Review

Animal models in systemic sclerosis

V. Rogai[1], R.J. Lories[2], S. Guiducci[1], F.P. Luyten[2], M. Matucci Cerinic[1]

[1]*Department of Biomedicine, Division of Rheumatology, University of Florence, AOUC, Florence, Italy;*
[2]*Laboratory for Skeletal Development and Joint Disorders, Division of Rheumatology, Department of Musculoskeletal Sciences, Katholieke Universiteit Leuven, Belgium.*

Veronica Rogai, MD
Rik J. Lories, MD, PhD
Serena Guiducci, MD
Frank P Luyten, MD, PhD
Marco Matucci Cerinic, MD, PhD

Please address correspondence to:
Dr. Veronica Rogai,
Department of Biomedicine,
Division of Rheumatology,
University of Florence, AOUC,
Villa Monna Tessa,
Viale Pieraccini 18,
50139 Florence, Italy.
E-mail: vrogai@hotmail.com

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ABSTRACT

Systemic sclerosis (SSc) is a complex systemic disease. It is characterised by fibrosis, of the skin and internal organs, as lungs, kidney, heart and gastrointestinal tract. As the aetiology of SSc is unknown, numerous animal models have been developed to better understand the pathogenesis of the disease and to test potentially useful therapeutic interventions. Although several animal models have been described, predominantly in mice, none reproduces precisely all manifestations of SSc. However, all animal models display tissue fibrotic changes similar to those present in SSc. This review is focused on the principal animal models and the molecular pathways involved. Animal models can be divided into two groups. In one group the pathologic phenotype is the result of a genetic mutation (tight skin 1 and tight skin 2,UCD 200). In the second group, the pathologic alterations are induced in normal animals by manipulation of their immune system (sclerodermatous graft-vs-host disease (Scl GVHD) induced by transplantation, or by administration of exogenous substances). In the future, the development of additional animal models may become important in the further understanding of the alteration of the molecular pathways that regulate a physiologic processes to induce tissue fibrosis, the hallmark of this disease, and to identify and test targeted therapeutic agents.

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Introduction

Systemic Sclerosis (SSc) is a systemic disease leading to organ fibrosis, involving the microvascular, immune and connective tissue systems (1). In the past, several attempts have been made to create animal models that may mimic the disease, but, given the multifactorial nature of SSc, it has been difficult to develop an animal model fulfilling the full clinical picture of SSc. Animal models have been developed, including genetic models (tight skin mouse) and induced disease models (bleomycin or vinyl chloride injection into skin of mice and graft-versus-host [GVH]-induced disease by transfer of lymphocytes between certain strains of mice). All these models develop dermal thickening and fibrosis, the most obvious feature of human SSc, but they often exhibit only a part of the clinical spectrum of the disease or include additional abnormalities not associated with SSc in humans (Table I).

1. Genetic models

Tight skin 1 (Tsk1)

Initial genetic studies mapped the *Tsk1* mutation to mouse chromosome 2 (2). Siracusa *et al*. (3) identified the *Tsk1* mutation as a large in-frame duplication of exons 17 through 40, inserted between exons 40 and 41 within the fibrillin-1 gene. The mutation results in duplication of numerous important domains in the encoded mutant protein including epidermal growth factor (EGF) Ca^{++}-binding domains, one fibrillin, one RGD integrin, and two TGF-β binding domains. *Tsk1* is a spontaneous dominant mutation that occurs in the inbred B10.D2 (58N)/Sn strain (2-4). The most striking feature of heterozygous animals (*Tsk1*/+) is the presence of thickened and tight skin that is firmly bound to the subcutaneous and deep muscular tissues. The *Tsk1* mutation is lethal and homozygous embryos degenerate *in utero* at 8-10 days of gestation. *Heterozygote* mice display cutaneous and some visceral changes, as well as biochemical and molecular abnormalities that closely resemble those present in patients with SSc (5). In addition to a cutaneous fibrosis similar to that observed in human SSc, heterozygous mice (tsk/+) present with an enlarged heart and their lungs

display a histologic picture similar to human emphysema. These results were confirmed by Bona *et al*. (6) who, in addition, found four amino acid differences between the two fibrillin-1 gene copies in the *Tsk1*/+ mouse. The studies of Siracusa *et al*. (3) showed that *Tsk1*/+ mice express both the normal 11 kilobase (kb) fibrillin-1 transcript and a mutant 14 kb transcript encoded by the mutated gene. The 14 kb mutant transcript is translated into a larger than normal fibrillin-1. Dermal fibroblasts from *Tsk1*/+ mice synthesized and secreted both the normal and the mutant fibrillin-1 molecule in approximately equal amounts and both types of fibrillin-1 molecules are incorporated into the extracellular matrix (ECM). It is unclear how this mutation alters the matrix metabolism (7): it was hypothesized that the abnormal fibrillin-1 recruits a greater number of EGF and TGF-β molecules leading to excessive production of collagen. Saito *et al*. (8) provided support for this hypothesis and reported that the mutant fibrillin-1 binds greater amounts of TGF-[beta] than the normal protein. Lemaire *et al*. (9) showed that Tsk mutant fibrillin 1 increases ECM incorporation of microfibril-associated glycoprotein 2 (MAGP-2) and type I collagen. The authors also reported an increase in MAGP-2 deposits in skin lesion SSc and concluded that alterations in the microfibril structure or deposition may contribute to cutaneous fibrosis in SSc. Another characteristic of these mice was reported by the Denton group (10). They found an overexpression of monocyte chemoattractant protein 3 (MCP3), a proinflammatory chemokine and chemotactic for monocytes, eosinophils and basophils. It is noted that high levels of MCP3 are present in SSc patients (11). Many studies showed the importance of the immune system in the development and in the maintenance of the Tsk fibrotic phenotype. Ong *et al*. reported the involvement of CD4+ T cells and IL-4 (12,13). They showed that neutralizing antibodies to IL-4 or a null mutation in this gene could prevent the development of dermal fibrosis in mice and suggested that a specific usage of a portion of the T cell repertoire could prevent the development of fibrosis in *Tsk1*/+ mice. Kodera *et al*. (14) showed that embryos carrying the normally lethal *Tsk1/Tsk1* genotype could be rescued by disrupting either one (+/-) or both (-/-) alleles of the IL-4 gene. These mice did not develop cutaneous hyperplasia, although they exhibited pulmonary emphysema. They also reported that IL-4 could increase the levels of TGF-β mRNA in fibroblasts and that the levels of TGF-β in the lungs of *Tsk1*/+, IL-4(-/-) mice were lower than those in the lungs of *Tsk1*/+, IL-4(+/+) animals. McGaha *et al*. (15) reported that crossing the *Tsk1*/+ mouse to an IL-4 receptor α-deficient mouse prevented cutaneous fibrosis in the F1 generation but did not prevent the emphysematous changes in the lungs. Mononuclear inflammatory cell infiltration of affected organs is not seen in the *Tsk1*/+ mouse. In fact the most important difference with SSc is the absence of the inflammatory and vascular involvement. However, fluorescent antinuclear antibodies (ANA) have been detected in about 50% of *Tsk1*/+ mice at 8 months of age, and anti-Scl70 antibodies were demonstrated in supernatants from hybridomas established from *Tsk1*/+ mice splenocytes (16). These antibodies have been extensively characterized (17). Shibata *et al*. (18) demonstrated autoantibodies to the 190 kilodalton (kD) subunit of RNA polymerase 1, which is considered to be a specific marker for SSc. Saito *et al*. showed that B cell functional defects caused by the loss of CD 19 significantly decreased skin fibrosis in Tsk/1+ mice, suggesting that B cells play an important role (19). In Tsk/1+ mice overexpressing CD19, anti Topo-I antibody (Tsk/1+ mice produce autoantibodies like Topo-I) levels were significantly increased, although skin thickness was not enhanced. The role of cellular immunity in the pathogenesis of tissue fibrosis in *Tsk1*/+ mice was further examined by Phelps *et al*. (20). These authors showed that infusion of bone marrow cells or T and B lymphocytes from *Tsk1*/+ mice into pallid mice led to tissue fibrosis, cellular infiltration, autoantibody production, and increased transcription of the α_1(I) collagen gene. The results were the first demonstration that immunocompetent cells played a role in the activation of collagen synthesis leading to fibrosis.

Table I. Animal models of SSc.

	Vascular	Fibrosis	Inflammation	Autoimmunity
Tsk1	-	+	-	+
Tsk2	-	+	+	+
Donor B10.D2 and recipient BALB/c	-	+	+	-
Donor LPJ and recipient C57Bl	-	+	+	-
Donors B10.D2 and recipient RAG-2KO	+	+	+	±
bleomycin	-	+	+	-
UCD200	+	+	+	±

Tight skin 2 (Tsk2)

Peters *et al*. in 1986, described for the first time the tight skin 2 mutation as a chemically (ethylnitrosourea) induced autosomal dominant mutation. This mutation was localized to mouse chromosome 1, indicating that it was clearly different from the *Tsk1* mutation which resides on chromosome 2; it is inherited as an autosomal dominant trait, and only heterozygous (*Tsk2*/+) animals survive. *Tsk2*/+ mice develop a tight skin phenotype that becomes apparent at 3-4 weeks of age (21). In addition, previous biochemical studies show increased amounts of collagen and an increase in the steady-state level of α1(I) and α1(III) procollagen mRNA in *Tsk2*/+ dermal fibroblasts compared to normal fibroblasts (22). These findings indicate that the *Tsk2*/+ mouse is a good model for the study of the fibrotic process which is the hallmark of human SSc, because similar increases in collagen gene expression have been reported in dermal fibroblasts obtained from SSc patients (23-25). Increase in the transcription rates of type I and

III collagen genes and elevated steady state levels of their corresponding transcripts were reported for fibroblasts isolated from SSc patients (24, 26, 27). However, in contrast to *Tsk1*/+ mice, a prominent mononuclear cell infiltration is present in the dermis and adipose tissue of *Tsk2*/+ mice. These mice had an increased thickness in the adventitia of vessels in the heart and lungs along with some distortion of alveolar spaces (21, 28).

2. Transplantation induced models

Sclerodermatous graft versus host disease induced by transplantation

Patients who have haematologic malignancy and severe combined immunodeficiency treated with ionizing irradiation and heterologous bone marrow transplantation may develop chronic GvHD with skin and visceral fibrosis that resembles scleroderma, so called Scl GvHD. In 1963, Stastny *et al*. (29) first generated chronic GvHD in rats and pointed out the similarities between this disease and scleroderma. After twenty years, Jaffee and Claman suggested murine chronic GvHD models as models for human scleroderma (30). Two strains are typically used in this model, donor LPJ and recipient C57Bl; donor B10.D2 and recipient BALB/c. In contrast, most animals transplanted across minor histocompatibility loci develop cytotoxic GvHD with alopecia and injury to epithelia (skin, lung, gut, liver), leading to death. Challenge of the skin with profibrotic substances does appear to result in good reproduction of the skin disease of humans; however, these challenges do not result in widespread progression of the fibrosis to internal organs. Chronic GvHD typically resembles connective tissue autoimmune-like immunologic disorders such as SSc characterized by sclerodermoid lesions of the skin, along with joint contractions, eosinophilia, circulating autoantibodies, hypergammaglobulinemia, and plasmacytosis of viscera and lymph nodes.

Donor B10.D2 and recipient BALB/c

B10.D2 mice provide cells for transplantation and Balb/c mice serve as the recipient. Recipient Balb/c mice are lethally irradiated and receive spleen and bone marrow cells prepared from B10.D2 donors. Typically male mice serve as donors and females as recipients to follow-up donor cells through a polymerase chain reaction designed to detect Y specific sequences. Lakos *et al*. (31) present a detailed description of these transplantation procedures. Skin thickening and pulmonary fibrosis develop in the recipient mice 14 to 21 days after transplantation: pulmonary fibrosis is associated with a loss of alveolar spaces and extensive dermal thickening is associated with elevated levels of type I collagen mRNA and protein. This fibrotic process is preceded by an infiltration of immune cells of donor origin (32). Zhang *et al*. in 2002 (33) observed that macrophages expressing markers of activation and Ag presentation (CD11b, I-A, SR-A, and VLA-4) were predominant in the skin and infiltrated during early time points in murine Scl GVHD, when skin thickening is first apparent. In skin samples elevated levels of various cutaneous cytokines and chemokines, that attract macrophages, were observed including TGF-β1, macrophage chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1α (MIP-1α) and RANTES. TGF-β1 (but not TGF-β2 and TGFβ3) up-regulated in the lesional skin of Scl GvHD mice seems to lead to fibrosis at early phases. Gilliam et al. reported that anti-TGF-β Abs prevent the progression of skin and lung fibrosis in Scl GVHD at day 21 (34). In this model it was shown that C-C chemokines as CCL2, macrophages chemoattractant protein-1 (MCP-1) and RANTES may play important roles in early pathogenesis of skin fibrosis; in mice Scl GvHD: these molecules were increased, actively involved attracting monocyte/macrophages and T cells into the skin and possibly interacting with TGF-β1(35).

Donor LPJ and recipient C57Bl

Scl GvHD can be produced also by transplanting allogenic (C57BL/6J) bone marrow and spleen cells into lethally irradiated recipients (LP/J). Onset of GvHD starts 7 days after transplantation, with epidermal injury and round cells infiltrating in the dermis and subcutis with mononuclear cell exocytosis. By day 14 after bone marrow transplantation, the dermis and subcutis of GvHD mice becomes sclerotic with compressed and atrophic pilosebaceous units. Extracutaneous involvement includes periportal liver mononuclear cell infiltrates and interstitial round cell influx to lungs and kidneys (36).

Donors B10.D2 and recipient RAG-2KO

Recently Ruzek *et al*. (37) described a modified model of Scl GVHD. In this model RAG-2KO mice genetically deficient in mature T and B cells were used as recipients for transfer of donor B10.D2 spleen cells to induce the Scl GvHD. The recombination-activating gene 2 (Rag2) is required for proper rearrangement of immunoglobulin and T cell receptor genes, and thus mice lacking a functional Rag2 gene have a severe immune deficiency. Part of the rationale for using the Rag2 null mice is that it eliminates the need for irradiation prior to transferring donor cells. To induce Scl GVHD, spleen cells from B10.D2 mice were injected without prior recipient irradiation into Balb/c mice null for Rag2 (Rag2-/-). Dermal thickening, developed in 3 -5 weeks, appear different between trunk and extremities and they noted progressive internal organ fibrosis during the course of disease. In addition, an examination of blood vessels in skin and kidneys revealed vasoconstriction and elevated levels of endothelin-1 (ET-1), similar to those detected in SSc patients.

3. Bleomycin induced

Bleomycin, produced by fungus Streptomyces verticillus, is an antibiotic used in the treatment of cancer. In animals bleomycin induces pulmonary fibrosis through iv injection and generates skin and lung fibrosis through subcutaneous injection (38). Dermal sclerosis induced by local injections of the drug has biochemical and histologic features similar to the dermal fibrosis observed in SSc (39). Yamamoto *et al*. established a model for SSc by local bleomycin treatment (40-45). Dermal sclerosis was induced by repeated subcutaneous injections of bleomycin in various mice

strains (42), although there is some variation among strains in the intensity and the periods required to induce dermal sclerosis. 100μl of bleomycin, at a concentration of more than 10 μg/ml, was injected into the shaved back skin every day or every other day for 3-4 weeks. Dermal sclerosis was characterized by thickened collagen bundles, deposition of homogenous material in the thickened dermis with cellular infiltrates, which mimicked the histologic features of human scleroderma. Dermal thickness gradually increased up to two-fold when sclerosis was developed. In these animals kidney, liver, heart and remote regions from the site of injection (as fingers or abdominal skin) were spared. Yamamoto *et al*. (42) evaluated the responsiveness of eight different inbred strains of mice. While all strains responded and developed a dermal thickening after 4 weeks of injections, four strains, C3H/He, DBA/2, B10.A, and B10.D2, displayed particularly robust responses with a greater than two-fold increase in dermal thickness compared with phosphate-buffered saline (PBS) injection controls. Two strains, Balb/c and B10.A, displayed substantial increase in mast cell number. Further, C3H/He mice responded after only 3 weeks of local injections. These data identified two strains, C3H/He and B10.A, as the most useful murine models for bleomycin induced scleroderma. Within a few weeks the skin shows mast cell degranulation at the side of cutaneous injection, early inflammatory infiltrates of mononuclear cells, and thickened homogeneous dermal collagen bundles. Activated macrophages are the first cells found in increased numbers at the early stages of fibrosis, and they release a number of proinflammatory and fibrogenic mediators such as TGF-β and PDGF (46). In the early stage Nishioka *et al*. (47) showed mast cells were increased in number in the lesional skin, and suggested that mast cells were one of the initiators of scleroderma. Mast cells produce cytokines, growth factors and mediators that are capable of activating fibroblasts and endothelial cells. In the bleomycin model, mast cells were increased in number in parallel with the induction of dermal sclerosis. Among cytokines, TGF-β increases the synthesis of collagen type I and type III or fibronectin by many cell types, modulation of cell-matrix adhesion protein receptors and regulation of production of proteins that can modify the extracellular matrix by proteolytic action (48). In the bleomycin-treated skin, TGF-β was detected on the infiltrating cells; Yamamoto and Nishioka have recently observed increased expression and synthesis of TGF-β in a bleomycin susceptible mouse strain (49). A recent study of Takagawa *et al*. (50) observed TGF-β/Smad signaling during the induction of Fibrosis: in the early stages of the response (3 days) to bleomycin, Smad3 expression was associated with infiltrating mononuclear cells and at later stages (3 weeks) with resident fibroblasts. Significantly elevated levels of phospho-Smad2/3 were also evident in lesional fibroblasts. Interestingly, Smad7, an endogenous inhibitor of Smad signaling, was not upregulated, but downregulated. In patients with SSc there are elevated levels of chemokines, as CCL2. CCL2/monocyte chemoattractant protein-1 upregulates type I collagen mRNA expression in fibroblast. According to one of our studies (51) stimulation with PDGF determines a significant increase in CCL2 mRNA and protein. In the bleomycin model, expression of CCL2 and CCR2, a receptor of CCL2, was elevated at both protein and mRNA levels in the lesional skin following bleomycin treatment. In 2001 Moore et al. demonstrated that CCR2-deficient mice are protected from pulmonary fibrosis, suggesting that CCR2 signaling promotes a profibrotic cascade. Bleomycin in vivo produces reactive oxygen species such as superoxide and hydroxyl radicals, which may be important initiators of tissue and vascular injury that lead to sclerosis. Pulmonary fibrosis is thought to be caused by the overproduction of oxidants because increased free radical production is also found in lungs of patients with scleroderma (52).

4. UCD 200

University of California at Davis line 200 (UCD 200) chickens, a well-established animal model for human SSc, spontaneously develop an inherited fibrotic syndrome with features similar to those observed in human scleroderma, which permits study of the initial stage of the disease (53-56).

UCD 200 chickens develop an inherited scleroderma-like disease with an acute inflammatory stage 1–3 weeks after hatching. The clinical signs of this stage, *i.e*., erythema, edema and necrosis of the comb, are readily apparent markers of disease inception. Three to four weeks later, this chicken model develops the characteristic histological features of SSc, *i.e*., vascular occlusion, perivascular mononuclear cell (MNC) infiltration and fibrosis of the comb, skin and internal organs (54, 55, 57-61). Finally, in the skin and viscera of these chicken an excessive accumulation of collagen types I, II and III is found. Subsequent tissue fibrosis occurs later in the course of the disease, usually when the inflammatory infiltrates begin to decrease. At 6 months of age, the majority of chickens have extensive pathology of visceral organs, including oesophagus, lung, heart, kidney and testes. In addition, the affected birds develop multiple serological abnormalities with production of a variety of autoantibodies, *i.e*., rheumatoid factors, anticardiolipin, anti-endothelial cell antibodies (AECA), anticytoplasmic and antinuclear antibodies (53, 62, 63), but non specific SSc antibodies.

Conclusions

Systemic Sclerosis is a complex disorder that involves multiple organs. Several animal models mimic aspects of the disease, but they are all defective for some feature. Skin fibrosis is the only characteristic that is present in all animal models. Yet no animal model carries all clinical characteristics (skin and visceral fibrosis, vascular damage, immune activation). The development and the course of the disease is characterized by a variety of factors, exogenous and endogenous, not yet completely understood. Therefore it is not realistic in a model to reproduce a process that has not been clearly defined. On the other hand animal models already give us opportunities to better understand some of the disease aspects.

Animal models remain an important tool in discovering new mechanisms of disease. The study of similarities in the pathogenetic mechanisms in different animal models provides an opportunity to better understand the cellular and molecular basis of systemic sclerosis.

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