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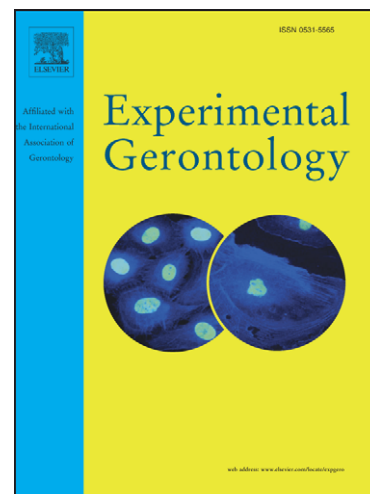
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In contrast to lung fibroblasts - no signs of senescence in skin fibroblasts of patients with emphysema

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Running title: Ageing markers in emphysema fibroblasts

Key words: ageing; beta-galactosidase; COPD; fibroblasts; IGFBP; lung emphysema; proliferation; senescence

Abstract

Smoking is known to be linked to skin ageing and there is evidence for premature senescence of parenchymal lung fibroblasts in emphysema. To reveal whether the emphysema-related changes in cellular phenotype extend beyond the lung, we compared the proliferation characteristics of lung and skin fibroblasts between patients with and without emphysema. Parenchymal lung fibroblasts and skin fibroblasts from the upper torso (thus limiting sun exposure bias) were obtained from patients without, or with mild, or with moderate to severe emphysema undergoing lung surgery. We analysed proliferation rate, population doublings (PD), staining for senescence-associated beta-galactosidase (β -gal) and gene expression of IGFBP-3 and IGFBP-rP1. Population doubling time of lung fibroblasts differed between control, mild, and moderate to severe emphysema (median (IQR) 29.7(10.0), 33.4(6.1), 44.4(21.2) h; $p=0.012$) and staining for β -gal was elevated in moderate to severe emphysema. Compared to control subjects, skin fibroblasts from patients with emphysema did not differ with respect to proliferation rate, PD and β -gal staining, and showed a lower abundance of mRNA for IGFBP-3 and -rP1 ($p<0.05$, each). These results suggest that the induction of a senescent fibroblast phenotype by cigarette smoke, as observed in emphysema, primarily occurs in the lung.

Introduction

The destruction of alveolar walls and resultant reduction of elastic recoil are hallmarks of lung emphysema (GOLD 2006). Fibroblasts provide an important part of the lung's structural support and extracellular matrix that is essential for its integrity (Absher 1995). Based on clinical and experimental data it has been hypothesized that the long-term exposure to noxious agents or cigarette smoke, the major cause for emphysema, could result in premature ageing of lung fibroblasts (Holz et al. 2004; Nyunoya et al. 2006). Such a change in fibroblast phenotype could be responsible for an impaired structural maintenance of the lung and could thereby contribute to the development of emphysema (Nyunoya et al. 2006; Tudor et al. 2006).

In line with this, lung fibroblasts from patients with emphysema exhibit a reduced proliferation rate, lower number of population doublings in serial passaging (Holz et al. 2004), and altered growth factor response (Holz et al. 2004; Noordhoek et al. 2003). In addition, we found an increased staining for senescence-associated beta-galactosidase (β -gal, (Dimri et al. 1995)), as well as increased mRNA expression of the insulin-like growth factor-binding proteins (IGFBP)-3 and -rP1 in these cells (Müller et al. 2006). Both proteins have been shown earlier to be senescence-associated (Goldstein et al. 1991; Swisshelm et al. 1995; Wilson et al. 2002).

As a clinical sign of ageing, the extent of skin wrinkling has been evaluated and, in fact, shown to be associated with emphysema (Patel et al. 2006). Skin wrinkling ranges among smoking-induced alterations which also comprise a rise in the proportion of elastic fibres (Frances et al. 1991; Just et al. 2007). Noteworthy enough, this increase was found to be associated with lung function impairment, suggesting a common effect of smoking on elastic fibres in both lung and skin (Just et al. 2005). This might be linked to disturbances in protease/anti-protease balance, as elevated MMP-1 gene expression has been observed in skin biopsies of active smokers compared to non-smokers (Lahmann et al. 2001). Moreover, in vitro experiments revealed that cigarette smoke extract can raise the expression of MMP-1 and -3 in skin fibroblasts of healthy individuals (Yin et al. 2000). As fibroblasts are basically involved in the maintenance of the extracellular matrix (Absher 1995), alterations of this function, as observed in ageing, could well be associated with an altered, particularly aged, cellular phenotype.

In the present study we therefore aimed to reveal, whether skin fibroblasts of patients with emphysema showed evidence for cellular senescence in terms of proliferation rate and capacity, as well as staining for β -gal and gene expression of IGFBPs. For this purpose we compared parenchymal lung and skin fibroblasts within individuals in patients with and without lung emphysema.

Material and Methods

Patients

The study population (Table 1) comprised 18 control patients (C) who did not show signs of emphysema, 13 patients with mild emphysema (ME), and 11 patients with moderate to severe emphysema (E). Patients were undergoing surgery for lung tumour resection, except two who had lung volume reduction surgery. Patients were assigned to their respective group by two independent investigators (RAJ, HW) who were blinded with respect to the *in vitro* results and took into account all available information, including the patient's history, symptoms, lung function, chest X-ray/CT, bronchoscopic, and histological findings. When evaluating expiratory flow-volume curves and body plethysmographic resistance loops, special attention was paid to their characteristic form in emphysema, as well as to lung hyperinflation. In addition, data on single-breath diffusing capacity for carbon monoxide were available in a number of patients (9 C/ 12 ME/ 6 E). The study was approved by the local Ethics Committee and all patients gave their written informed consent.

Fibroblasts

Lung fibroblasts were derived from surgical tissue (Holz et al. 2004). Briefly, the macroscopic appearance of the specimen was examined and pleura-free parenchymal samples were taken from non-cancerous areas. Samples were washed, minced (1-2 mm²) and transferred into 25 cm² culture dishes (Sarstedt, Nürnberg, Germany) for primary explant culture in Dulbecco's modified Eagle's medium (DMEM) containing 10 % foetal calf serum (FCS, same batch used throughout the study), 100 U/mL Penicillin, 100 µg/mL Streptomycin, and 50 µg/mL Gentamycin (all cell culture reagents: Invitrogen). Following primary culture, cells were trypsinized, resuspended in culture medium plus 10 % dimethylsulfoxide (DMSO), stored for 24 h at -80°C and transferred into liquid nitrogen. Skin samples of size 2-8 mm² were taken during surgery from the wound edge located at the patients' upper torso. The tissue was minced, immediately transferred to explant culture and treated as described for lung fibroblasts.

Assessment of proliferation

Cell proliferation and serial passaging were performed as previously described (Holz et al. 2004). Proliferation rate was assessed in passage 2 (about 3-4 population doublings after primary culture) in 24 well-dishes. Cell number was determined in triplicate after 24, 48, 72, and 96 h. Mean cell numbers were logarithmically transformed and the linear regression slope was calculated to derive the doubling time (DT). For serial passaging, cells were thawed and cultured

for about 5 days before transfer to passage 2. Cells were seeded at a density of 100 000 cells per 25 cm², grown for one week, harvested, and seeded again. When the number of harvested cells dropped below the seeded number, the culture was terminated and the cumulative number of population doublings (PD) calculated.

Staining for senescence-associated beta-galactosidase (β -gal)

Following a previously described procedure (Müller et al. 2006), 10 000 - 15 000 fibroblasts were incubated for 24 h on glass slides. Slides were then stained for β -gal activity according to the manufacturer's instructions (Cell Signaling Technologies, Beverly, MA, USA). The analysis was performed in cells of passage 4 or 5 after primary culture. Cells positive for the blue stain were counted under visible light, while counter-staining with DAPI enabled the unambiguous determination of cell numbers under UV light.

Gene expression analysis

The abundance of mRNA for IGFBPs was analysed by qPCR using the Lightcycler Instrument (Roche, Mannheim, Germany). Cells were thawed, cultured up to passage 3, harvested and stored frozen until RNA isolation and cDNA transcription. Primers (Müller et al. 2006) and protocols (Erpenbeck et al. 2003) were those used previously. Abundance of mRNA was determined via the ratio of target to house-keeping gene (PBGD, porphobilinogen deaminase).

Data Analysis

Owing to skewed data distributions in some variables, median values and quartiles or interquartile ranges (IQR) were chosen for all variables to achieve a homogeneous description. For non-normally distributed variables the Kruskal-Wallis test was employed for the comparison of groups and the Mann-Whitney U-test for specific post hoc comparisons. The Wilcoxon test was used for paired comparisons. Additionally, analysis of variance (ANOVA) was performed on log-transformed data of non-normally distributed variables to utilise the power of post hoc comparisons by the Newman-Keuls-statistics. Correlation analysis was performed by computation of linear correlation coefficients. P-values <0.05 were considered statistically significant.

Results

In both skin and parenchymal lung fibroblasts proliferation rates were determined in 18 control patients, 13 patients with mild, and 11 patients with moderate to severe emphysema. (Table 1A). The doubling time of lung fibroblasts differed between the three groups analysed (ANOVA,

$p=0.012$, Figure 1A) with the longest mean doubling time in patients with moderate to severe emphysema. No such difference was detected in skin fibroblasts of the same patients (ANOVA, $p=0.54$; Figure 1B). In control patients the doubling time of skin fibroblasts was higher compared to lung fibroblasts (t-test, $p=0.005$), while there was no such difference in the emphysema groups. The mean (95%-CI) ratio between doubling times of lung relative to skin fibroblasts was 84.8 (73.2; 96.4) % in control subjects, 100.9 (85.0; 116.8) % in mild emphysema, and 130.3 (85.0; 175.5) % in moderate to severe emphysema (ANOVA, $p=0.02$).

Neither skin nor lung fibroblasts showed a correlation between doubling time and the donors' age or number of packyears, neither in the separate groups nor the total group. The correlation between doubling times of skin and lung fibroblasts was significant in control ($r=0.50$, $p=0.04$), but not in mild ($r=-0.10$, $p=0.74$) or moderate to severe emphysema ($r=-0.19$, $p=0.57$).

As the group of patients with mild emphysema did not show a pronounced difference in doubling time compared to control patients, the subsequent experiments were performed with all available fibroblasts strains from patients with moderate-severe emphysema and randomly selected fibroblast strains from a matched number of control patients (Table 1B).

Cumulative population doublings (PD) of skin fibroblasts were assessed until the number of harvested cells fell below the number of seeded cells. The median (quartiles) number of PD in control patients was 8.3 (7.4; 9.0), in emphysema 8.1 (5.9; 13.0). These values were not significantly different from each other.

In control patients the median (quartiles) percentage of β -gal positive skin fibroblasts was 2.9 (1.9; 3.4) %, in emphysema 4.9 (3.1; 5.7) % ($p=0.12$). The corresponding lung fibroblasts showed percentages of 5.4 (4.3; 6.8) % in control patients, and of 17.0 (13.0; 20.9) % in emphysema ($p=0.012$). In both groups the proportion of cells staining for β -gal was lower in skin compared to lung fibroblasts ($p<0.05$, each). Doubling time and β -gal staining were not correlated with each other, neither in skin nor in lung.

In skin fibroblasts of patients with emphysema the median (quartiles) relative abundance of mRNA for IGFBP-3/PBGD and for IGFBP-rP1/PBGD was lower (Mann-Whitney U-test, $p=0.04$, $p=0.02$) than in control patients (Figure 2). In lung fibroblasts, the relative abundance of mRNA for IGFBP-3/PBGD and for IGFBP-rP1/PBGD was higher (Figure 2). Taken overall, there were significant correlations between the expression of IGFBP-3 and IGFBP-rP1 in skin fibroblasts ($r=0.56$, $p=0.03$) and in lung fibroblasts ($r=0.75$, $p=0.003$). The ratio of normalised mRNA abundance of skin to lung fibroblasts for IGFBP-3 was 1.9 (1.7; 2.3) in control patients and 1.3 (1.1; 1.5) in severe emphysema ($p=0.014$). The corresponding ratios were lower for IGFBP-rP1 (1.1 (1.0; 1.9) vs. 1.0 (0.8; 1.1)) and not significantly different ($p=0.26$).

Discussion

In the present study we compared the *in vitro* proliferation of lung and skin fibroblasts from the same individuals between patients with and without emphysema. Parenchymal lung fibroblasts showed a reduced proliferation rate in moderate to severe emphysema, which is in line with our previous findings (Holz et al. 2004). There was, however, no such difference for skin fibroblasts. The proportion of skin fibroblasts positive for senescence-associated beta-galactosidase was similar between groups and lower than in lung fibroblasts. The abundance of mRNA for senescence-associated IGFBP-3 or -rP1, was lower in skin fibroblasts of emphysema compared to control patients. These findings suggest that smoking-related changes in fibroblast phenotype as found in emphysema occur primarily in the lung (Table 2).

Smoking has long been known to affect skin appearance, in particular by promoting facial skin wrinkling (Daniell 1971; Kadunce et al. 1991; Koh et al. 2002; Patel et al. 2006). The effect is enhanced by sunlight (Yin et al. 2001), but alterations of elastic tissue properties also occur in sun-protected areas (Frances et al. 1991; Helfrich et al. 2007). Accordingly, MMP-1 which can act on elastic fibres has been found up-regulated in the buttock skin of smokers (Lahmann et al. 2001). However, the effect of smoking on skin wrinkling is not unopposed (Malvy et al. 2000; O'Hare et al. 1999), and in some cases the effect of UV light may outweigh that of smoking. Both smoking and UV-light induce changes in the morphology of elastic fibres (Frances et al. 1991; Just et al. 2007). Remarkably, increased amounts and morphological abnormalities of elastic fibres in the reticular dermis of the skin were found to be associated with lung function impairment (Just et al. 2005). Thus there might be a common effect of smoking on tissue properties of both lung and skin.

Fibroblasts could be involved in the increased MMP-1 gene expression, as *in vitro* experiments revealed that cigarette smoke extract increased the expression of MMP-1 and -3 in skin fibroblasts of healthy individuals (Yin et al. 2000). Moreover, it blocked the cells' responsiveness to TGF- β 1 (Yin et al. 2003). As TGF- β directly stimulates fibroblasts to produce extracellular matrix and collagen, this could mediate the observed lower collagen production in smokers (Jorgensen et al. 1998). Interestingly, lung fibroblasts of patients with emphysema exhibit reduced responsiveness to exogenous TGF- β 1 (Togo S. et al. unpublished data).

While lung fibroblasts are probably more or less directly affected by smoking, cells of other body compartments might be exposed to tobacco smoke compounds or metabolites carried by the bloodstream. Water-soluble compounds of cigarette smoke exert effects on lung fibroblasts (Carnevali et al. 1998; La Rocca et al. 2007) and these effects can be lasting, apparently altering their phenotype (Nyunoya et al. 2006). Based on this, the comparison of fibroblasts from lung and skin of the same individuals seemed reasonable to address the question whether smoking exerts

parallel effects in different compartments and induces a common fibroblast phenotype.

However, the skin fibroblasts studied exhibited similar proliferation characteristics in control and emphysema patients. This is unlikely to have been the result of differential cell culture bias, as the lung fibroblasts showed a difference, confirming our previous findings (Holz et al. 2004) in newly recruited patients (only lung fibroblasts of four patients were included in our previous study (Holz et al. 2004)). It has also been shown previously, that the reduced proliferation rate in lung fibroblasts of patients with emphysema is not associated with an increase in apoptosis or necrosis (Holz et al. 2004). The fact that we did not observe a relationship between donor age and proliferation rate is compatible with data from other studies (Cristofalo et al. 1998).

Foetal skin fibroblasts appear to show a lower proliferation rate than lung fibroblasts (Schneider et al. 1977). In line with this, the ratio of doubling times between lung and skin was <1 in control patients, whereas in emphysema the slow proliferation of lung fibroblasts raised this ratio to values >1 . Skin fibroblasts of both groups did not differ in their number of total population doublings. Compared to lung fibroblasts (Holz et al. 2004), values were lower in skin fibroblasts, in line with previous findings (Schneider et al. 1977). Moreover, the proportion of skin fibroblasts positive for β -gal was not significantly elevated in emphysema. Levels of staining were much lower than in parenchymal lung fibroblasts. In accordance with our previous results (Müller et al. 2006), staining in lung fibroblasts was more pronounced in patients with moderate to severe emphysema as compared to the control group, suggesting that a senescent phenotype is limited to lung fibroblasts in patients with emphysema. Both doubling time and β -gal staining were significantly increased in emphysema but did not correlate with each other. Although in general replicative age correlates with β -gal staining (Dimri et al. 1995), the interindividual variability of both markers was likely to be responsible for a lack of correlation in our study. In a previous study we had observed such a correlation within serial passages of the same strains of fibroblasts (Müller et al. 2006).

IGFBP-3 and -rP1 can indicate cellular senescence (Goldstein et al. 1991; Swisshelm et al. 1995; Wilson et al. 2002) and their mRNA abundance has been shown to be elevated in lung fibroblasts of emphysema (Müller et al. 2006). Skin fibroblasts of control patients showed a relative mRNA abundance of IGFBP-3 about two-fold higher than lung fibroblasts. This might explain their lower proliferation as compared to lung fibroblasts, as IGFBP-3 is known to be a proliferation inhibitor (Oh et al. 1993). In emphysema this ratio was close to one, due to lower levels in skin and higher levels in the lung. Abundance of mRNA for IGFBP-rP1 was similar in skin and lung fibroblasts. As two control patients showed exceptional values, the difference between groups regarding lung fibroblasts was less pronounced than found previously (Müller et al. 2006). Taking into account the similar proliferation characteristics of skin, in contrast to lung,

fibroblasts in the groups, the expression patterns of IGFBP-3 and -rP1 could not be considered as unequivocal markers of cellular senescence.

The recent observations (Patel et al. 2006) on a correlation between emphysema and facial wrinkling are of great interest with regard to the factors involved. Facial skin is regularly exposed to UV-light, whereas the skin fibroblasts used in the present study were not, as they were derived from the upper torso. Probably induced senescence requires an appropriate, sufficient exposure, which might be cigarette smoke in the lung or UV-light in the skin. Moreover, effects by smoking or UV-light comprise alterations of elastic fibres. MMP up-regulation in smokers (Lahmann et al. 2001) might represent an acute effect *in vivo*, in parallel to acute *in vitro* effects of cigarette smoke extract (Yin et al. 2000). However, skin fibroblast proliferation was assessed after at least 4 weeks in culture, thus acute effects of smoking-related compounds were excluded. The proliferation data did not indicate persistent alterations in skin fibroblast phenotype, in contrast to lung fibroblast data as reported here and previously (Holz et al. 2004; Müller et al. 2006). The most plausible explanation is that the induction of acute versus persistent changes in cell function depends on the strength of the exposure, and that persistent effects, especially those on proliferation behaviour, need higher local doses than temporary effects on cell function.

Taken together, the findings of our study indicate that fibroblast phenotype was not grossly altered in samples of sun-protected skin of patients with lung emphysema compared to control smokers. This suggests that smoking induced cellular senescence is primarily found in the lung. Future studies might compare cells from skin areas differently exposed to UV-light, as such comparisons might reveal a potential disposition for induced senescence associated with lung emphysema.

Disclosure Statement

The authors declare that they have no competing interests. The interpretation and presentation of these results do not influence the personal or financial relationship of any of the authors with other people or organisations.

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Legends

Figure 1 - Fibroblast proliferation

Population doubling time (DT) of fibroblasts (h); open circles represent single data points for individual patients, closed circles and whiskers represent geometric mean values and SEM for each group. (A) lung fibroblasts (ANOVA, $p=0.012$), (B) skin fibroblasts (ANOVA, $p=0.54$).

Figure 2 – Relative mRNA abundance of IGFBP-3 and –rP1

Relative mRNA abundance of IGFBP-3 and IGFBP-rP1 each in relation to PBGD. Circles / whiskers represent the respective group median values / quartiles; open circles: fibroblasts from patients with moderate to severe emphysema; closed circles: fibroblasts from control patients.

Table 1 - Patient characteristics

- A) Patient characteristics (all patients): The table shows median values and quartiles (in parentheses). ANOVA, Newman-Keuls-post-hoc analysis: * $p < 0.05$, ** $p < 0.01$: control vs. mild or severe emphysema. ⁺ $p < 0.05$, ⁺⁺ $p < 0.01$: mild vs. severe emphysema. BMI = body mass index, VC = vital capacity, FEV₁ = forced expiratory volume in one second, ITGV = intra-thoracic gas volume, RV = residual volume, TLC = total lung capacity, yr = years, ¹reliable data available in 14 control subjects, 7 mild emphysema and 5 moderate-to-severe emphysema patients; all patients were current or ex-smokers except for one never-smoking control patient. Predicted values were taken from ERS guidelines (Quanjer et al. 1993), stages of COPD according to GOLD guidelines (GOLD 2006). ² n.a. = not applicable for patients with FEV₁/FVC > 70% but FEV₁ < 80% as lung function impairment was most likely due to lung tumour. ³ One subject was included who was functionally not limited in terms of GOLD criteria but showed clear macroscopic evidence for emphysematous alterations in the resected lung tissue.
- B) Patient characteristics (subgroups): Main characteristics and case numbers of patients subgroups included in the analysis of the respective markers are stated here. Age and FEV₁ are displayed as median (IQR); for all markers analysed these subgroups did not differ in age, BMI or smoking history.

Table 2 – Alterations in fibroblasts from patients with emphysema

The table summarises the results obtained in different studies on cultures of primary fibroblasts from patients with emphysema. The effects are presented as relative changes as compared to fibroblasts from control patients without emphysema. The repair capacity model comprises the ability to contract collagen gels and the extend of migration towards different stimuli.

¹ Holz et al. 2004; ² S. Togo, unpublished data; n.d.: not determined

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Table 1

A) Patient characteristics (all patients)

| | | Control | Mild emphysema | Moderate to severe emphysema |
|----------------------|-----------------------------|-------------------|-------------------|-------------------------------------|
| n | | 18 | 13 | 11 |
| Sex | m / f | 14 / 4 | 9 / 4 | 10 / 1 |
| Age | yr | 65 (58; 73) | 66 (61; 72) | 62 (53; 71) |
| BMI | kg/m ² | 25.4 (22.9; 27.6) | 23.5 (21.1; 27.8) | 22.8 (17.7; 26.3) |
| VC | %pred | 90 (81; 108) | 99 (87; 118) | 89 (72; 106) |
| FEV ₁ | %pred | 80 (75; 101) | 73 (65; 83) | 48 (34; 63) ** ⁺⁺ |
| FEV ₁ /VC | %pred | 96 (82; 102) | 82 (74; 83) * | 54 (41; 68) ** ⁺⁺ |
| ITGV | %pred | 99 (85; 122) | 124 (114; 156) * | 182 (155; 219) ** ⁺⁺ |
| RV | L | 2.48 (2.23; 2.60) | 2.92 (2.26; 3.27) | 5.00 (4.00; 6.13) ** ⁺⁺ |
| TLC | L | 5.59 (5.24; 6.39) | 6.26 (5.37; 6.72) | 8.60 (7.24; 10.15) ** ⁺⁺ |
| RV/TLC | % | 41 (36; 45) | 48 (42; 53) * | 58 (50; 70) ** ⁺ |
| Packyr ¹ | | 45 (30; 52) | 45 (15; 75) | 40 (30; 40) |
| GOLD | -/I/II/III/VI/ ² | 9/0/4/0/0/5 | 2/3/6/0/0/2 | 1 ³ /0/3/7/0/0 |

B) Patient characteristics (subgroups)

| | | N | Age (yr) | FEV ₁ (%pred) |
|--------------------|---|---|----------|--------------------------|
| Serial Passaging | C | 9 | 59 (18) | 85 (19) |
| | E | 8 | 62 (15) | 37 (24) * |
| beta-Galactosidase | C | 9 | 61 (7) | 88 (19) |
| | E | 9 | 63 (11) | 37 (29) * |
| Gene expression | C | 8 | 59 (15) | 91 (25) |
| | E | 7 | 58 (14) | 36 (29)* |

Table 2:

| | lung fibroblasts | skin fibroblasts |
|--------------------------|------------------|------------------|
| Population doubling time | ↑ | ↔ |
| Serial passaging (cumPD) | ↓ ¹ | ↔ |
| β-gal staining | ↑ | ↔ |
| Repair capacity | ↓ ² | n.d. |
| IGFBP-3 , IGFBP-rP1 | ↑ | ↓ |
| PGE2 | ↑ ² | n.d. |

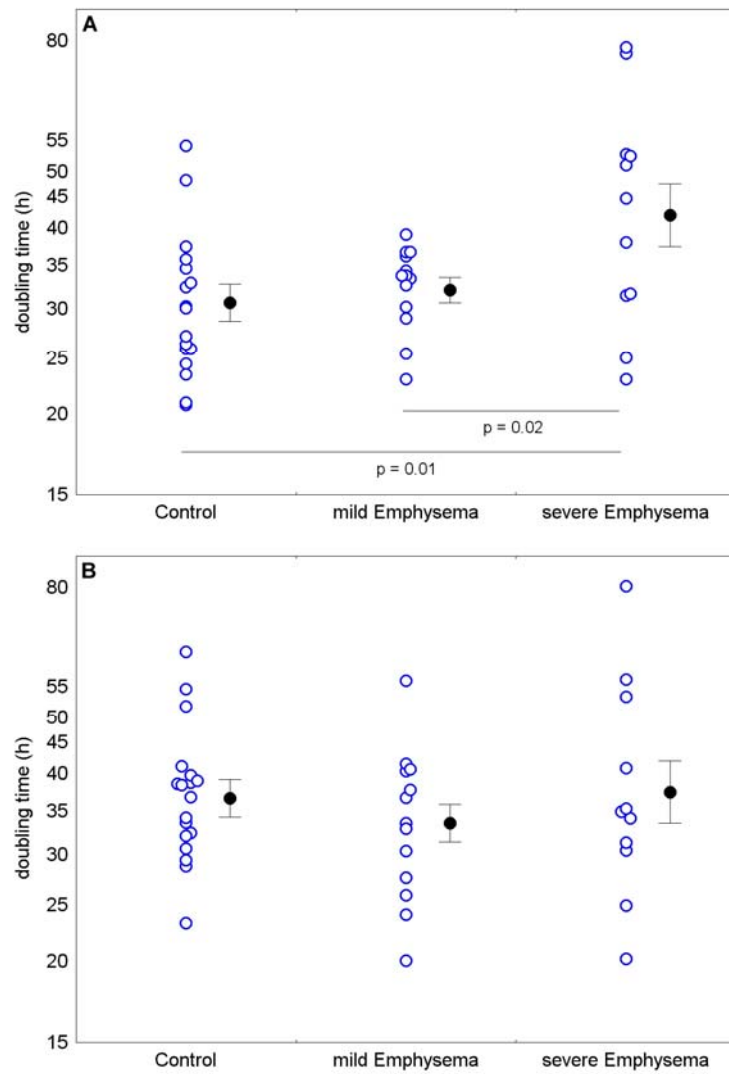


Fig.1

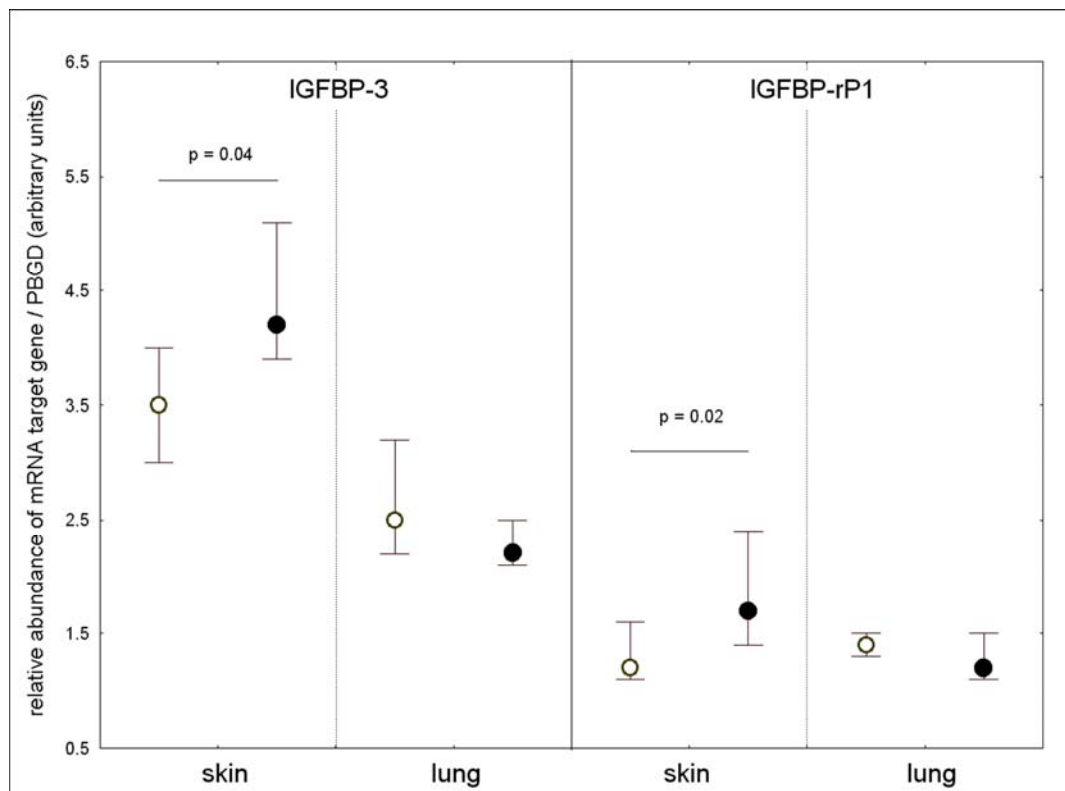


Fig.2