and designed the study, performed data analysis and prepared the final manuscript for publication.

Ethics approval and consent to participate
The present study was approved by the Jagiellonian University’s Ethics Committee (decision no. 122.6120.69.2015) and written informed consent was obtained for the use of HBF cell lines in basic research, which had previously been obtained from all donors.

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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