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Chapter 8

Animal Models of Systemic Sclerosis

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Abstract

Systemic sclerosis (SSc) is a rare, chronic connective tissue disease affecting the skin, vessels, musculoskeletal system, and internal organs. Despite advances in pharmacotherapy of organ manifestations and new knowledge about the pathogenesis of SSc, there is still no effective universal treatment of this serious disease. The aim of this chapter is to introduce traditional, most commonly used experimental animal models of SSc, clarify their basic pathological mechanism, describe their advantages and limitations, and outline their use in preclinical tests of potential therapeutic agents with subsequent clinical trials in patients with SSc. The existing models have already contributed significantly to preclinical testing of several available biological agents and small molecules, some of which achieved promising results in early clinical studies, and could provide better prospects for patients with this incurable disease.

Keywords: systemic sclerosis, experimental models, biological therapy, small molecules

1. Introduction

Systemic sclerosis (SSc) or scleroderma is a rare, chronic connective tissue disease affecting the skin, vessels, musculoskeletal system, and internal organs. The name of this disease is derived from the Greek words “scleros” and “derma” meaning tough skin and was first used by Gintrac in 1847. The first mention of a stiff skin comes from Hippocrates around 400 BC. SSc affects women more often, usually begins in their 40s, and overall survival is shorter (10-year survival of around 70%). Despite advances in pharmacotherapy of organ manifestations and new knowledge about the pathogenesis of SSc, there is still no effective treatment of this serious disease [1]. The etiology of SSc is still unclear, although there are long known associations between some external factors (mainly silicon compounds and organic solvents) and the development of SSc.

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The possibility of biopsy sampling of affected tissues in patients with SSc, in particular as a skin biopsy, helped to a large degree to elucidate the pathogenesis of this disease. Histological analysis of biopsy samples of tissues in different stages of the disease has identified three basic pathological processes and their relative time sequence: vasculopathy, inflammation, and fibrosis. The first pathological changes can be detected at the level of microcirculation, in which damage to the endothelium leads to progressive development of inflammation caused by the activation of cells of both the innate and, subsequently, acquired immunity. Activated cells produce pro-fibrotic cytokines, especially transforming growth factor beta (TGF-β), connective tissue growth factor (CTGF), and platelet-derived growth factor (PDGF). These cytokines activate resident fibroblasts, which increase production of extracellular matrix (ECM) components that lead to remodeling of a functional tissue to a fibrotic one. The fibrotic phase of the pathogenesis of SSc and its severity and extent determine the morbidity and mortality of this disease [2]. The material from skin biopsies has also enabled the analysis of gene and protein expression, epigenetic modifications, and signaling pathways of different target molecules. The potential importance of these candidate molecules must always be first tested and subsequently confirmed, pending their therapeutic use in SSc. For these purposes, we may use in vitro experiments on tissue or cell cultures isolated from tissues explanted from SSc patients and healthy individuals or in vivo experiments using experimental animal models of SSc [3].

Today, there are numerous animal models of SSc. However, none of the available models mimics the full range of pathologies and clinical manifestations of SSc. Experimental animal models help clarify certain pathological mechanisms and only mimic some aspects of SSc. Each model has its advantages and disadvantages. Selection of an appropriate animal model for the analysis of target genes or candidate molecules for therapy must be carefully considered beforehand [4]. Since the skin fibrosis is a dominant and common feature in most patients with SSc, this pathological process is not only a starting point but also the key aspect for the majority of experimental models. With the progress in scientific knowledge and technologies, new models were developed with other dominant features such as activation of immunity and inflammation, vasculopathy, and specific pathological processes in the involved organs, especially the lungs. However, most of available models provide an overlap of the abovementioned pathological mechanisms and a concurrent involvement of several organs characteristic of SSc.

The aim of this chapter is to introduce traditional, most commonly used experimental animal models of SSc, clarify their basic pathological mechanism, describe the advantages and limitations, and outline their use in preclinical tests of potential therapeutic agents with subsequent clinical trials in patients with SSc.

2. Experimental murine models of fibrosis

2.1. Tight skin 1 (Tsk1) mice

Tsk1 mice possess an autosomal dominant mutation, tandem duplication in the gene for fibrillin 1, which is an important regulator of the TGF-β signaling and fibrogenesis. In heterozygous

mice (labeled Tsk1), this mutation leads to hyperplasia and thickening of the subcutaneous tissue and fascial layers of the skin without striking thickening of the dermis itself (**Figure 1**). Similarly, hyperplasia of subcutaneous fascia and lipoatrophy can also be detected in the skin of patients with SSc. Tsk1 mice also produce autoantibodies (anti-topoisomerase I, anti-RNA polymerase I, anti-dsDNA) [5–9]. Fibrosis in these mice develops from excessive production of ECM by activated fibroblasts during the activation of TGF-β pathway. The exact mechanism has not yet been elucidated. The progression of fibrosis in Tsk1 mice is mediated by an increased expression of CD19, chronic B-cell activation leading to increased secretion of IL-6, and mast cells. These mechanisms are also applied in the progression of SSc in humans. Tsk1 mice are commonly used as a model mimicking later stages of SSc that are independent of inflammatory infiltrates [10].

The advantage of this model is a detailed documentation and endogenous activation of fibroblasts similar to SSc in humans. The limitations to this model include insufficiently elucidated molecular mechanisms, the absence of vascular phenotype, dominant histological changes in the hypodermis, and some other changes, such as emphysema and kyphosis, which are not part of SSc in humans [4].

2.2. Tight skin 2 (Tsk2) mice

In 1986, a tight skin 2 (Tsk2) mouse model was first described, which is induced by a chemical compound ethylnitrosourea. This compound induces an autosomal dominant mutation localized on the chromosome 1. Only heterozygous individuals (Tsk2/+) survive [11]. The development of phenotypic features of this model occurs mainly between the third and fourth weeks after birth. The gene responsible for the mutation has not yet been precisely identified [12]. However, using in vivo and in vitro genetic tests, previous studies have demonstrated a significant role of missense mutations in the gene for type III collagen alpha 1 (Col3a1) [13]. The Tsk2/+ mice showed elevated levels of type III collagen and changes in the ECM in the

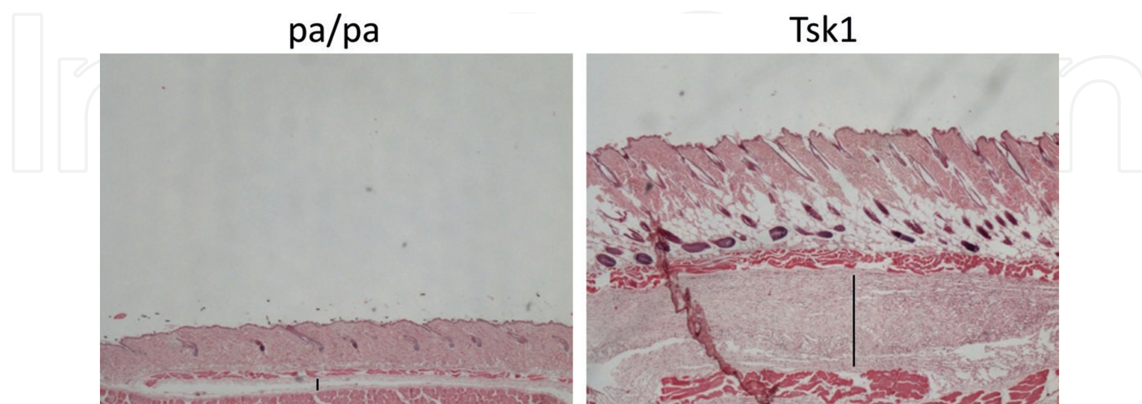

Figure 1. Hyperplasia and thickening of the subcutaneous tissue and fascial layers of the skin without striking thickening of the dermis in Tsk1 mice compared to the wild-type littermates (pa/pa). The sections are stained with hematoxylin-eosin (magnification 20-fold; the black vertical bar represents the thickness of the hypodermis).

dermis, especially during 10 days after birth, which were still prevalent in seventh to eighth month compared with the wild-type littermates. Furthermore, the Tsk2/+ mice demonstrated the presence of mononuclear inflammatory infiltrates [11]. Cultured fibroblasts isolated from Tsk2 mice showed elevated mRNA expression of Col1a1 and Col3a1 gene and overproduction of collagen fibers [14]. Compared with Tsk1 model, the Tsk2 mice are characterized by abundant mononuclear cells infiltrating the dermis and adipose tissue. These mice have excessive thickening of adventitia of the vessels in the heart and lungs along with changes in the alveolar compartment [14, 15].

These findings indicate a significant use of these models for research of fibrotic processes, such as SSc in humans, as this disease is also characterized by an increased expression and accumulation of collagen in dermal fibroblasts [16, 17]. The Tsk2 mouse model has a large number of very similar clinical manifestations to those seen in SSc patients, including skin thickening, increased production of collagen and ECM, and autoimmune responses [11, 18].

2.3. Deletion of the kinase domain of the type II TGF-β receptor in murine fibroblasts (TβRIIΔk-fib)

An experimental mouse model for deactivating the TGF-β signaling has been developed, in which the fibroblasts express a non-signaling mutant type II TGF-β receptor that lacks the intracellular kinase domain (TβRIIΔk) [19]. In in vitro experiments, TβRIIΔk was demonstrated to have inhibitory properties on TGF-β signaling and was characterized as a competitive antagonist of TGF-β1 [20]. TGF-β signaling is mediated through type I and type II TGF-β receptors (TβRI and TβRII), which have a serine/threonine kinase activity and trigger subsequent Smad-dependent or Smad-nondependent signaling. Binding of TGF-β1 to TβRII leads to subsequent phosphorylation of TβRI, which then mediates the activation of downstream signaling pathways [21]. Authors of this model hypothesized that only fibroblasts expressing TβRIIΔk would have distorted TGF-β signaling leading to suppression of genes regulated by this pathway and thus achieve reduced pro-fibrotic effects of TGF-β signaling in these fibroblasts. In in vivo experiments, even though fibroblasts cultured from mouse models expressing TβRIIΔk exhibited resistance to exogenous TGF-β, the TβRIIΔk mice paradoxically and surprisingly developed progressive cutaneous, intestinal, and pulmonary fibrosis [19, 22]. The TβRIIΔk model showed phenotypic properties resembling the properties of the activated TGF-β pathway. Fibrosis was demonstrated in both sexes. From the sixth week after birth, TβRIIΔk mice experienced weight loss and developed pathological changes in the lung, in terms of reduced lung capacity and increased presence of connective tissue and ECM components, compared to wild-type littermates [19]. In 10% of these animals, the acquired damage led to death of an adult mouse around the 16th week of age. At week 12, abnormal thickening of the dermis with the loss of subcutaneous adipose tissue can be demonstrated, which can be especially evident in the lower back of these mice. Furthermore, the TβRIIΔk mice are also characterized by elevated levels of collagen.

This model is used not only for understanding the regulatory effects of non-signaling TGF-β receptors and constitutive activation of TGF-β signaling in vivo but also as a model for genetically determined fibrosis [19].

2.4. Spontaneous age-related organ fibrosis in sirtuin 3-deficient mice

Sirtuin 3 (SIRT3) belongs to the large family of class III histone deacetylases, which alone requires nicotinamide adenine dinucleotide (NAD^+) as a cofactor for its proper enzymatic activity [23]. In mammals, there are seven isoforms/classes of sirtuins (SIRT1-7), which differ in specific binding substrates, different biological functions, and various locations in the cell [24]. SIRT3 is found primarily in the mitochondria, and elevated levels were detected during reduced food intake, so-called caloric restriction, and endurance sports [25]. Proper function of SIRT3 in murine models prevents the development of a number of diseases such as cancer, metabolic syndrome, etc. [26, 27]. Experimental mice deficient for SIRT3 (Sirt3-KO) develop cardiac hypertrophy and contractile dysfunction in adulthood [28]. An association between SIRT3 deficiency and aging was analyzed initially on the development of cardiac fibrosis in three age categories of Sirt3-KO mice. Results showed aggravation of fibrotic cardiac impairment depending on higher age of Sirt3-KO mice. Furthermore, formation of tissue fibrosis in a number of other organs again depending on the age was demonstrated in the Sirt3-KO mice. The older the Sirt3-KO mouse, the more affected organs were observed, including the lung, kidney, and liver, in contrast to the corresponding age-matched control group [29]. Mice that lack or have reduced expression of SIRT3 also develop pulmonary arterial hypertension. Nevertheless, malfunction of SIRT3 is not accompanied by the presence of inflammatory infiltrates, even though mitochondrial damage and the presence of oxidative stress have been detected [30]. Studies on SIRT3 also showed that increased expression of SIRT3 prevents the development of experimentally induced organ fibrosis and promotes the essential role of SIRT3 in maintaining cellular homeostasis of tissues during aging [29, 31].

The model of SIRT3-deficient mice allows for deeper examination of cellular mechanisms involved in the development of organ fibrosis and pulmonary arterial hypertension (PAH) in the absence of inflammatory infiltrates, depending on the age of the mouse.

2.5. Vinyl chloride

Vinyl chloride is a colorless, toxic gas of sweet fragrance, which is an important component in the production of the polymer polyvinyl chloride (PVC). Some individuals, who are exposed to repeated doses of vinyl chloride, develop cutaneous and pulmonary fibrosis. The development of fibrosis is often preceded by the Raynaud's phenomenon [32]. Animal models, particularly mice and rats that are exposed to vinyl chloride, develop the same disease as humans. Injections of vinyl chloride to BALB/cJ retired breeder mice lead to the development of cutaneous fibrosis and dermal inflammation with a substantial presence of mononuclear infiltrates similar to SSc in humans [33].

2.6. $TBRI^{CA}$; Cre-ER mice

In order to study a variety of genes in in vivo environment with activated TGF-β signaling, which is a central pro-fibrotic cytokine in the pathogenesis of SSc, the $TBRI^{CA}$ mouse model was developed with the mutated form of the TGF-β type I receptor ($TBRI^{CA}$) in fibroblasts, which leads to its sustained activation independent of the ligand TGF-β. Specific expression in fibroblasts was achieved using a transcription enhancer of proα2(I) collagen gene, which

directs the expression exclusively in fibroblasts [34–36]. The attempt to create transgenic mice despite the specific expression of this mutation only in the fibroblasts was not successful, and the mice died during the gestation. Thus, a postnatally induced sustained activation of TBRI using tamoxifen inducible Cre/loxP system was introduced, which leads to progressive generalized dermal fibrosis with overproduction of type I and type III collagen and adnexal atrophy [35]. This model is also characterized by thickening of the walls of the small arteries of the lungs, kidneys, and adrenal glands, as well as the pulmonary artery. Technical and economic demands of this model led to the introduction of alternative solution, in which local skin fibrosis is achieved by subcutaneous injection with a weakened adenovirus with sustained activation of TBRI, which is no longer limited to fibroblasts [35].

The advantages include a well-described pathological mechanism and a possibility of a detailed in vivo analysis of TGF-β signaling in fibroblasts. The limitations comprise only a minimal representation of inflammation and autoimmunity [4].

3. Experimental murine models mimicking immunologic aspects of SSc

3.1. Bleomycin-induced skin fibrosis

This model is a widely used model mimicking the in vivo inflammatory changes present in the early stage of SSc. Bleomycin was originally isolated from *Streptomyces verticillus* and is used in the treatment of various types of tumors [37]. High doses of bleomycin used in cancer therapy have well-known side effects. They cause lung disease, fibrosis, and even SSc-like skin alterations. Therefore, bleomycin is used in murine models of both lung and skin fibroses. Repeated subcutaneous injections in the defined area for 4 weeks lead to dermal fibrosis, which persists even 6 weeks after their administration (**Figure 2**) [38]. Apart from the development of inflammatory infiltrates and fibrosis in the affected skin, there is also a mild lung involvement with fibrosis. Furthermore, this model is characterized by the presence of antinuclear antibodies (ANA), anti-topoisomerase-I, anti-U1 RNP, and anti-histone antibodies, suggesting

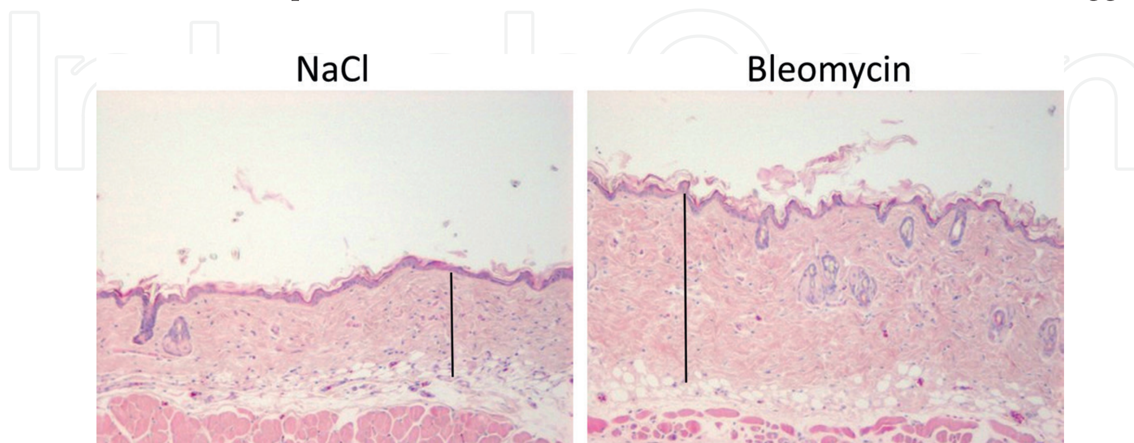

Figure 2. Fibrosis and inflammatory infiltrates in the dermis of mice locally injected with bleomycin compared to control mice injected with saline (NaCl). The sections are stained with hematoxylin-eosin (magnification 100-fold; the black vertical bar represents the thickness of the dermis).

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for systemic involvement [39]. Bleomycin injections in the skin induce production of reactive oxygen species, resulting in impairment of endothelial cells and other cell types, and expression of adhesion molecules. This results in increased infiltration of the affected skin with polymorphonuclear cells, T and B lymphocytes, macrophages, eosinophils, and mast cells. Inflammatory infiltrates release increased amounts of pro-fibrotic and pro-inflammatory cytokines (TGF-β, PDGF, MCP-1, IL-4, IL-6, and IL-13), which activate the resident fibroblasts to produce excessive amounts of ECM components leading to cutaneous fibrosis [40].

The model of bleomycin-induced dermal fibrosis is very well documented and described, can be applied to numerous strains of mice, and is easy to use. A limitation of this model is that it is artificially induced, it is not associated with significant systemic involvement, and it tends to overestimate anti-fibrotic effect of anti-inflammatory agents [4].

3.2. Model of chronic sclerodermatous graft-versus-host diseases (SclGvHD)

Chronic graft-versus-host disease (GvHD) occurs in 40–60% of long-term survival patients following hematopoietic cell transplantation. Sclerodermatous (SclGvHD) and cytotoxic GvHD types are the two main subtypes of chronic GvHD [41]. SclGvHD is the fibrosing type, and its clinical manifestations are similar to the symptoms of the early inflammatory phase of diffuse cutaneous SSc. Induction of SclGvHD in mice is carried out in (a) a standard manner, when hematopoietic cells are transplanted into sublethally irradiated BALB/c mice or (b) a modified method, when hematopoietic cells are transplanted into immunodeficient recombinase-activating gene-2 (RAG-2) mice [42]. In both cases, SclGvHD is induced by a transplantation of hematopoietic cells of a donor to a recipient with identical major histocompatibility complex (MHC) but with different minor histocompatibility antigens. After reconstitution of hematopoiesis, SclGvHD is induced in the recipient with inflammation and fibrosis of the skin, lung, liver, kidney, gastrointestinal tract, and parotid gland. Besides inflammation and fibrosis, this model is also characterized by production of autoantibodies against nuclear antigens. In the pathogenesis of chronic SclGvHD, alloreactive CD4 T lymphocytes play a key role [43–47].

The advantage of this model is again a good documentation and systemic manifestations. The disadvantage is the need for sophisticated techniques and experience in dealing with problematic immunocompromised mice [4].

3.3. MRL/lpr murine model with deficient interferon gamma (IFN-γ) receptor (MRL/lpγR−/−)

For understanding and examining immunological aspects of SSc, a mouse model was developed, based on the murine model of MRL/lpr, which primarily mimics the manifestations of systemic lupus erythematosus (SLE) and other pathological mechanisms of other systemic autoimmune diseases. The MRL/lpr mice are characterized by development of inflammatory involvement of tissues, such as immune complex-mediated vasculitis, arthritis, skin disease, and glomerulonephritis, which are accompanied by the production of autoantibodies [48]. On a molecular level, this model is based on a mutation in the gene encoding the Fas receptor belonging to a tumor necrosis factor (TNF) receptor family [49]. However, to induce

symptoms of SSc in this model, a subsequent deletion of the interferon gamma (IFN-γ) receptor was introduced. MRL/lpr mice lacking IFN-γ receptor (MRL/lpγR−/−) are protected from the development of glomerulonephritis, which is often the cause of death of MRL/lpr mice. In fact, previous studies demonstrated the essential role of functional IFN-γ receptor for the development of glomerulonephritis in MRL/lpr mice [50]. In MRL/lpγR−/− mice, there is a number of pathological processes and clinical manifestations characteristic of SSc in humans. This mouse model (MRL/lpγR−/−) promotes the development of proliferative vasculopathy, particularly in the lung, and fibrosis in many organs including the skin, lungs, and kidneys [51]. Furthermore, this model is characterized by the presence of autoantibodies and mononuclear infiltrates in the skin, lungs, liver, and heart, accompanied by increased accumulation of collagen. Increased activation of fibroblasts was also observed in MRL/lpγR−/− mice [51].

This mouse model thus mimics the most typical symptoms for SSc, such as vasculopathy, inflammation, and the presence of autoimmunity.

3.4. Fra-2 transgenic mice

Fra-2 is a member of a family of transcription factors called activator protein 1 (AP-1). AP-1 is a heterodimer composed of protein subunits belonging to the family of Fos (c-Fos, Fra-1, Fra-2, FosB) and Jun family (c-Jun, JunB, JunD), which regulate cell proliferation, inflammation, and wound healing via binding of AP-1 to the promoters of target genes [52]. Recent studies have reported a significant role of two members of the AP-1 family, Fra-2, and c-Jun on production of TGF-β and on its autocrine signaling in SSc fibroblasts [53, 54]. Overexpression of Fra-2 was also reported in patients with SSc, specifically in myofibroblasts, endothelial cells, and smooth muscle cells, suggesting a potential importance of Fra-2 in the pathogenesis of SSc [54, 55]. Increased expression of Fra-2 in mice leads to the development of systemic inflammation and fibrosis, especially in the skin and lungs. Fra-2 transgenic mice are also characterized by a vascular phenotype with increased apoptosis of endothelial cells of the capillaries, which appear in the ninth week after birth and lead to serious dysfunction of the small arteries of the skin and lungs. Subsequently, at 12 weeks of age, there is a significant development of dermal fibrosis, which correlates with progressive capillary loss [55]. Fra-2 is also highly expressed in the skin tissue of animal models of SSc. High expression of Fra-2 was detected in myofibroblasts in SSc skin lesions, suggesting a specific role of Fra-2 in fibroblast activation and subsequent transdifferentiation into myofibroblasts. Furthermore, it was demonstrated that silencing of Fra-2 gene leads to reduced production of type I collagen and inhibition of apoptosis but also to the development of angiogenesis in human microvascular endothelial cells [54, 55]. Histological changes of proliferative vasculopathy in the lungs of mice resemble pulmonary arterial hypertension in SSc in humans. Lung fibrosis and pulmonary arterial hypertension then lead to pulmonary insufficiency and increased mortality of mice [54, 55]. Pathological processes that are shared between pulmonary vascular involvement in Fra-2 transgenic mice and SSc in humans are further documented by constitutively activated PDGF signaling in murine pulmonary arteries. The key role of this pathway has been demonstrated by the administration of nilotinib, a tyrosine kinase inhibitor of the PDGF receptor, which prevents the development of proliferative vasculopathy and pulmonary fibrosis in Fra-2 transgenic mice [55].

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The advantage of this model is the integration of characteristic vascular and fibrotic manifestations and a similar course of the disease as of SSc in humans. The disadvantages of Fra-2 transgenic mice comprise inadequate characterization of the model and the absence of autoimmunity [4].

3.5. uPAR−/− mice

Urokinase receptor belongs to glycoproteins anchored in the cell membrane via glycosylphosphatidylinositol (GPI) anchor, which is expressed on the surface of fibroblasts, endothelial cells, and lymphohematopoietic cells. The main task of the urokinase-type plasminogen activator receptor (uPAR) is binding the ligand urokinase-type plasminogen activator (uPA) at the interface of cells and matrix. uPA is an important part in the conversion of plasminogen to plasmin and activation of growth factors and matrix metalloproteinases. uPA/uPAR system plays a significant role in fibrinolysis, maintaining cellular homeostasis and angiogenesis. It also participates in many biological processes including differentiation, proliferation, and migration of cells through its interaction with membrane proteins and components of the ECM [56]. Relationship between SSc and uPAR was studied in dermal fibroblasts and endothelial cells obtained from SSc skin lesions, where a decrease in uPAR was detected compared to healthy control cells [57]. Recent studies have demonstrated the effect of inactivation of uPA/uPAR on transdifferentiation of fibroblasts into myofibroblasts and structural and functional changes in vasculature in SSc [58, 59]. uPAR−/− mice mimic fibrotic and vascular manifestations of SSc, which supports a significant role of the uPA/uPAR in the pathogenesis of SSc. In the 12th week, mice with inactivated uPA/uPAR system (uPAR−/−) develop progressive thickening of the skin with increased amount of collagen fibers and the number of activated fibroblasts, as well as the increased presence of perivascular inflammatory infiltrate compared to wild-type littermates. At the same time, fibrosis and perivascular fibrosis in the subcutaneous tissue develop [57]. In the skin of uPAR−/− mice, elevated levels of pro-fibrotic mediators, such as TGF-β and CTGF, were detected. Similar to Fra-2 murine models, uPAR−/− mice have an increased number of apoptotic endothelial cells, reduced number of functional blood vessels, and subsequent development of fibrosis, but do not develop fibroproliferative changes in arteries. During the 24th week, the skin changes stabilize and do not deteriorate [57]. The lung tissue is characterized by interstitial damage, infiltration of inflammatory cells, and excessive deposition of collagen, which is similar to the involvement in SSc patients with interstitial lung disease. Pulmonary involvement is already evident at 12 weeks, with progressive tendencies until the 24th week. This model is also characterized by cardiac involvement, which is also typical for SSc-related cardiomyopathy. This involvement is characterized by damage to cardiomyocytes, differentiation of myofibroblasts, apoptosis of endothelial cells, collagen accumulation, and myocardial fibrosis. However, cardiac involvement occurs later compared to the aforementioned manifestations [57].

Murine models with inactivated uPAR may be used as another preclinical model that mimics vascular changes, fibrosis of tissues similar to those in SSc but again lacks immunological processes typical of this disease [57].

4. Experimental murine models of lung fibrosis

In pulmonary fibrosis, the original functional lung tissue is being replaced by connective tissue which deteriorates the exchange of respiratory gases. The pathogenesis is characterized by damage to epithelial cells and alveolar hyperplasia, accumulation of inflammatory infiltrates, fibroblast hyperplasia, deposition of ECM components, and scarring [60]. Interstitial lung disease in SSc is most commonly characterized by nonspecific interstitial pneumonia (NSIP) or usual interstitial pneumonia (UIP) or organizing pneumonia with later development into NSIP [2]. The most frequently used experimental models of pulmonary fibrosis, which mimic some aspects of the human disease, include induction by bleomycin, silica, fluorescein isothiocyanate, and radiation.

4.1. Bleomycin-induced lung fibrosis

To induce pulmonary fibrosis, bleomycin can be administered intratracheally or intranasally directly into the airways but also by subcutaneous, intraperitoneal, or intravenous injection. The principle of action of bleomycin is described in bleomycin-induced skin fibrosis [61]. The main advantage of the use of bleomycin is its availability, ease of administration, and yet the most accurate induction of symptoms of pulmonary fibrosis within 14–28 days (**Figure 3**). A limitation of this model is the fact that lung fibrosis wanes 28 days after intratracheal administration of bleomycin [61]. Many studies have confirmed that bleomycin-induced experimental pulmonary fibrosis is reversible, unlike pulmonary fibrosis in humans, and after 6 weeks, the animal presents with almost normal lung findings [62].

4.2. Lung fibrosis induced by silica

The instillation of silica into the lungs of mice causes the formation of fibrotic nodes and fibrosis, similar to lesions in humans exposed to long-term inhalation of silica dust and aerosol particles. Silica may be administered via an aerosol or intratracheally via oropharyngeal aspiration [60]. Induction of lung fibrosis is based on the activation of macrophages that phagocytose silica particles and begin to produce the pro-fibrotic cytokines PDGF and

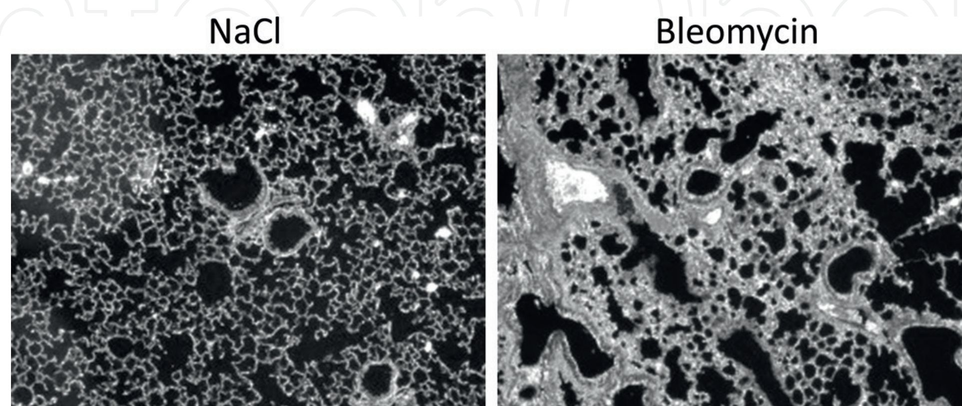

Figure 3. Fibrotic lung tissue (grey colour) of mice after intratracheal administration of bleomycin compared to control mice after intratracheal instillation of saline (NaCl). The sections are stained with sirius red (magnification 100-fold).

TGF-β [61]. The fibrotic response is dependent on the particular strain of mice [63]. The main benefit of this model is that the silica particles are not easily removed from the lungs and cause permanent fibrotic stimuli. The disadvantage of this model is that it lacks the characteristic signs of UIP. The experiment is also expensive and time-consuming due to the need for highly specialized equipment for administration of silica via aerosol and due to the development of the disease only during the 12th to 16th week after exposure [61].

4.3. Lung fibrosis induced by fluorescein isothiocyanate (FITC)

Other chemicals used to induce pulmonary fibrosis include fluorescein isothiocyanate (FITC). FITC is administered directly into the respiratory tract, where it acts as a hapten that binds to the protein present in the lungs, resulting in a new persistent antigen and the subsequent formation of antibodies [61]. This association is used in immunofluorescence for localization of pulmonary fibrosis, which correlates closely with the areas of occurrence of the bound FITC. Administration of FITC increases infiltration of mononuclear cells and neutrophils, in particular in the area of the respiratory tract. In terms of pathogenesis, FITC is linked with the occurrence of acute lung injury, the development of edema, inflammation, and the subsequent development of fibrosis [63]. Induction of lung fibrosis by FITC is based on activation of chemokine receptor type 2 (CCR2) signaling [64]. Release of chemokines CCL12, and to a lesser extent CCL2, causes an increase in the number of fibrocytes expressing CCR2 in the affected lung, which leads to pulmonary fibrosis [65, 66]. FITC further induces increased production of pro-fibrotic cytokine IL-13 [67]. The advantage of this model is the visualization of fibrosis using the characteristic green fluorescence. Pulmonary involvement occurs within 14–28 days and persists for at least 6 months. Unfortunately, this model of pulmonary fibrosis has no characteristic findings of UIP [60].

4.4. Radiation-induced lung fibrosis

Irradiation of mice also leads to the formation of pulmonary fibrosis, without the use of chemicals. Radiation induces direct cell death of type I and type II pneumocytes and the subsequent accumulation of macrophages at the site of damage. Macrophage activation triggers the production of pro-inflammatory and pro-fibrotic cytokines tumor necrosis factor alpha (TNF-α) and TGF-β, which participate in the formation of fibrosis [61]. Radiation-induced fibrosis has findings consistent with UIP and can only be performed in the C57B1/6 mouse model. The disadvantages of this method are the financial and time demands, since the interval from exposure to the development of the first symptoms is 30 weeks [60].

5. Experimental murine models of pulmonary arterial hypertension

Pulmonary arterial hypertension (PAH) is a disease of pulmonary arteries caused by contraction and proliferation of smooth muscle cells of the vascular wall, which may occur alone (primary PAH) or accompany interstitial lung disease (secondary PAH) in SSc [2]. The most frequently used experimental models of PAH include a model induced by semaxanib with chronic hypoxia and monocrotaline and a model of athymic rats.

5.1. PAH induced by chronic hypoxia

Chronic hypoxia, normally induced by the half fraction of inspired oxygen (i.e., FiO_2 10%) for 21 days, leads, unfortunately, only to slight PAH, which is also reversible, unlike PAH in humans, and less pronounced in mice than rats. Furthermore, in mice hypoxia induces perivascular inflammatory infiltrates with the secretion of various pro-inflammatory cytokines and chemokines. Induction of more pronounced and irreversible PAH is achieved by the simultaneous administration of semaxanib (SU5416), a tyrosine kinase inhibitor of the type 1 receptor (Flt) and type 2 receptor (KDR) of vascular endothelial growth factor (VEGF), which was initially developed for cancer treatment [68]. SU5416 is an inhibitor of proliferation and triggers apoptosis of endothelial cells [69]. Paradoxically, however, blocking of VEGFR leads to an expansion of endothelial cells instead of inhibition of their proliferation. This is explained by a compensatory mechanism, when the inhibition of VEGFR is followed by upregulation of other growth factors such as fibroblast growth factor (FGF), and platelet-derived growth factor (PDGF) securing the proliferation of endothelial cells [69]. This model is frequently used for a sufficient understanding of the mechanism of hyperproliferation of endothelial cells, which is a characteristic of plexiform lesions of PAH in humans [70].

5.2. PAH induced by monocrotaline

Monocrotaline (MCTP) is a toxic alkaloid found in the plant *Crotalaria spectabilis*. Monocrotaline must be activated in vivo by liver oxidases to a reactive bifunctional MCTP component that contributes to the development of vascular disease. The response to MCTP depends on the species of animal, since it differs in hepatic metabolism of cytochrome P-450. The preferred animal models include rats, to which MCTP is injected either subcutaneously or intraperitoneally [71]. Subcutaneous injection of MCTP induces changes in the endothelium and smooth muscle cell proliferation in the blood vessels, which are involved in the development of PAH during 3 weeks [72]. The exact mechanism of pathogenesis of PAH induced by MCTP is still not fully understood. A possible cause could include the damage to the endothelium, which triggers rampant progression of severe pulmonary hypertension. Increased pulmonary artery pressure and vascular remodeling causing accumulation of mononuclear inflammatory cells in the tunica adventitia may represent another stimulus to the formation of PAH. This model is used mainly for modeling acute involvement which is easily treatable, that is, unfortunately, completely different from PAH in humans [71].

5.3. PAH in athymic rats

Another commonly used model of PAH is SU5416 treatment of athymic rats. The initial assumption was that the models without T lymphocytes (athymic naked rnu/rnu rats) will exhibit less inflammation with less pronounced PAH. There was, however, paradoxically exactly the opposite, and these models have developed a far higher degree of involvement. Inflammation of the pulmonary artery is induced by activation of macrophages and B cells and the presence of anti-endothelial antibodies. The cause of the development of severe forms of pulmonary hypertension is the absence of anti-inflammatory regulatory T cells, which explains a predisposition for PAH in athymic rats [69].

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6. Experimental chicken models of SSc (UCD-200 and UCD-206 chickens)

Chickens of the line UCD-200 were originally isolated from the University of California Leghorn chickens, which had atrophic abnormalities of the comb. This line has systemic manifestations similar to the symptoms of SSc: skin involvement of the comb, neck, and back manifested as edema, Raynaud's phenomenon-like changes, loss of skin adnexa, and skin thickening leading to necrosis [73]. This involvement develops between the first and second weeks after birth and culminates around the second to fourth week of life. Ischemic finger lesions occur in 20% of chickens, and digital ulcerations develop in later stages. There is also an internal organ involvement, particularly of the esophagus, heart, lung, and kidneys and an increased mortality. At around sixth week of age, the chickens develop glomerulonephritis and pericarditis [73, 74]. Histopathological changes are similar to those in SSc and include massive perivascular mononuclear infiltrates, particularly of T and B lymphocytes and deposition of ECM components by activated fibroblasts, signs of obliterative vasculopathy, and apoptosis of endothelial cells with loss of functional capillaries [73]. Subcutaneous adipose tissue is often replaced by collagen. UCD-200 chickens also produce autoantibodies, especially antinuclear, anti-centromere, and anti-phospholipid antibodies, rheumatoid factor, and antibodies to endothelial cells [74, 75]. UCD-206 chickens are similar to the chickens of the line UCD-200. However, they can develop more serious organ involvement [76].

The advantage of this model is the significant systemic involvement covering almost the complete spectrum of pathological processes and the disease course, which is similar to SSc in humans. While this model of SSc affects both sexes equally, in patients with SSc, women are affected more often. Another difference is the composition of mononuclear infiltrates,which consist, in particular, of monocytes/macrophages and T lymphocytes in humans [77]. The limitations of this model include the avian genetic background, which restricts the molecular studies, and, in particular, a very difficult and costly breeding [4].

7. Testing of biological agents and small molecules in preclinical models and clinical setting

One of the first available biological drugs with proven effects in other systemic rheumatic diseases, which were tested in preclinical models of SSc, was the anti-TNF agents. In in vitro experiments, a direct anti-fibrotic effect of TNF-α itself prevailed, while in in vivo experiments, on the contrary, using the TNF inhibitors prevented the development of bleomycin-induced fibrosis, which may be partly explained by the dominant inflammatory component in the model [78, 79]. Results of clinical trials of anti-TNF agents in SSc rather support the anti-fibrotic role of TNF-α itself, judging from progression of fibrosis in anti-TNF-treated SSc patients [80, 81]. Also other clinical trials failed to show significant improvement in skin scores and lung function using etanercept and infliximab, and, thus, the EUSTAR workgroup (EULAR Scleroderma Trials and Research) did not recommend the use of anti-TNF therapy in patients with SSc outside clinical trials [82–84].

The role of B lymphocytes and their depletion has also been studied in experimental models of SSc. Administration of rituximab to 3-day-old Tsk1 mice resulted in a reduction of skin fibrosis, whereas the same application to older, 56-day-old Tsk1 mice did not cause any change [85]. Similar reduction of the skin and pulmonary fibrosis induced by bleomycin was achieved in mice with genetic inactivation of CD19 [86]. Both studies document the effect of depletion of B cells on reduction of fibrosis rather in the early inflammatory phase of experimental SSc. The phase 2 clinical trial and observational study by EUSTAR documented the beneficial effects of rituximab on reduction of skin scores and lung function, but the results will need to be confirmed in a placebo-controlled randomized trial [87–92].

Another, in rheumatology, widely used type of therapy that has been tested in mouse models of SSc was the inhibition of interleukin (IL)-6. Numerous in vitro data demonstrated an increased production of IL-6 by SSc fibroblasts, and its important role as an inducer of activation of fibroblasts and collagen production, and ultimately point to the effective reduction of collagen synthesis through its inhibition [93–95]. In vivo inhibition of IL-6 with antibody against IL-6 receptor reduced the cutaneous fibrosis in a model of bleomycin-induced fibrosis and chronic GvHD, whereas in Tsk1 mice, no improvement was observed, which indicates rather anti-fibrotic effect in the early, inflammatory stage of experimental models of SSc [96–98]. The results of the phase 2/3 double-blind randomized placebo-controlled study faSScinate evaluating efficacy of tocilizumab in 87 patients with an active form of diffuse cutaneous SSc after 48 weeks of treatment showed a trend of reduction in skin scores ($p = 0.06$) and an encouraging stabilization of FVC (forced lung capacity) compared to placebo ($p = 0.09$) [99].

Tyrosine kinases such as the PDGF receptor (platelet-derived growth factor) constitute another interesting target of therapy in SSc. Numerous experimental studies demonstrate the anti-fibrotic effects of imatinib mesylate, a multikinase inhibitor (inhibitor of the kinases PDGFR, c-Abl, and c-Kit). In vitro, imatinib reduced the expression of pro-inflammatory and pro-fibrotic genes and collagen production by SSc fibroblasts [100, 101]. In vivo imatinib therapy prevented the development of cutaneous fibrosis induced by bleomycin and in Tsk1 mice and prevented the development of pulmonary, renal, and hepatic fibrosis, as well as reduced established dermal fibrosis [100, 102]. Similar results were obtained in vivo with other PDGFR inhibitors, such as nilotinib and dasatinib [103]. The first promising results from an open clinical trial with imatinib in SSc demonstrated significant improvement in skin scores and FVC after 12 months, however, with numerous side effects, particularly edema. Unfortunately, these findings were not confirmed in two other randomized placebo-controlled clinical trials, one of which was closed for numerous early side effects, and the other failed to show significant improvement in skin scores, and was accompanied by numerous side effects as well [104–106].

Another interesting target of anti-fibrotic therapies investigated in in vitro and in vivo experiments in SSc is a vasoactive small molecule riociguat, a stimulator of soluble guanylate cyclase (sGC). Treatment with riociguat led to a significant improvement in the primary outcome (6-min walking test) after 16 weeks of treatment of pulmonary arterial hypertension in the phase 3 PATENT-1 trial and also met a number of secondary outcomes [107]. In vitro experiments with riociguat resulted in a reduction of collagen synthesis by SSc fibroblasts

in a dose-dependent manner. In in vivo experiments, riociguat prevented the development of cutaneous fibrosis induced by bleomycin, in Tsk1 mice, in a TβRI mouse model, and in SclGvHD in which it even reduced the gastrointestinal fibrosis. In the bleomycin-induced skin fibrosis model, riociguat was also shown to reduce the established fibrosis [108–110]. Effectiveness of riociguat in skin and pulmonary involvement is currently being evaluated in an ongoing phase 2 placebo-controlled randomized clinical trial RISE in patients with early diffuse cutaneous SSc.

8. Conclusion

Currently, there are several well-characterized experimental models through which we can study various pathological processes of SSc. The knowledge gained through animal models of SSc may bring new information and clarify previously unexplained pathogenic mechanisms of SSc. Animal models also serve as a promising tool for developing and testing new candidate molecules for the treatment of SSc. It must be stressed that none of the currently available experimental models includes all aspects of SSc in humans. Most of established models represent several pathologic mechanisms at once. Almost all models, except for a few focused only on vasculopathy, are characterized by severe tissue fibrosis. Conversely, only a few models are acceptable for studying damage to small arteries. It is therefore very important to carefully consider the selection of a suitable model or a combination of several models before commencing specific in vivo experiments. When the aim is to completely analyze the pathogenesis of the disease, it is suitable to prioritize models that mimic a wide spectrum of pathologies of SSc, such as Fra-2 transgenic mice or chicken lines UCD-200/206. For testing a new anti-fibrotic agent, it is preferable to start with a well-characterized model, such as bleomycin-induced fibrosis for inflammation and Tsk1 mice for fibrosis independent of inflammation. Ideally, both abovementioned models should be used for testing, and, subsequently, the efficiency should be checked on a more complex model, such as Fra-2 transgenic mice [4].

Systemic sclerosis represents a great unmet medical need due to its substantial morbidity and mortality and, to date, still missing efficient disease-modifying therapies. Finding novel effective therapeutic approaches requires better and more complex understanding of the pathogenesis of this heterogeneous multisystem disorder. The currently existing animal models of SSc, with their abovementioned limitations, still continue to serve as the basis for the vital preclinical studies of presently available and novel candidate targeted therapies before they can be used in clinical trials in humans. Furthermore, they will be essential for further in-depth analysis of the hallmark pathogenic mechanisms in SSc and for progress in hypothesis-driven and discovery research. In addition, with recent advances in genetic approaches, mapping of the human and murine genome, and novel data from the high-throughput sequencing of the biopsy samples from different subsets of SSc patients and healthy volunteers, there is a new window of opportunity for currently existing animal models to be further explored and used in more suitable and elaborate way and thus lead to vital progress in understanding the pathogenesis of SSc and providing new candidate therapies. Furthermore, the recent use of conditional genetic strategies in murine SSc models, through

which particular genes of interest can be turned on/off in determined cell lineages at defined points of time, enables also for assessment of genes, which would have had lethal consequences if manipulated on germ-line level. These approaches also provide novel possibilities to examine modifications to specific cell lineages only and to assess their effect in experimental settings other than prevention (of induction of particular pathology), such as reversal or treatment (of established pathology). Progress in research strategies and new developments in existing animal models will inevitably lead to discovery of novel and more sophisticated animal models of SSc. In the future, a combination of genetic and induction strategies can lead to creation of experimental models which accurately, and to a greater extent, mimic SSc in humans and could lead to substantial clarification of pathological mechanisms and the discovery of a universal causal treatment of SSc. Nevertheless, the existing models have already contributed significantly to preclinical testing of several available biological agents and small molecules, some of which achieved promising results in early clinical studies and could provide better prospects for patients with this incurable disease.

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