VILNIUS UNIVERSITY

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THE PERFORMANCE OF ANTIFIBROTIC AGENTS IN PRECLINICAL MODELS OF SYSTEMIC SCLEROSIS

Summary of Doctoral thesis
Biomedical science, medicine (07B)

Vilnius-2010

The thesis was prepared during the period of 2008-2010 at Clinics of Rheumatology, traumatology-orthopedics and reconstructive surgery at Vilnius University.

The laboratory part was performed at University of Erlangen-Nürnberg, while working as ARTICULUM fellow.

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VILNIAUS UNIVERSITETAS

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ANTIFIBROZINIŲ PRIEMONIŲ PAIEŠKA PREKLINIKINIUOSE SISTEMINĖS SKLEROZĖS MODELIUOSE

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Biomedicinos mokslai, medicina (07B)

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ABBREVIATIONS

c-Abl – Abelson kinase
DMEM – Dulbecco's modified Eagle's medium
DMSO – Dimethyl sulfoxide
ECM – extracellular matrix
FCS – fetal calf serum
HMEC-1 – Immortalized human microvascular endothelial cell 1
ICAM-1 – Intercellular adhesion molecule 1
IM – imatinib mesylate
MMPs – matrix metalloproteinases
MTT – microtiter tetrazolium
NaCl – sodium chloride
PDGF – platelet-derived growth factor
PDGFr – PDGF receptor
SSc – systemic sclerosis
TGF-β – transforming growth factor β
TIMP – tissue inhibitor of metalloproteinases
Tsk-1 – tight skin 1
TUNEL – Terminal uridine deoxynucleotidyl transferase dUTP nick end labeling
VCAM-1 – vascular cell adhesion molecule-1
VEGF – vascular endothelial growth factor
αSMA – α-smooth muscle actin

INTRODUCTION

Systemic sclerosis (SSc) – is one of the most complicated and fatal systemic diseases. It's a chronic fibrotic disorder of unknown etiology that affects the skin and various internal organs including the heart, lungs, and gastrointestinal tract.
SSc is characterized by three major components: microangiopathy with progressive loss of capillaries, tissue fibrosis and autoimmunity.

In early stages of disease while the clinical picture is not clearly developed the autoimmunity is not pronounced, that's why the course of disease cannot be controlled by classical anti-inflammatory therapy. Differently from rheumatoid arthritis, spondylophaties, vasculitides where substantial progress has been achieved in recent years, the same can't be applied for SSc. In above mentioned rheumatic diseases the biological therapy allows controlling the autoimmunity and the course of diseases in

most of cases. This does not suit to SSc patients and once again confirms that the autoimmunity is not a major issue in pathogenesis of this disease, however.

The most obvious histological features of SSc are progressive microangiopathy with loss of capillaries and a massive accumulation of extracellular matrix components (ECM). Increased apoptosis of microvascular endothelial cells as one of the earliest manifestations of vasculopathy occurs usually before tissue fibrosis becomes evident. The ongoing endothelial cell damage results in a progressive loss of capillaries, the narrowing of the lumen with decreased capillary blood flow, lack of nutrients and severe tissue hypoxia.

The angiopathy manifests clinically not only as fingertip ulcers or even gangrene, but also as pulmonary hypertension or renal crisis and strongly contributes to the morbidity and mortality of SSc patients.

The overproduction of ECM components in SSc is mediated by activated fibroblasts, which produce increased amounts of glycosaminoglycans, fibronectins, and types I, III, VI, and VII collagen. Profibrotic cytokines such as transforming growth factor β (TGF-β) and platelet derived growth factor (PDGF) play key roles in the pathogenesis of SSc. Both cytokines are up-regulated in the skin of SSc patients and strongly stimulate matrix synthesis by dermal fibroblasts. Accordingly, blockade of TGF or PDGF signaling has been shown to reduce the development of fibrosis in various experimental models. However, substances that specifically inhibit PDGF pathways are not yet available for clinical application. Similarly, antibodies against TGF-β and other strategies to block TGF-β signaling are only at the early stage of clinical development and have uncertain efficacy for slowing the development of fibrosis in patients with SSc.

The tyrosine kinase inhibitor imatinib mesylate was successfully introduced to oncology for treatment of BCR-Abl positive chronic myeloid leukemia. Imatinib mesylate (IM) effectively blocks the activity of not only pathological, but also some of physiological tyrosine kinases. It bindes to the ATP-binding pocket of c-Abl and efficiently blocks its tyrosine kinase activity. Notably, c-Abl has recently been identified as an important downstream molecule in TGF-β signaling. Its ability to interfere with PDGF signaling by blocking the tyrosine kinase activity of PDGF receptors was also

noticed. Considering its specific dual inhibition of TGF-β and PDGF signaling pathways, the longstanding clinical experience in other disease applications, and its good tolerability with rare side effects, it was hypothesized that imatinib mesylate might be useful as a potent antifibrotic drug for the treatment of dermal fibrosis in SSc. Distler et al has demonstrated that imatinib effectively blocked collagen synthesis in fibroblasts in vitro and prevented the development of dermal fibrosis upon challenge with bleomycin in murine model of SSc. Though idea of dual inhibition of TGF-β and PDGF in fibroblasts has been worked out in experiments, some clinical issues are still to be answered before initiating clinical trials with Imatinib.

The prevention of fibrosis might be an objective only in early stages of disease. However, most patients with diffuse SSc are seen by the rheumatologist when significant tissue fibrosis is already present. Thus, the aim of therapy for such patients would be to stop disease progression and even induce regression of preexisting fibrosis. Ideal antifibrotic drugs should sufficiently decrease the production of collagen to shift the balance between matrix synthesis and matrix degradation toward matrix degradation, with a subsequent reduction in the preexisting accumulation of ECM. No drug has shown to induce regression of established fibrosis yet. Considering the potent anti-fibrotic effects of imatinib on prevention of fibrosis in inflammatory models, we hypothesized that imatinib might also be effective in models mimicking longstanding disease.

The effect of IM on TGF-β and PDGF signaling is not specific nor for fibrosis nor for fibroblasts. TGF-β and PDGF are multipotent cytokines, regulating many physiological processes in many cells. Imatinib might block TGF-β and PDGF signaling not only in fibrogenesis, but also in angiogenesis, not only in fibroblast, but also in endothelial cells. Anti-angiogenic side effects might complicate the use of novel anti-fibrotic drugs in SSc patients as it might worsen the vascular manifestations of SSc.

We found important to evaluate effectiveness of imatinib mesylate for the treatment of pre-established tissue fibrosis and to exclude that the anti-fibrotic effects of imatinib are complicated by inhibitory effects on endothelial cell functions.

AIM OF THE STUDY

Assess the effect of imatinib mesylate on the process of fibrosis and endothelium in experimental models of systemic sclerosis and cell cultures.

OBJECTIVES OF THE STUDY

1. Assess the effectiveness of imatinib mesylate on non-inflammatory SSc model and on model of established fibrosis.
2. Evaluate if therapeutic concentrations of imatinib mesylate has an effect on basal functions of endothelial cells in vitro and in vivo.
3. Assess effect of imatinib mesylate on the process of angiogenesis in vitro.

STATEMENTS DEFENDED

1. Therapeutic concentration of imatinib has antifibrotic effect on Tsk-1 mice and established bleomycin-induced dermal fibrosis model.
2. Therapeutic concentration of imatinib has no negative effect nor for angiogenesis, nor for viability of endothelial cells.

PRACTICAL VALUE OF THE STUDY

Carrying out the research projects was a necessary step before the IM clinical studies in SSc. If this product would come to clinical practice of rheumatology, it would be an important step in to solving very complex problems in SSc.

Finally, while writing the thesis it was brought to our knowledge that several clinical trials, using the material published in relation to this research project, were launched.

Even if IM would not be introduced (eg. due to toxicity) in to the clinics, our projects have established the theoretical assumptions for search of other, multiple inhibitory properties on intracellular kinases having, agents.

SCIENTIFIC NOVELTY OF THE STUDY

It was shown for the first time that IM not only prevents fibrosis from occurring, but also induces regression of pre-established skin fibrosis.

Scientific data was collected to propose that IM has no negative effect nor for basal, nor for angiogenic functions of endothelial cells.

METHODS

Bleomycin induced dermal fibrosis

Skin fibrosis was induced in female 6 week-old DBA mice by local injection of bleomycin. One hundred microliters of bleomycin dissolved in 0.9% sodium chloride (NaCl) at a concentration of 0.5 mg/ml was administered every other day for 24 days, by subcutaneous injection into defined areas of the upper back. Subcutaneous injections of 100 µl 0.9% NaCl were used as controls. Two subgroups were additionally treated with imatinib mesylate. Imatinib mesylate was dissolved in 0.9% NaCl and administered by daily intraperitoneal injection, at a concentration of 50 mg/kg/day or 150 mg/kg/day in a total volume of 0.1 ml. After 4 weeks, animals were killed by CO2 asphyxiation. The injected skin was removed and processed further for histological analysis.

Treatment of established bleomycin induced dermal fibrosis with imatinib

Skin fibrosis was induced in 6-weeks-old DBA mice by local intracutaneous injections of bleomycin as described previously. The injection schemes for the six different groups are summarized in Figure 1b. Briefly, one group (group 2) of mice was sacrificed after 3 weeks of treatment with bleomycin to analyze the fibrotic changes before treatment with imatinib. Another group (group 4) of mice was sacrificed after 6 weeks of injections with bleomycin. Another group (group 3) was injected for 3 weeks with bleomycin and then for next 3 weeks with NaCl to control for spontaneous regression of fibrosis. Group 5 mice were challenged with bleomycin for 6 weeks and treated in parallel with imatinib at doses of 150 mg/kg/d for the last 3 weeks (as described previously). Group 1 and 6 mice were used as controls.

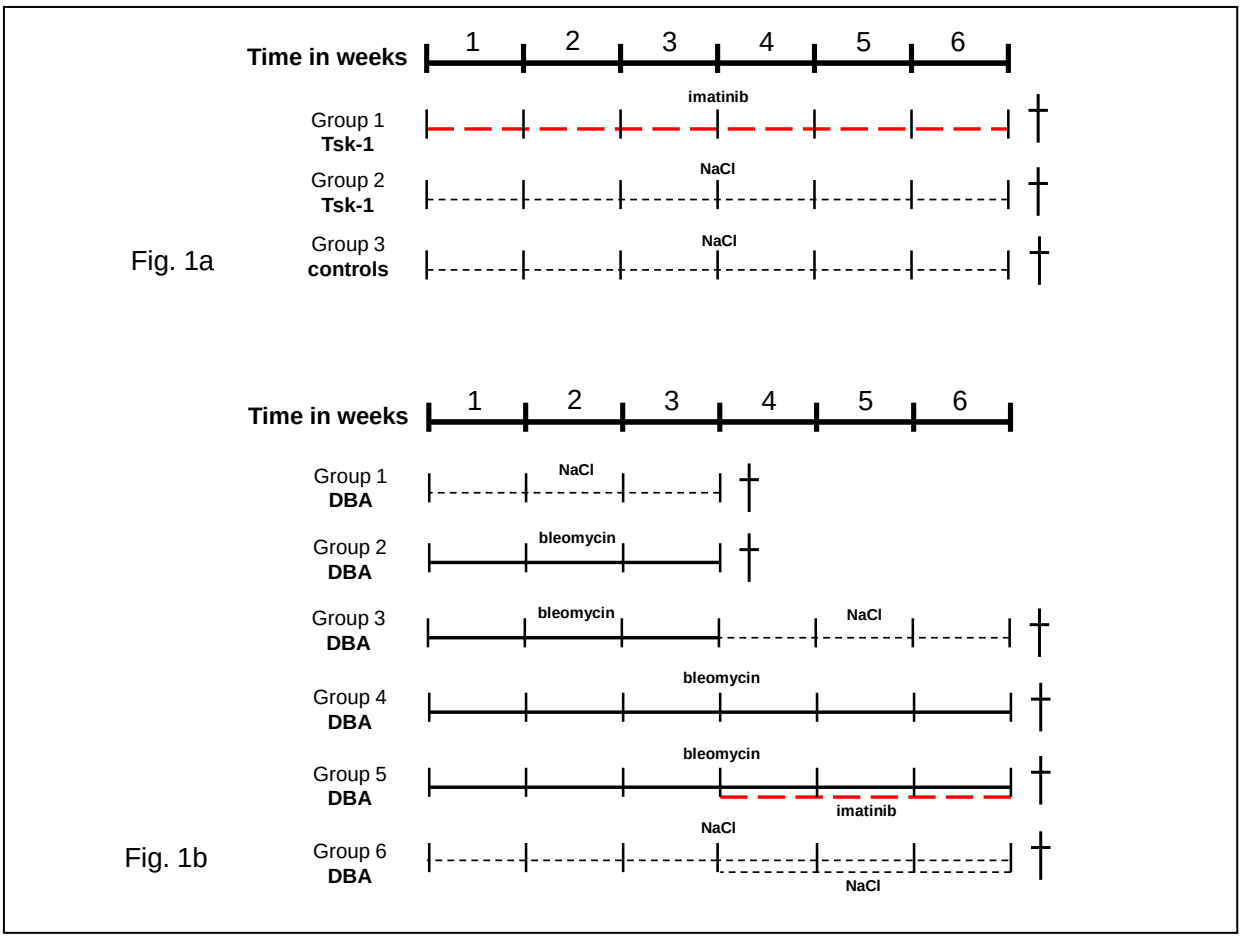


Fig.1 Experimental design for the treatment of Tsk-1 mice (**Figure 1a**) and bleomycin induced, established dermal fibrosis (**Figure 1b**) with imatinib. † = sacrifice

Prevention of fibrosis in tight skin 1 mice by imatinib

The anti-fibrotic potential of imatinib was evaluated in the tight-skin-1 (Tsk-1) mouse model of SSc. Due to a dominant mutation of the fibrillin-1 gene, the phenotype of Tsk-1 is characterized by an increased dermal and hypodermal thickness as well as an atrophy of the hypodermis. Imatinib was dissolved in 0.9 % NaCl and injected intraperitoneally in a total volume of 100 µl. Three groups with a total of 27 mice were analyzed. One group of Tsk-1 mice was treated with imatinib at a dose of 150 mg/kg/d, another Tsk-1 group was injected with the solvent NaCl. The last group consisted of pa/pa mice not carrying the Tsk-1 mutation, which also received intraperitoneal injections of NaCl. The treatment was started at an age of five weeks. After five weeks of treatment, mice were sacrificed by cervical dislocation and the skin processed further for histological analysis. The injection scheme is summarized in Figure 1a.

Terminal uridine deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay

Paraffin-embedded tissue sections from lesional skin of mice challenged with bleomycin were used to quantify the number of apoptotic endothelial cells. TUNEL staining was performed using the In situ cell death detection kit (Roche, Mannheim, Germany) including positive and negative controls.

Histological analysis

Skin sections were stained with hemalaun/eosin for better visualization of the tissue structure. The dermal thickness was analyzed with a Nikon Eclipse 80i microscope (Nikon, Badhoevedorp, Netherlands) by measuring the maximal distance between the epidermal-dermal junction and the dermal-subcutaneous fat junction at four different skin sections. The hypodermal thickness was determined by measuring the thickness of the subcutaneous connective tissue beneath the *panniculus carnosus* at four different sites in each mouse.

Detection of myofibroblasts

For quantification of myofibroblasts, skin sections were deparaffinized and incubated with 5% bovine serum albumin for 60 min. α-smooth muscle actin (αSMA) positive cells in mouse sections were detected by incubation with monoclonal anti-αSMA antibodies (clone 1A4, Sigma-Aldrich) for two hours at room temperature followed by incubation with 3% hydrogen peroxide for 10 min. Goat-anti-rabbit antibodies labeled with horseradish peroxidase were used as secondary antibodies (Dako). The expression of αSMA was visualized with 3,3-diaminobenzidine tetrahydrochloride (DAB) (Sigma-Aldrich). Monoclonal mouse IgG antibodies (Calbiochem) were used for controls.

Cell culture

Immortalized human microvascular endothelial cells (HMEC-1) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal calf serum (FCS), 25 mM HEPES, 100 units/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, and 2.5 µg/ml amphotericin B (all from Gibco BRL, Basel, Switzerland).

Incubation with imatinib mesylate

HMEC-1 were incubated with imatinib mesylate in concentrations from 0.001 to 1.0 μg/ml for 24 to 96 h. These concentrations correspond to mean plasma peak and through concentrations in humans after administration of standard doses of imatinib. Medium treated cells were used as controls.

Caspase 3 activity assay

The activities of caspase-3-like proteases were determined using the EnzChek caspase-3 assay kit (Invitrogen).

Quantification of apoptotic cells by staining for annexin V

HMEC-1's were stained with fluorescein isothiocyanate-labeled annexin V (BD Bioscience). Staining with annexin V in the absence of calcium was used for control. The number of cells positive for annexin V was quantified with the FACSCalibur flow cytometer.

Microtiter tetrazolium (MTT) assay

The metabolic activity of HMEC-1's incubated with imatinib for 24 to 96 h was assessed using the MTT [3, (4,5-dimethylthiazol-2-yl) 2,5-diphenyl-tetrazolium bromide] method. Fresh medium and imatinib mesylate were added every other day. Untreated HMEC were used as controls.

Quantitative real-time PCR

Total RNA was isolated with the NucleoSpin RNA II extraction system (Machery-Nagel) and reverse transcribed into complementary DNA (cDNA) with random hexamers. Gene expression was quantified by SYBR Green real-time PCR using the ABI Prism 7300 Sequence Detection System (Applied Biosystems). Specific primer pairs were used for each gene.

Generation of proliferation curves

HMEC-1's were seeded onto 6 well plates at a density of 1.3 x 104 cells/well and incubated with imatinib at concentrations from 0.1 to 1.0 µg/ml. Fresh medium and imatinib mesylate were added every other day. Untreated HMEC-1's were used as controls. After 48, 72 and 96 hours, cells were detached using trypsin and counted with a BD FACS Calibur flow cytometer.

Scratch assay

Confluent monolayer of HMEC-1's was wounded by scratching the surface uniformly with a pipette tip. This initial wounding and the movement of the cells in the scratched area were photographically monitored using the Spot Insight QE camera. Shortening of the distance between the two borders of the scratch was determined at three defined sites per sample after 0, 24 and 48 hours.

Chemotaxis assay

The effect of imatinib on the chemotaxis of HMEC-1 was determined with a transwell chemotaxis assay using 24 well cell culture inserts with the bottom sealed by an 8 µm pore polycarbonate-filter. The number of cells that migrated through the filter to the lower compartment was counted after 36 h with a BD FACS Calibur flow cytometer.

In-vitro capillary morphogenesis assay

24-microwell plates were filled with 300 µl Matrigel/well and polymerized for 1 h at 37°C. 5 x10^3 HMEC-1's were seeded into each well.
Tube forming was analyzed after 24 and 48 h. Representative images were obtained using an Axiovert 25 microscope and a Spot Insight QE camera (Zeiss, Jena, Germany).

Statistics

Data are expressed as mean ± standard deviation. A p-value of less than 0.05 was considered statistically significant.

RESULTS

Imatinib induces regression of established fibrosis

To prevent progression of fibrosis is a major aim in SSc. However, optimally, anti-fibrotic drugs should prevent fibrosis, but also induce regression of pre-existing tissue fibrosis. To evaluate the efficacy of imatinib for the treatment of established fibrosis, a modified model of bleomycin induced dermal fibrosis was used. The dermal thickness increased strongly to 1.50 ± 0.05 fold after 3 weeks of injections with bleomycin (P<0,0001 compared to controls). Prolonged injections of bleomycin increased further the dermal thickness. When the challenge with bleomycin was continued for additional 3 weeks to a total of 6 weeks, the dermal thickness increased to 1.67 ± 0.06 fold (P = 0,0025 compared to 3 weeks and P < 0,0001 compared to controls). The progression of dermal thickness is summarized in Fig. 2.

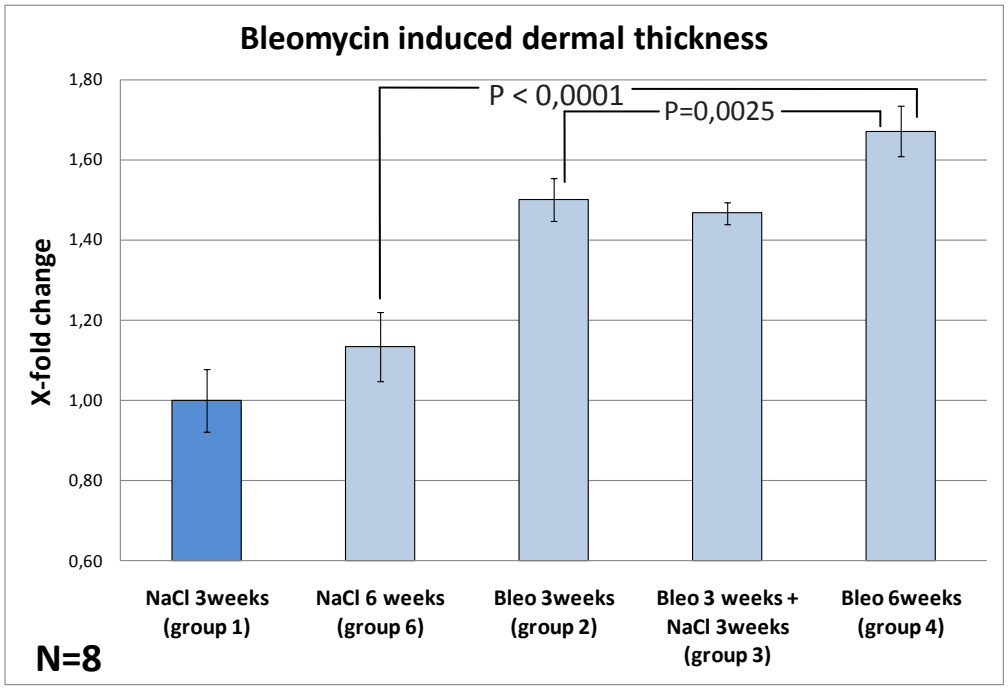


Fig.2 Progression of dermal thickness in various mice groups in modified bleomycin induced dermal fibrosis model.

Treatment with imatinib for the last 3 weeks of bleomycin injection did not only stop the progression of fibrosis, but induced regression of pre-existing matrix accumulation and decreased the dermal thickness below pre-treatment levels (Figures

3 and 4). The dermal thickness in mice treated with imatinib was significantly reduced to 1.07 ± 0.08 fold compared to mice injected with bleomycin for 6 weeks (P < 0,0001). The dermal thickness in mice injected with bleomycin for 6 weeks and treated with imatinib for 3 weeks was also significantly lower than in mice challenged with bleomycin for 3 weeks (1.07 ± 0.08 fold vs. 1.29 ± 0.03 fold compared to controls, P < 0,0001). These findings suggest that imatinib does not prevent the development of fibrosis, but can also induce regression of pre-existing fibrotic damage.

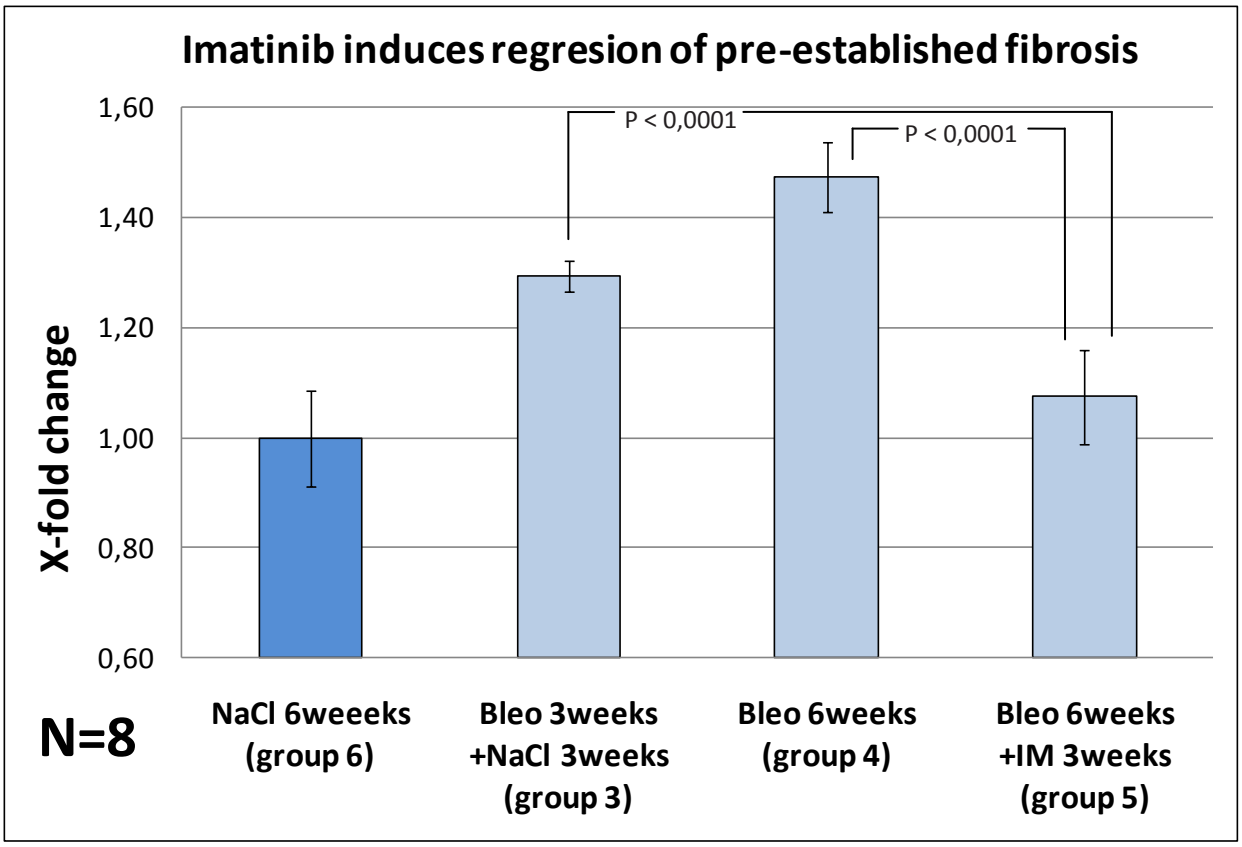


Fig.3 Imatinib-induced regression of preexisting skin fibrosis in a modified model of Bleomycin induced fibrosis. Treatment with imatinib decreased dermal thickness to below baseline levels.

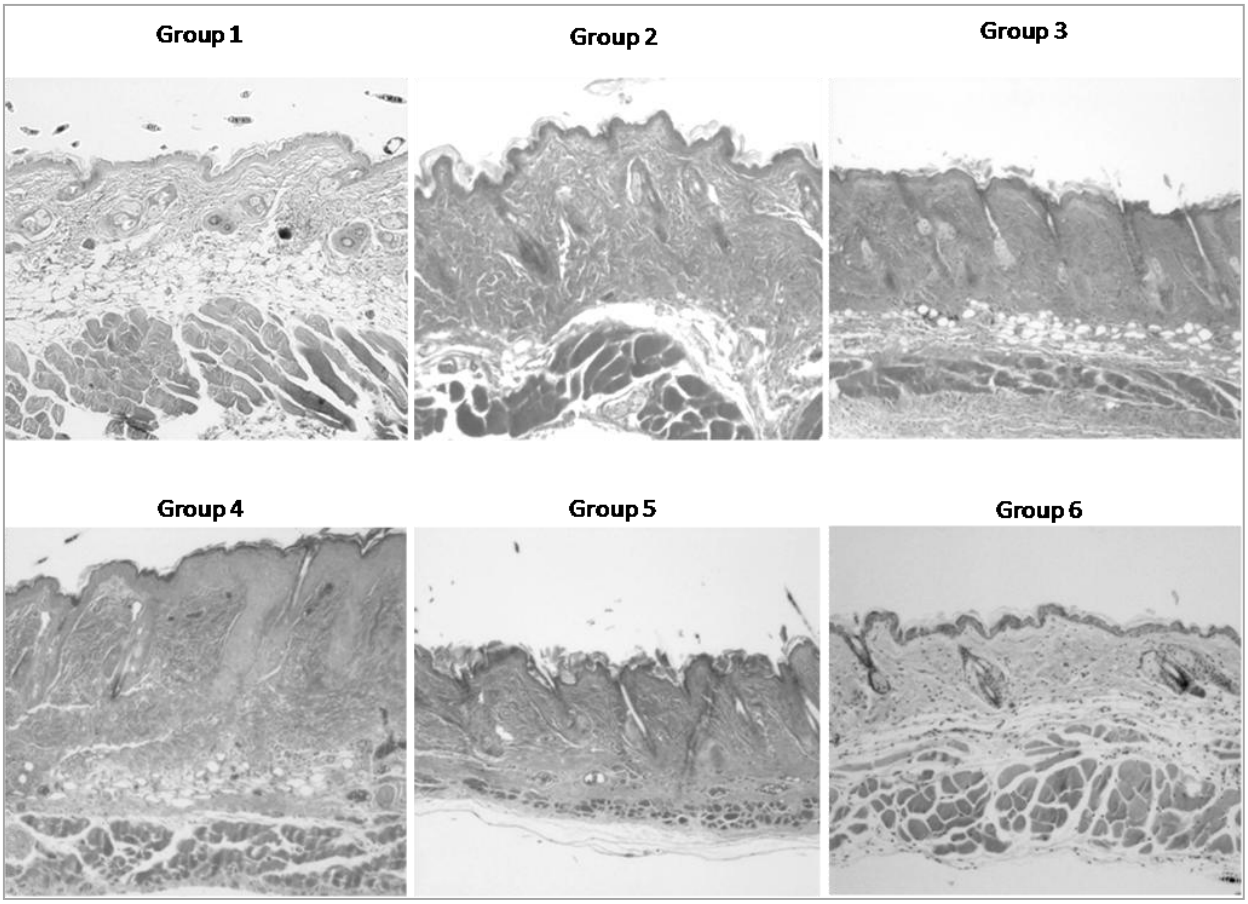


Fig.4 Representative pictures of dermal thickness in skin fibrosis in a modified model of bleomycin induced fibrosis.

Imatinib corrects the tight skin-1 phenotype

After it was demonstrated previously that imatinib prevents dermal fibrosis upon bleomycin challenge, we wanted to confirm the efficacy of imatinib in another, less inflammatory animal model of SSc. Thus, we evaluated the efficacy of imatinib in the Tsk-1 mouse model. Tsk-1 mice are characterized by a modest increase in dermal thickness and increased hypodermal thickness. Treatment with imatinib almost completely prevented the histological changes in Tsk-1 mice. The dermal thickness in Tsk-1 mice was increased by 1.39 ± 0.06 fold compared to pa/pa mice. Treatment with imatinib reduced the dermal thickness to 0.94 ± 0.09 fold (Fig.5). The increased thickness of the hypodermis in Tsk-1 mice was also significantly reduced from 2.89 ± 0.09 fold to 1.71 ± 0.06 fold by imatinib ($P < 0,0001$) (Fig.5 and 6).

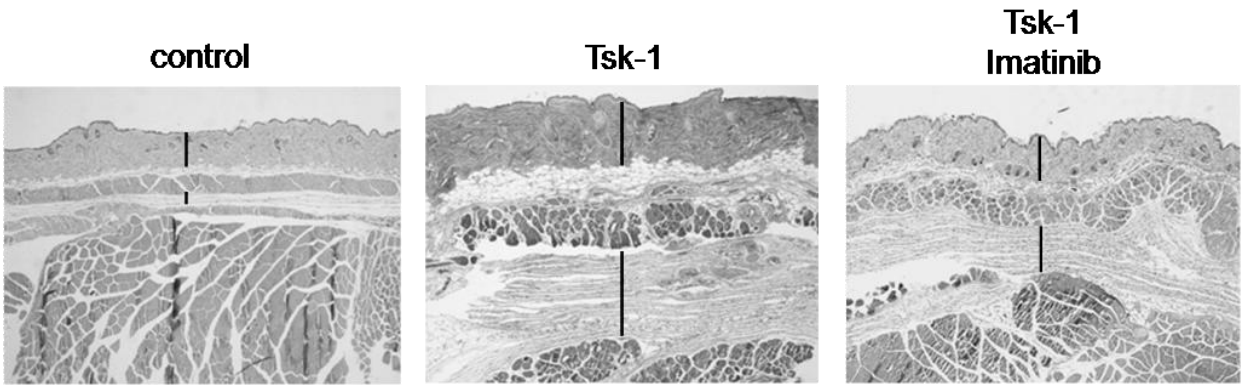


Fig.5 Antifibrotic imatinib effects in TSK-1 mice. Dermal and hypodermal thickening in Tsk-1 mice treated with imatinib was significantly reduced compared with that in nontreated TSK-1 mice. Representative sections are shown. Top vertical bars show dermal thickness; lower vertical bars show hypodermal thickness.

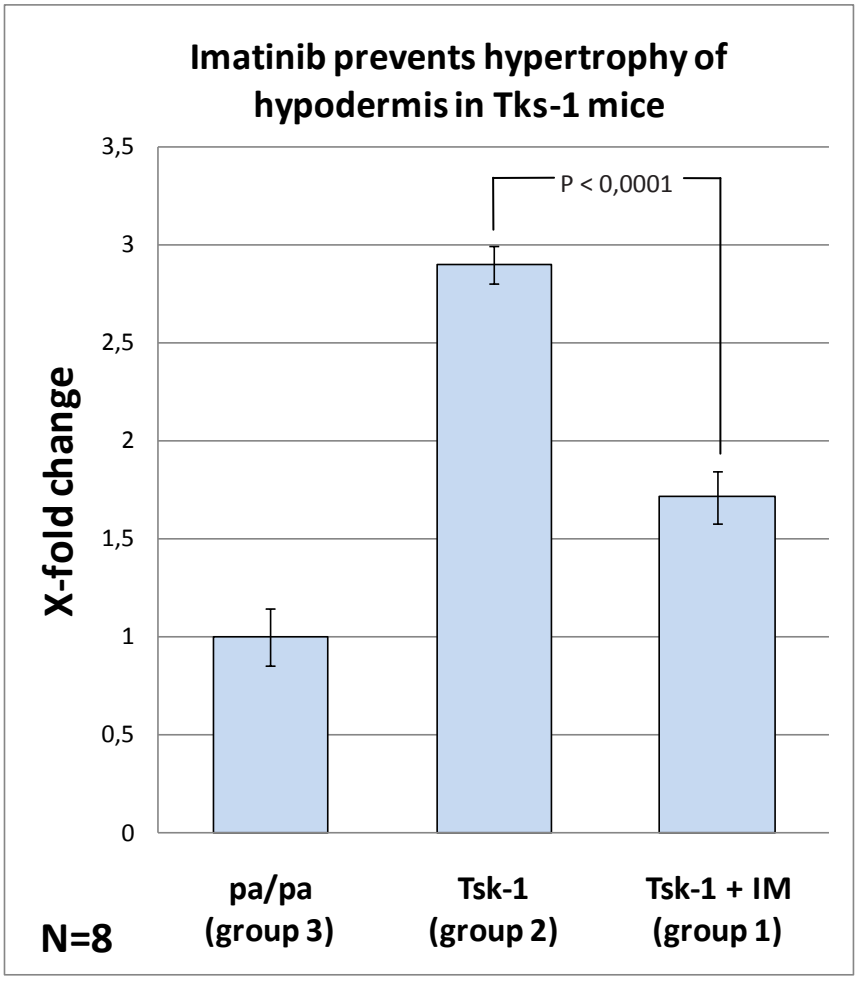


Fig.6 Hypodermal thickness was decreased in TSK-1 mice treated with imatinib.

Myofibroblasts are considered as major effector cells for fibrosis. Imatinib significantly reduced the differentiation of resting fibroblasts into myofibroblasts in Tsk-1 mice (Fig.7). The number of myofibroblasts was reduced from 2.98 ± 0.12 fold in untreated tsk-1 mice to 0.95 ± 0.16 fold in tsk-1 mice treated with imatinib (P < 0,0001).

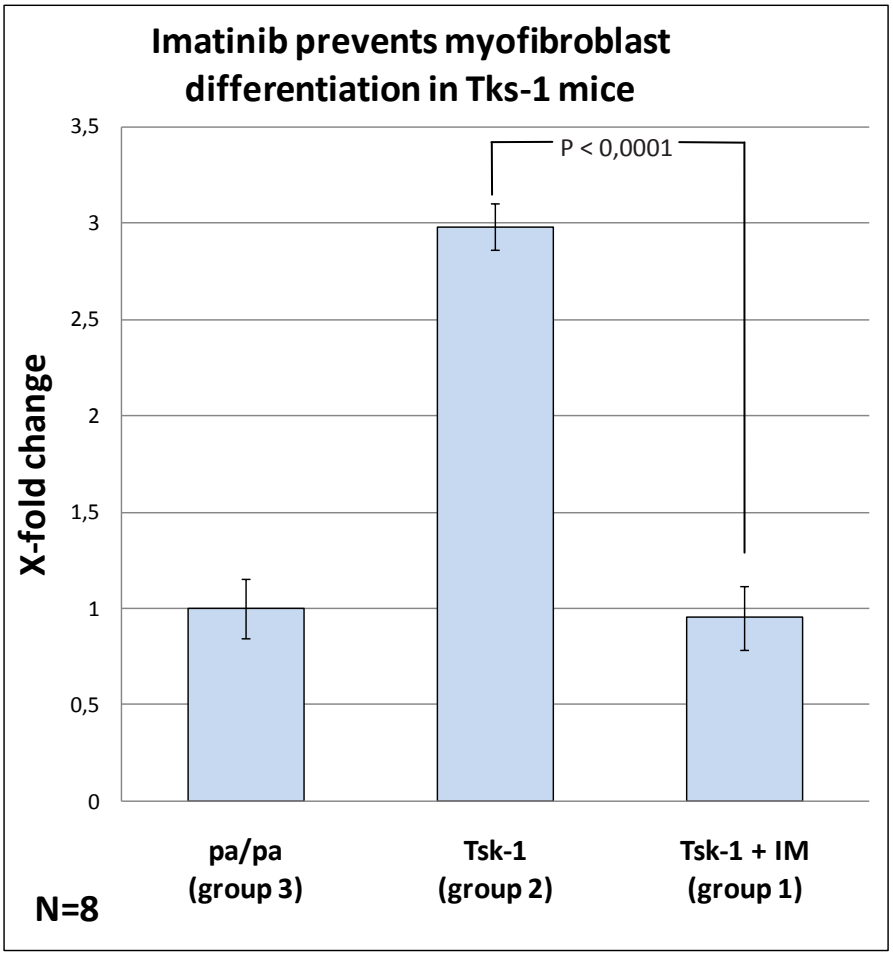
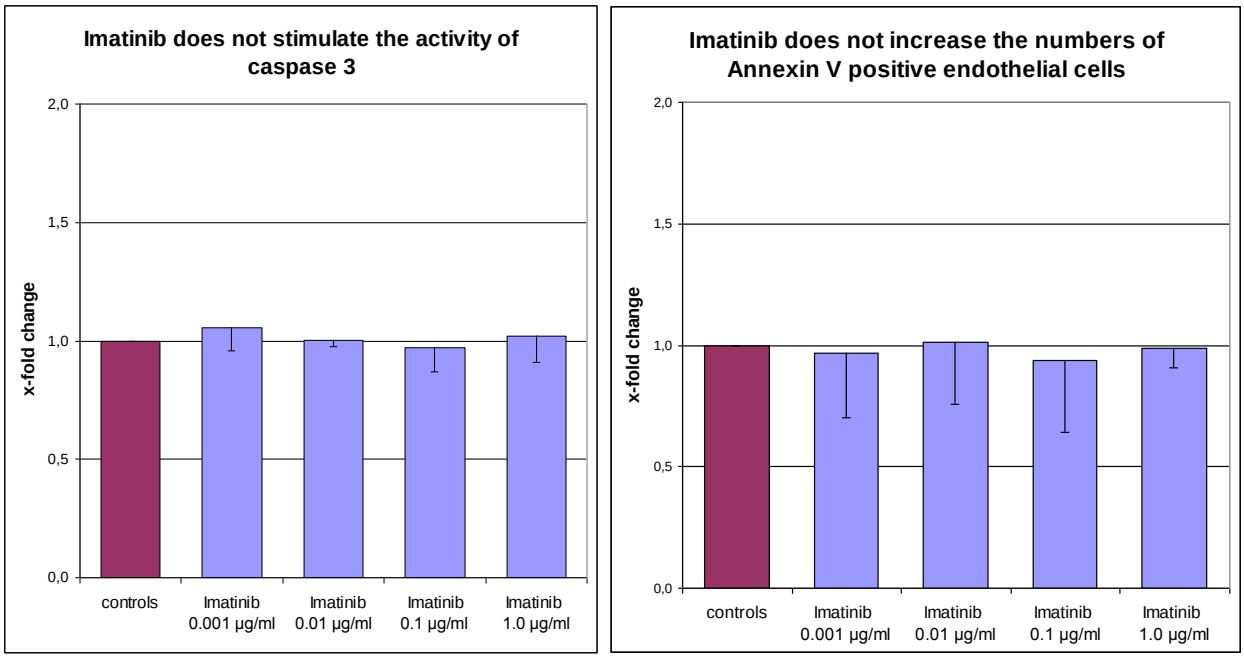


Fig.7 Myofibroblast differentiation was completely prevented by imatinib

Imatinib does not reduce the viability of endothelial cells

The effect of imatinib on apoptosis of endothelial cells was analyzed by quantification of caspase 3 activities and staining for annexin V. After incubation with imatinib mesylate in concentration from 0.001 - 1.0 µg/ml, no changes of the activity of caspase 3 were detectable (Fig.8a). Incubation of HMEC-1's with increasing concentrations of imatinib did also not increase the number of annexin V positive, apoptotic cells (Fig. 8b). To investigate, whether imatinib augments pre-existing endothelial cell damage, HMEC-1's were serum starved and then incubated with imatinib.



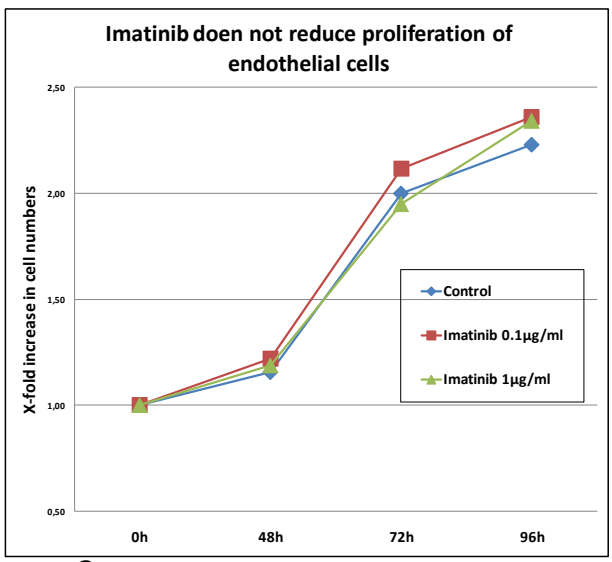
a **b**

Fig.8 Imatinib does not induce apoptosis of endothelial cells. Incubation with imatinib in concentrations from 0.001 – 1.0 µg/ml for 24 h did not increase the activity of caspase 3 (n=6) (**Fig. 6a**) or the number of annexin V positive HMEC-1's (n=6) (**Fig. 6b**).

As under normal culture conditions, imatinib did not induce the activity of caspase 3 or increase the number of apoptotic cells.

Imatinib does not decrease proliferation of endothelial cells

Potential effects of imatinib mesylate on the metabolic activity and proliferation of endothelial cells were analyzed by MTT assay and by generation of proliferation curves. No differences in metabolic activity were observed with the MTT assay between HMEC-1's treated with imatinib for 24 to 96 h and untreated controls (Fig.9b). Incubation with imatinib in pharmacologically relevant concentrations for up to 96 h also did not alter cell counts (Fig.9a). Imatinib did not increase the time period needed to reach 100% confluence also.



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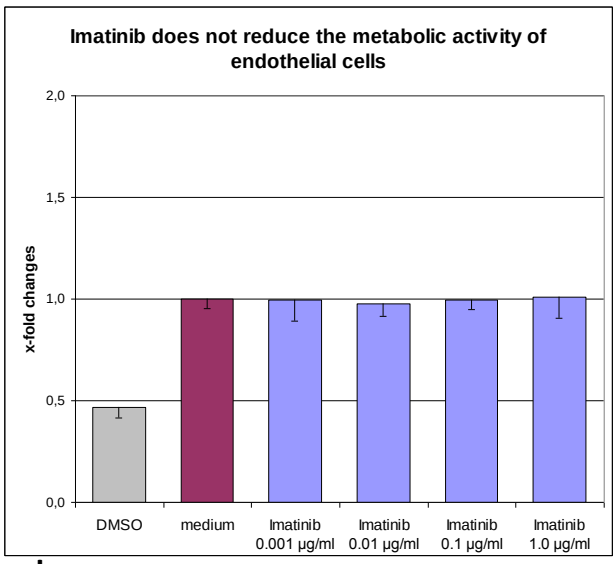


b

Fig.9 Imatinib does not induce apoptosis, reduce the metabolic activity or decrease proliferation of endothelial cells **Figure9a:** Imatinib did not reduce proliferation of HMEC-1's as analyzed by direct cell counting (n = 6). **Figure9b:** Incubation with imatinib in pharmacologically relevant concentrations for 48 h did not reduce the metabolic activity of HMEC-1's compared to cells incubated with medium only (n = 16). In contrast, incubation with 50 % dimethyl sulfoxide (DMSO) profoundly reduced the metabolic activity.

Imatinib does not alter the expression of markers for vascular activation

ICAM-1, VCAM-1, endothelin-1 and VEGF are differentially expressed in SSc and are common markers of endothelial cell activation. Incubation of HMEC-1's with imatinib did not change the expression of ICAM-1, VCAM-1, endothelin-1 and VEGF, suggesting that imatinib might not alter the activation of endothelial cells.

Imatinib does not inhibit migration or chemotaxis of endothelial cells

To assess, whether imatinib reduces the migration of endothelial cells, scratch assays were performed. No differences in the distance between the two borders of the scratch were observed in HMEC-1's incubated with imatinib compared to untreated controls after 24 h and 48 h (Fig.10a). The time until closure of the scratch was also not prolonged.

To the effects of imatinib on chemotaxis of endothelial cells, a mirco-chemotaxis assay was performed. Imatinib did not reduce the number of migrated HMEC-1's (Fig.10b).

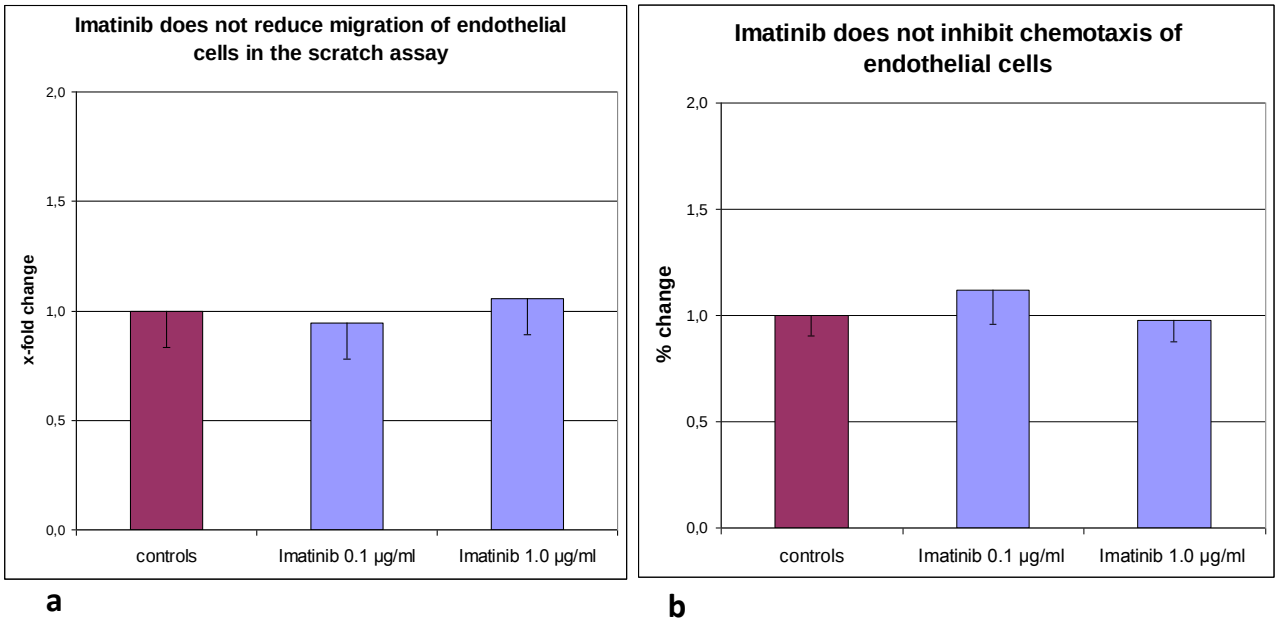


Fig.10 Imatinib does not affect migration or chemotaxis of endothelial cells. **Fig.10a**: Imatinib does not reduce migration in the scratch assay. The distances between the borders of the scratch were not increased in imatinib treated HMEC-1's at various time-points (n = 6). **Fig.10b** Imatinib does not inhibit chemotaxis of HMEC-1's in the transwell assay. The number of HMEC-1's that migrated through the 8 µm pores did not differ between HMEC-1's incubated with imatinib and controls (n = 6).

Imatinib does not inhibit the formation of capillary tubes

Next, we analyzed, whether imatinib inhibits tube formation using an in the in-vitro capillary morphogenesis assay. Imatinib did not reduce the number of tubes, the maximal length of the capillary tubes or the number of branching points in (Fig.11) suggesting that imatinib does not affect tube formation in-vitro.

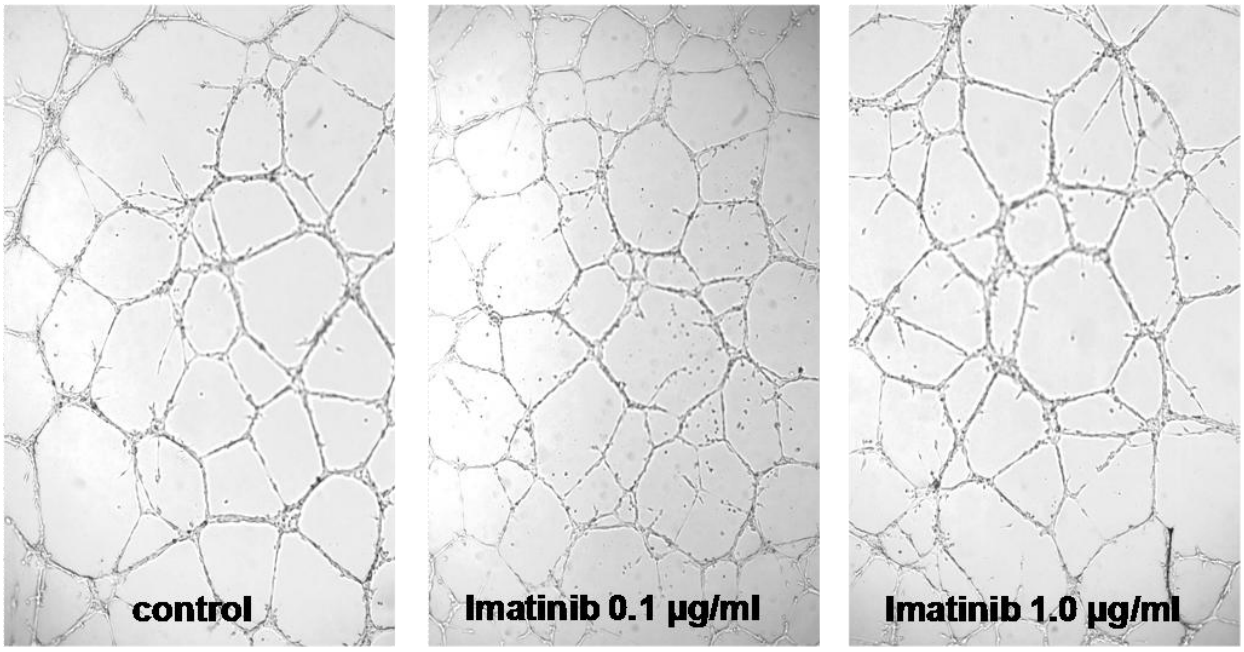
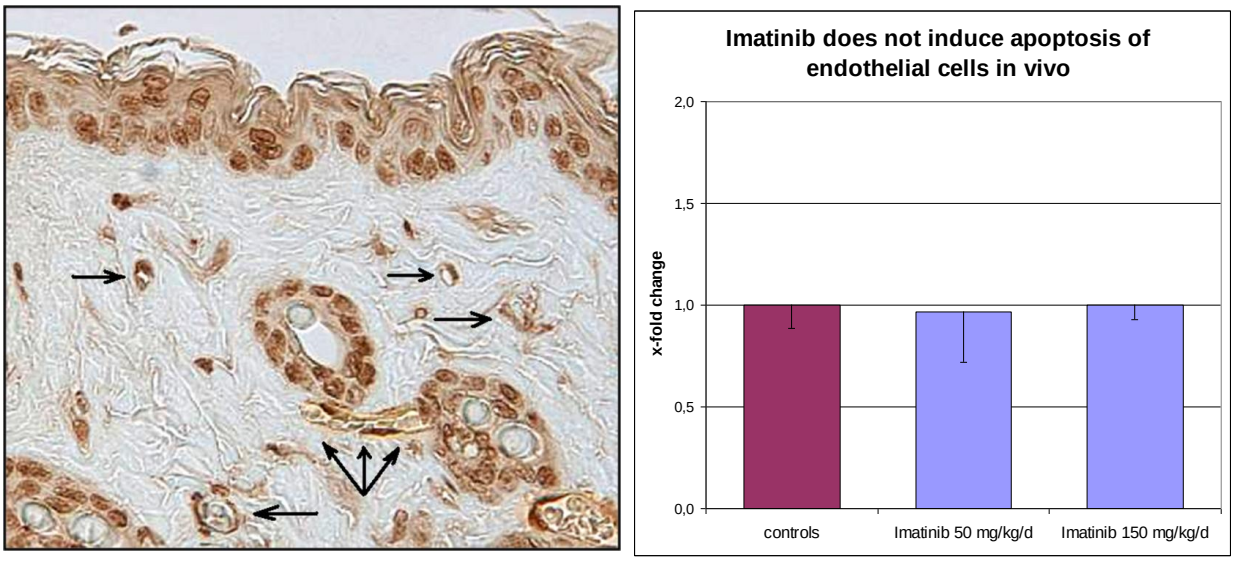


Fig.11 Imatinib does not inhibit the formation of capillary tubes. Imatinib did not reduce the number of tubes, the maximal length of the capillary tubes or the number of branching points. Representative images from controls and HMEC-1's treated with imatinib in concentrations of 0.1 µg/ml and 1.0 µg/ml are shown at 5 fold magnification (n = 15).

Imatinib does not induce apoptosis of endothelial cells in experimental dermal fibrosis

To evaluate, whether imatinib induces apoptosis of endothelial cells in-vivo under fibrotic conditions, the number of apoptotic endothelial cells in mice challenged with bleomycin were quantified by TUNEL staining. Treatment of mice with imatinib in doses of 50 mg/kg/d for 4 weeks did not increase the number of TUNEL positive apoptotic endothelial cells compared to bleomycin alone (Fig.12a and b). Similarly, no increase in apoptotic endothelial cells was detectable in mice treated with imatinib in concentrations of 150 mg/kg/d.



a **b**

Fig.12 Imatinib does not induce apoptosis of endothelial cells in vivo. The number of apoptotic endothelial cells (arrows) upon treatment with imatinib in vivo was quantified in the mouse model of bleomycin induced dermal fibrosis (**Fig.12a**). Treatment with imatinib at doses of 50 mg/kg/d and 150 mg/kg/d did not increase the numbers of apoptotic endothelial cells (n = 6 for each group) (**Fig.12b**).

Bleomycin induced *established* skin fibrosis model and Tsk-1 model results indicate that the combined inhibition of c-Abl and PDGFr might be effective for later, less inflammatory stages of SSc and for the treatment of established fibrosis. We also showed that imatinib does not inhibit activation, viability, proliferation, migration or tube forming of endothelial cells in vitro and in vivo. Thus, imatinib is an interesting candidate for clinical trials with patients with longstanding disease and pre-existing tissue fibrosis.

DISCUSION

It was previously shown that imatinib prevents the development of fibrosis in the mouse model of bleomycin induced dermal fibrosis which is characterized by dense inflammatory infiltrates in lesional skin. Thus, it mimics early stages of SSc, but is less suited for later stages of SSc, where inflammatory infiltrates are scarce. To evaluate the anti-fibrotic efficacy of imatinib in a model for later stages of SSc, we used the Tsk-1 mouse model, in which persistent overproduction of extracellular matrix proteins

occurs in the absence of inflammatory infiltrates. We demonstrate potent anti-fibrotic effects of imatinib in Tsk-1 mice with prevention of histological changes and inhibition of myofibroblast differentiation. Thus, our data obtained on Tsk-1 mice indicate that imatinib might be effective not only for prevention of fibrosis in early, inflammatory stages of SSc, but also under less inflammatory conditions as in later stages of SSc.

The data obtained on Tsk-1 mice in the present study demonstrate that imatinib prevents effectively the development of dermal fibrosis in preclinical model of SSc. If translated into clinics, prevention of fibrosis is mainly relevant for patients with very early, progressive SSc. In contrast, treatment of established fibrosis with reduction of pre-existing matrix accumulation is also relevant for SSc patients with long-standing extensive disease and high morbidity. In these patients treatment would remove pre-existing fibrotic tissue damage and potentially reduce organ dysfunction. Using a modified model of bleomycin induced dermal fibrosis with prolonged application of bleomycin and late onset of treatment, we demonstrate that imatinib does not only halter progression, but even reduces pre-existing dermal fibrosis and decreases dermal thickness below pre-treatment levels despite ongoing challenge with the pro-fibrotic stimulus bleomycin. Given the extremely long half-life of dermal collagen and a fact that imatinib has no direct effects on MMPs or TIMPs, we believe that imatinib does not only block the increased synthesis of collagen by SSc fibroblasts, but decreases the collagen synthesis potently enough to shift the balance between collagen release and collagen degradation towards degradation. Thus, the observed regression of fibrosis might be due to the potent suppression of the collagen production with relative overweight of matrix degradation rather than due to induction of MMPs or TIMPs.

Besides fibrosis, a progressive microangiopathy is a characteristic feature of SSc. Anti-angiogenic side effects might complicate the use of novel anti-fibrotic drugs in SSc patients as they might augment further the vascular manifestations of SSc. The aim of the present study was also to determine, whether imatinib mesylate interferes with endothelial cell functions and might thereby aggravate vascular disease in SSc. We demonstrate with in vitro and in vivo studies that imatinib does not induce apoptosis or inhibit proliferation of microvascular endothelial cells. Imatinib did also not affect

activation of endothelial cells, reduce migration and chemotaxis or decrease the formation of capillary tubes. Together, these data suggest that imatinib does not affect major functions of endothelial cells. Thus, treatment with imatinib might not be complicated by vascular side effects under physiological conditions. The lack of vascular side effects of imatinib observed herein is supported by clinical observations on patients with chronic myelogenic leukemia and gastrointestinal stromal tumors. No increased incidence of vascular side effects have been reported from the more than 100,000 patients treated so far.

In SSc, ongoing damage of endothelial cells occurs via several distinct mechanisms. Infection with cytomegalovirus, anti-endothelial cell autoantibodies and microparticles all have been implicated in the vascular pathogenesis of SSc. Although we did not observe inhibitory effects of imatinib on major functions of endothelial cells, we cannot exclude that imatinib might inhibit functions of endothelial cells that have previously been damaged by autoantibodies or microparticles. However, imatinib did not increase apoptosis of serum starved, pre-damaged HMEC-1's, indicating that imatinib does not enhance pre-existing damage of endothelial cells in general. Furthermore, first case reports of SSc patients treated with imatinib did not report exacerbations of vascular manifestations of SSc.

Capillaries compose of two different cell types, endothelial cells and pericytes. Pericytes have also been implicated in the pathogenesis of SSc. In contrast endothelial cells, pericytes are over-activated and their numbers are increased in SSc. Recent studies indicate that increased activation of pericytes might contribute directly to tissue fibrosis in SSc. Pericytes might transdifferentiate into myofibroblasts, as pericytes and myofibroblasts share the expression of several phenotypic markers. Pericytes might also augment vascular disease in SSc. Pericytes negatively regulate proliferation of endothelial cells. Increased inhibition of microvascular endothelial cells by hyperplastic and overactivated pericytes might thus prevent the necessary proliferation of endothelial cells upon damage and might contribute to the progressive microangiopathy in SSc. Thus, therapeutic strategies should aim for inhibition of the pathologically increased pericyte function rather than for further activation. Activation

and proliferation of pericytes depends on PDGF. As imatinib inhibits the tyrosine kinase activity of PDGF receptors, imatinib might normalize pericyte activation, thereby stimulating endothelial cell functions in SSc.

In summary, we have shown that imatinib exerts potent anti-fibrotic effects in two different models of SSc with different underlying pathologic mechanisms. Imatinib was effective for prevention of fibrosis and for treatment of established dermal fibrosis. We've demonstrated that imatinib does not inhibit major functions of endothelial cells. Thus, the anti-fibrotic activity of imatinib does not seem to be accompanied by unwanted side effects on endothelial cell and imatinib might not augment further the preexisting vascular damage in SSc.

Conclusions

1. In the model of pre-established dermal fibrosis, the effect of imatinib is not only limited by halting the progression of fibrosis, but also inducing the regression of pre-existing dermal fibrosis with reduction of the dermal thickness below pre-treatment levels.
2. Treatment with imatinib reduced the dermal and hypodermal thickening in Tsk-1 mice and prevented the differentiation of resting fibroblasts into myofibroblasts.
3. Imatinib mesylate had no effect on viability, apoptosis and activation of microvascular endothelial cells in vitro.
4. Imatinib did not induced apoptosis of endothelial cells in the model of bleomycin induced experimental dermal fibrosis in vivo.
5. Imatinib mesylate had no negative effect on the major stages of angiogenesis: proliferation, migration, tube formation.

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2. "Effect of Imatinib mesylate on Endothelial cells functions"– ARTICULUM, Germany 2008

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SUMMARY IN LITHUANIAN

ANTIFIBROZINIŲ PRIEMONIŲ PAIEŠKA PREKLINIKINIUOSE SISTEMINĖS SKLEROZĖS MODELIUOSE

SANTRUMPOS

c-Abl – Abelson‘o kinazė
DMEM – Dulbecco modifikuota Eagle terpė (*Dulbecco’s modified Eagle's medium*)
ELM – ekstraląstelinis matriksas
FCS – embrioninio galvijų serumas (*fetal calf serum*)
HMEC-1 – žmogaus mikrovaskulinės endotelio ląstelės – 1
IM – imatinibo mezilatas
MTT – 3, (4,5-dimethyl thiazolyl -2) 2,5-diphenil-tetrazoliumo bromidas
PDGF – trombocitų išskirtas augimo faktorius (*Platelet derived growth factor*)
SSc – sisteminė sklerozė
TGF-β – transformuojantis augimo faktorius beta
TUNEL – galinės deoksinukleotidiltransferazės dUTP liekanos galo žymėjimas (*Terminal deoxynucleotidyl transferase dUTP nick end labeling*)
αSMA – lygiųjų raumenų alfa aktinas

ĮVADAS

Sisteminė sklerozė (SSc) – viena sunkiausių ir fatališkiausių autoimuninių sisteminių reumatinių ligų. Didelei daliai ligonių sisteminė sklerozė pasireiškia progresuojančia odos, plaučių, inkstų, širdies, virškinamojo trakto fibroze.
SSc patogenezėje galima išskirti tris svarbiausius, tarpusavyje susijusius komponentus: autoimuninį uždegimą, fibrozę ir vaskulopatiją.

Būtent kraujagyslių sienelių peraugimas jungiamuoju audiniu ir todėl atsiradęs spindžio susiaurėjimas sąlygoja kraujagyslines komplikacijas (plautinė hipertenzija, inkstinė krizė, bei daugelio audinių išemiją). Išemija atgalinio ryšio principu stimuliuoja fibrozės procesą. Susidaro ydingas ratas, kurį labai sunku paveikti net naujausios

terapijos priemonėmis. Tai lemia ligos fatališkumą ir blogą prognozę. Todėl ieškant terapinių sprendimų ir toliau būtina gilintis į ląstelinius ir molekulinius fibrozės veiksnius bei ieškoti specifinių priemonių šiam procesui stabdyti.

Jau gana seniai žinoma, jog fibrogenezės procese citokinai – transformuojantis augimo faktorius beta (TGF-β) ir iš trombocitų kilęs augimo faktorius (PDGF) – atlieka svarbiausius vaidmenis.

Pastaruoju metu į onkologinę praktiką buvo įdiegti tirozinkinazių inhibitoriai, tarp jų ir imatinibo mezilatas (IM). Pastebėtas IM gebėjimas blokuoti ne tik patologinės, Filadelfijos chromosomos sąlygotos kinazės, bet ir nepatologinių tirozinkinazių aktyvumą.

TGF-β intraląstelinis signalas ląstelėje, nuo receptoriaus iki kolageno sintezę inicijuojančių transkripcijos faktorių, perduodamas proteinkinazių dėka, o centrinį vaidmenį atlieka Abelsono kinazė (c-Abl). Į c-Abl panaši kinazė yra ir PDGF receptoriaus sudedamoji dalis. Neseniai tapo žinoma, kad imatinibas gana selektyviai blokuoja c-Abl kinazę. Taigi buvo galima daryti prielaidą, kad imatinibas, slopindamas c-Abl, slopins ir TGF-β bei PDGF signalo perdavimą fibroblastuose, taip pat fibrozės procesą.

Fibrozės prevencija aktuali tik ankstyvoje SSc stadijoje tiems pacientams, kurių ligos eiga agresyvi, vyksta aktyvi ekstraląstelinio matrikso (ELM) akumuliacija audiniuose ir vystosi įvairių organų fibrozė. Tačiau klinikinėje praktikoje SSc pacientai į reumatologo akiratį patenka ne pradiniuose ligos etapuose. Tuo atveju klinicistui iškyla užduotis ne tik sustabdyti tolesnį jungiamojo audinio išbujojimą, bet ir siekti fibrozės regresavimo. Buvo neaišku ar ją galima išspręsti į praktiką įdiegus imatinibą. Todėl mums buvo svarbu išsiaiškinti, ar imatinibas gali turėti įtakos jau susiformavusiai fibrozei.

Žinoma, kad TGF-β ir PDGF yra multipotentiniai, daugelį ląstelių veikiantys citokinai. Tai reiškia, jog imatinibas galėtų blokuoti TGF-β ir PDGF vaidmenį ne tik fibrogenezėje, bet ir angiogenezėje, ne tik fibroblastuose, bet ir endotelio ląstelėse.

Neoangiogenezės slopinimas sisteminės sklerozės atveju galėtų reikšti vaskulopatijos – vieno svarbiausių patogenezės veiksnių gilėjimą ir taip riboti daug žadančio fibrozės inhibitoriaus Imatinibo mezilato naudojimą gydant SSc.

Mums atrodė labai svarbu įvertinti imatinibo mezilato įtaką susiformavusiai fibrozei bei patikrinti, ar antifibrozinis IM efektyvumas nėra lydimas proangiogenetinių ir bazinių endotelio funkcijų slopinimo.

Darbo tikslas

Įvertinti imatinibo mezilato poveikį fibrozės procesui ir endoteliui sisteminės sklerozės eksperimentiniuose modeliuose ir ląstelių kultūrose.

Uždaviniai

1. Įvertinti imatinibo efektyvumą neuždegiminiame SSc modelyje ir patikrinti imatinibo mezilato efektyvumą uždegiminiame *suformuotos* fibrozės modelyje.
2. Ištirti, ar terapinės imatinibo mezilato koncentracijos daro neigiamą poveikį gyvybinėms endotelio funkcijoms in vitro ir in vivo.
3. Įvertinti imatinibo mezilato poveikį angiogenezės etapams in vitro

Ginamieji teiginiai

1. Terapinės imatinibo mezilato koncentracijos pasižymi antifibroziniu poveikiu neuždegiminio modelio Tsk-1 ir bleomicino sukeltos, *suformuotos* fibrozės modelio pelėms.
2. Terapinės imatinibo mezilato koncentracijos nedaro neigiamo poveikio nei endotelio gyvybingumui, nei angiogenezei.

Mokslinis naujumas

1. Pirmą kartą buvo įrodyta, kad IM ne tik slopina fibrozės formavimąsi, bet ir sąlygoja jau susiformavusios fibrozės regresiją.
2. Buvo gauti moksliniai įrodymai, leidžiantys teigti, kad IM nedaro neigiamo poveikio bazinėms endotelio funkcijoms ir neįtakoja angiogenezės proceso.

Praktinė nauda

Atlikti tyrimai yra būtinas etapas prieš pradedant IM klinikines studijas. Jeigu šis vaistas bus vartojamas reumatologijos klinikinėje praktikoje, tai bus svarbus žingsnis sprendžiant labai sudėtingas SSc gydymo problemas.

Baigiant rašyti disertaciją mus pasiekė žinia, kad mūsų atliktų projektų pagrindu, pradėtos net kelios klinikinės studijos, skirtos IM efektyvumo ir toksiškumo tyrimams, gydant SSc.

Net jeigu dėl kurių nors priežasčių (pvz. didelio toksiškumo) IM nebūtų įdiegtas į kliniką, mūsų tyrimas sukuria teorines prielaidas ieškoti kitų, dvigubu poveikiu (TGF-β ir PDGF) pasižyminčių, intraląstelines kinazes slopinančių, preparatų.

TYRIMO MEDŽIAGA

Eksperimentiniai odos fibrozės modeliai ir veikimas imatinibo mezilatu

Eksperimentuose naudoti trys odos fibrozės modeliai: bleomicino sukeltos odos fibrozės modelis; modifikuotas bleomicino sukeltos (*suformuotos*) odos fibrozės modelis, Tsk-1 mutacijos sąlygotos odos fibrozės modelis.

Bleomicino sukeltos odos fibrozės modelyje odos fibrozė buvo sukelta vietinėmis bleomicino injekcijomis 6 savaičių DBA linijos pelėms. Bleomicinas tirpintas 0,9 % NaCl tirpale, gauta 0,5mg/ml koncentracija. Bleomicinas į poodį leistas kas antrą dieną, 4 savaites, į apibrėžtą odos zoną laboratorinės pelės nugaros viršuje. Imatinibas tirpintas 0,9 % NaCl ir kartą per dieną leistas į pilvaplėvės ertmę (dozės 50 mg/kg/d ir 150 mg/kg/d). Imatinibas ir bleomicinas pradėti leisti vienu metu.

Norėdami ištirti imatinibo mezilato poveikį susiformavusiai fibrozei, modifikavome bleomicino sukeltos odos fibrozės modelį taip, kad imatinibo mezilatas būtų leidžiamas pelėms, odos fibrozei jau susiformavus. Modifikuotame bleomicino sukeltos (*suformuotos*) odos fibrozės modelyje bleomicinas leistas 6 savaites, o gydymas imatinibo mezilatu pradėtas ketvirtos savaitės pradžioje ir tęstas likusias 3 eksperimento savaites. Bleomicinas ir imatinibas leisti taip, kaip aprašyta prieš tai.

Tsk-1 mutacijos modelyje eksperimentai atlikti su heterozigotinėmis Tsk-1/pa pelėmis (jos paprastumo dėlei vadinamos – Tsk-1 pelėmis). Šiame modelyje odos fibrozė vystosi spontaniškai. Homozigotinės pa/pa pelės naudotos kontrolei. Imatinibas tirpintas 0,9 % NaCl ir kartą per dieną leistas į pilvaplėvės ertmę (dozės 150 mg/kg/d).

Endotelio ląstelių kultūra ir inkubacija su imatinibo mezilatu

Eksperimentuose buvo naudotos ilgaamžės žmogaus mikrovaskulinės endotelio ląstelės (*Immortalized human microvascular endothelial cells* – HMEC-1), išskirtos iš žmogaus odos, plaučių, kepenų.

Disertacijoje aprašomam projekte, HMEC-1 kultivuotos Dulbecco modifikuotoje Eagle'o terpėje (*Dulbecco's modified Eagle's medium* - DMEM) su 10 % karščiu inaktyvuoto embrioninio galvijų serumo (*fetal calf serum* - FCS), 25 mM HEPES (*4-(2-hydroxyethyl)-1-piperazineethanesulfoninė rūgštis*), 100 U/ml penicilino, 100 µg/ml streptomicino, 2 mM L-glutamino ir 2.5 µg/ml amfotericino B.

HMEC-1 inkubuotos su skirtingos koncentracijos imatinibo tirpalais (koncentracijomis nuo 0.001 iki 1.0 µg/ml).

TYRIMO METODAI

Naudoti metodai *in vivo*

Įsisavinti ir naudoti šie metodai *in vivo*: apoptozės tyrimas galinės deoksinukleotidiltransferazės deoksiuridintrifosfato liekanos žymėjimo (TUNEL) metodu; histologinė odos ir paodžio storio analizė dažant hemalaunu ir eozinu; Miofibroblastų nustatymas imunohistochemijos metodu atlikus lygiųjų raumenų alfa aktino dažymus (αSMA).

Naudoti metodai *in vitro*

Įsisavinti ir naudoti šie metodai *in vitro*: kaspazės-3 aktyvumo eksperimentas; apoptotinių endotelio ląstelių dažytų Aneksinu V kiekio skaičiavimas; proliferacijos kreivių generavimas; MTT eksperimentas; realaus laiko polimerazės grandžių reakcijos eksperimentai; „brėžio" eksperimentas; chemotaksio eksperimentas; kapiliarų morfogenezės eksperimentas.

REZULTATAI

Imatinibo mezilato poveikis bleomicino sukeltos suformuotos fibrozės modeliui

Imatinibo mezilato poveikis jau egzistuojančiai fibrozei vertintas modifikuotame bleomicino sukeltos *suformuotos* odos fibrozės modelyje, naudojant histocheminius dažymo būdus.

Suformuotos odos fibrozės modelyje stebėjome, kad po 3 savaičių bleomicino injekcijų, odos storis padidėjo 50% ± 5,3%, lyginant su kontroline grupe, kuriai 3 savaites injekuotas NaCl ($P < 0,0001$). Galima teigti, kad, po trijų savaičių veikimo bleomicinu, odos fibrozė yra susiformavusi. Leidžiant bleomiciną dar 3 savaites (viso 6 savaitės) odos storis padidėjo lyginant su kontroline, 6 savaites gavusia fiziologinio tirpalo injekcijas, grupe ($P < 0,0001$), bei lyginant su tik 3 savaites bleomiciną gavusia grupe ($P = 0,0025$). Per 6 savaites susiformavęs odos storio lygis bus atskaitos taškas vertinant imatinibo poveikį fibrozės progresijai. Pelių, paskutines tris eksperimento savaites veiktų ir imatinibo mezilatu, oda buvo 27% ± 6% plonesnė, nei pelių veiktų 6 savaites tik bleomicinu ($P<0,0001$) .Tai rodo, jog trijų savaičių gydymas imatinibu sustabdė tolimesnę fibrozės progresiją.

Pelių, 6 savaites veiktų bleomicinu ir 3 paskutines savaites imatinibo mezilatu, oda buvo taipogi plonesnė nei pelių, tris savaites veiktų bleomicinu ($P < 0,0001$). Tai rodo, kad tris savaites gydant imatinibu odos storis regresavo žemiau pradinio (to momento, kai pradėtas leisti imatinibas) lygio.

Iš odos tyrimų histcheminio metodu matyti, kad Imatinibo mezilatas ne tik sustabdė fibrozės vystymąsi Bleomicino sukeltos odos fibrozės modelyje, bet ir paskatino jau egzistuojančios firbrozės regresiją.

Imatinibo mezilato poveikis Tsk-1 mutacijos sukeltos fibrozės modeliui

Tyrime siekta įvertinti imatinibo mezilato efektyvumą ir nuo uždegimo nepriklausomame odos fibrozės modelyje *in vivo*.

Tsk-1 pelėms būdingas padidėjęs ne tik odos, bet ir poodžio storis. Imatinibo mezilato poveikyje Tsk-1 pelių odos storis sumažėjo iki normalaus lygio ir nesiskyrė nuo kontrolinių pelių grupės ($P = 0,1693$).

Tsk-1 pelių poodis yra 190% didesnis nei mutacijos neturinčių pa/pa pelių (P<0,0001). Tsk-1 pelių poodžio storis imatinibo poveikyje sumažėjo 41% (P < 0,0001).

Miofibroblastai – pagrindinės efektorinės, fibrogenezės procese dalyvaujančios, ląstelės. Imatinibo mezilatas ryškiai sumažino fibroblastų diferenciaciją į miofibroblastus Tsk-1 pelėse (P < 0,0001 lyginant su imatinibo negavusiomis Tsk-1 pelėmis).

Fibrilino-1 mutaciją turinčių Tsk-1 pelių odos tyrimai histochemijos ir imunohistochemijos (dažant prieš alfa-SMA) metodais, rodo, kad imatinibo mezilatas ryškiai sumažino poodžio storį, visiškai normalizavo Tsk-1 pelių odos storį, taipogi normalizavo fibroblastų diferenciaciją į miofibroblastus.

IM poveikis bazinėms endotelio funkcijoms *in vitro* ir *in vivo*

Tyrimų kaspazės-3 aktyvumo ir aneksino-5 metodais, duomenys rodo, kad terapinės imatinibo mezilato koncentracijos neinicijavo apoptozės endotelio ląstelėse *in vitro*. Iš MTT eksperimentų ir augimo kreivių duomenų matyti, kad terapinės imatinibo mezilato koncentracijos neturėjo neigiamo poveikio endotelio metabolizmo aktyvumui, bei proliferacijai *in vitro*. Polimerazės grandžių reakcijos eksperimentų duomenys rodo, kad imatinibo mezilatas nesukelia endotelio aktyvacijos ar pažeidimo žymenų sintezės. Iš TUNEL tyrimo duomenų matyti, jog imatinibo mezilatas nesukelia endotelio ląstelių apoptozės eksperimentinės Bleomicino sukeltos odos fibrozės modelyje *in vivo*.

Galima daryti išvadą, kad terapinės imatinibo mezilato koncentracijos neturėjo poveikio endotelio ląstelių gyvybingumui, aktyvacijos markerių ekspresijai *in vitro* ir neinicijavo apoptozės tiek in vitro, tiek *in vivo.*

Imatinibo mezilato įtaka angiogenezės etapams

Inkubuojant endotelio ląsteles su imatinibo mezilatu iki 96h ląstelių proliferacijos inhibicija nestebėta. Iš transšulinėlinio ir „brėžio“ eksperimentų *in vitro* matyti, jog imatinibo mezilatas neslopina endotelio ląstelių migracijos ar chemotaksio. Kapiliarų morfogenezės eksperimento duomenys rodo jog imatinibo mezilatas neturėjo neigiamos įtakos endotelio tubulių formavimuisi.

Visi šie duomenys rodo, kad endotelio ląstelių inkubacija su terapinėmis imatinibo koncentracijomis neturėjo neigiamo poveikio pagrindiniams angiogenezės etapams: proliferacijai, migracijai, kapiliarų formacijai.

Išvados

1. Imatinibo mezilatas ne tik sustabdė fibrozės vystymąsi uždegiminiame, Bleomicino sukeltos *suformuotos* odos fibrozės modelyje, bet ir paskatino jau egzistuojančios fibrozės regresiją.
2. Veikimas imatinibo mezilatu visiškai normalizavo odos ir poodžio storį, bei miofibroblastų skaičių neuždegiminiame, fibrilino-1 mutaciją turinčių pelių, sisteminės sklerozės modelyje.
3. Terapinės imatinibo mezilato koncentracijos neturėjo poveikio endotelio ląstelių gyvybingumui, aktyvacijos markerių ekspresijai ir neinicijavo apoptozės in vitro
4. Imatinibo mezilatas neinicijavo endotelio ląstelių apoptozės bleomicino sukeltos odos fibrozės modelyje in vivo.
5. Endotelio ląstelių inkubacija su terapinėmis imatinibo koncentracijomis neturėjo neigiamo poveikio pagrindiniams angiogenezės etapams: proliferacijai, migracijai, kapiliarų formacijai.

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Educational background

2009	Qualification (license) of rheumatologist acquired
2005 – 2009	Residentship in rheumatology Vilnius University medical faculty
2005	Qualification (license) of medical doctor acquired
2004 – 2005	Internship in Vilnius University medical faculty
1998 – 2004	Studies at Vilnius University medical faculty

Scientific background

2008 – Now	PhD fellow at Vilnius University (Lithuania)
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Professional background

2010	Rheumatologist at Vilnius University, Santariskiu Clinics (Lithuania)
2009 – 2010	Medical doctor at Kong Christian X's Gigthospital (Denmark).
2005 – 2009	Resident in rheumatology at Vilnius University Santariskiu clinics
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Practical skills Capillaroscopy, joint injection, ultrasonography, DEXA scanning

Language skills Lithuanian – native; Danish, Russian, English – fluent; German – basic