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PROVA ORALE DEL 22/3/2021

TRACCIA 1

Quesito tecnico:

Fasi di preparazione di sezioni di polmone per analisi immunoistochimica di fibrosi.

Quesito su applicazioni informatiche:

Come si effettua in Excel la selezione di più celle tra loro non adiacenti ?
[A] Tenendo premuto il Tasto SHIFT e selezionando successivamente tutte le aree interessate.
[B] Non è possibile questa operazione.
[C] Tenendo premuto il Tasto CTRL e selezionando successivamente tutte le aree interessate.

Accertamento conoscenza lingua inglese:

Lettura e traduzione del testo allegato.

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The Fra-2 transgenic mouse model of systemic sclerosis

Britta Maurer [a], Jörg H.W. Distler [b], Oliver Distler [a,*]

[a] Department of Rheumatology and Center of Experimental Rheumatology, University Hospital Zurich, Gloriastrasse 25, CH-8091 Zurich, Switzerland
[b] Department of Internal Medicine III and Institute for Clinical Immunology, University of Erlangen-Nuremberg, Erlangen, Germany

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ABSTRACT

In systemic sclerosis, microvascular injury often precedes the development of fibrosis. Whereas the development of digital ulcers and skin fibrosis causes high morbidity, the affection of internal organs, in particular complications such as interstitial lung disease and pulmonary (arterial) hypertension, account for the high disease-associated mortality of these patients. Vascular animal models of systemic sclerosis are of utmost importance to study pathophysiological aspects, to identify molecular key players, and to perform interventional proof of concept-studies. So far, animal models of systemic sclerosis have mainly reflected the pro-fibrotic features of the human disease. The Fra-2 (Fos-related antigen-2) transgenic mouse model simultaneously displays both pro-fibrotic and vascular characteristics of human systemic sclerosis.

1. Introduction

Systemic sclerosis (SSc) is an autoimmune disease characterized by widespread vascular changes and progressive fibrosis of the skin and internal organs. Alterations of microvessels, preceded by endothelial cell damage and likely an increased apoptosis of endothelial cells (EC) (Sgonc et al., 1996), lead to clinical vascular manifestations such as digital ulcers, scleroderma renal crisis and pulmonary arterial hypertension (PAH) (Steen et al., 2009). Vascular manifestations such as Raynaud's phenomenon and digital ulcers might occur even before the onset of fibrosis suggesting a crucial role of microvascular injury for the overall disease process. In general, there is a destructive vasculopathy of capillaries with vasodilation and rarefication, as well as a proliferative vasculopathy of small arteries with intimal thickening (Table 1). Often these morphological changes occur in parallel and are often found even in the absence of clinical manifestations.

To date, PAH and interstitial lung disease (ILD) are the leading causes of death in SSc patients (Steen and Medsger, 2007). Patients with SSc-PAH have a worse prognosis than patients with idiopathic PAH (IPAH) or PAH related to other connective tissue diseases (Condliffe et al., 2009). The overall response to PAH-specific therapies is less favorable in patients with SSc-PAH than in IPAH patients (Mathai et al., 2009) which might be explained by histopathological differences of pulmonary changes in IPAH and SSc-PAH (Dorfmuller et al., 2007; Overbeek et al., 2009). The prognosis of SSc patients with co-existing PAH and ILD is even worse compared to patients with isolated SSc-PAH with a 3-year survival of 28% even when treated with targeted therapies for PAH (Condliffe et al., 2009). Apparently, (subclinical) ILD, detected by sensitive techniques such as histology, occurs in 70% of SSc patients (D'Angelo et al., 1969; Matucci-Cerinic et al., 2011) with non-specific interstitial pneumonia (NSIP) as the most frequent histological pattern (Bouros et al., 2002).

The observed clinical, prognostic and histopathological differences in SSc-PAH and the frequent co-existence of ILD support the concept of a specific pathophysiology of pulmonary hypertension (PH) related to SSc. However, research in SSc-PH is hampered by the lack of human biosamples and most animal models in SSc deal with fibrotic rather than vascular changes (Beyer, Schett et al. 2010). Given the high morbidity and mortality of vascular changes in SSc, animal models that reflect vascular features of SSc are of utmost importance to 1) study pathophysiological interactions of vasculopathy and fibrosis, to 2) identify molecular key players and potential therapeutic targets and for 3) preclinical proof of concept studies, especially in SSc-PH.

In this review, we will discuss the Fra-2 (Fos-related antigen-2) transgenic (tg) mouse model as a potential novel animal model of SSc that simultaneously displays features of vasculopathy with fibrosis of the skin and internal organs.

2. Characterization of the Fra-2 model

The transcription factor AP-1 (activator protein-1) is a heterodimeric molecule, composed of members of the Jun (c-Jun, JunB, JunD) and the Fos family (c-Fos, FosB, Fra-1, Fra-2). AP-1 family members are immediate early genes which are induced by a variety of stress signals and control subsequent stress responses including cell proliferation, apoptosis, inflammation, wound healing and tumorigenesis (Eferl and Wagner, 2003). Whereas Jun proteins and the majority of Fos proteins are well characterized, relatively little is known about Fra-2 and its functions in vivo (Foletta, 1996; Bozec et al., 2008). In the Fra-2 tg mouse model,

* Corresponding author. Tel.: +41 44 255 2977; fax: +41 44 255 4170.
E-mail address: oliver.distler@usz.ch (O. Distler).

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PROVA ORALE DEL 22/3/2021

TRACCIA 2

Quesito tecnico:

Vie di somministrazione di bleomicina per ottenere modelli di fibrosi tessutale e/o d'organo: vantaggi e svantaggi.

Quesito su applicazioni informatiche:

1 - In riferimento al contenuto di una cella, i dati possono essere:
[A] solo informazioni alfabetiche
[B] solo informazioni numeriche
[C] informazioni numeriche o alfanumeriche

Accertamento conoscenza lingua inglese:

Lettura e traduzione del testo allegato.

generated by Wagner and co-workers (Eferl et al., 2008), the murine Fra-2 gene is expressed under the control of the ubiquitous major histocompatibility complex class I antigen H2Kb promoter. In Fra-2 tg mice, the mRNA of the transgene is detectable in various tissues, but the extent and distribution of protein expression varies.

2.1. Peripheral manifestations

2.1.1. Microvasculopathy

We could show in one of our initial studies on the Fra-2 tg mouse model that Fra-2 tg mice display various features of the peripheral vasculopathy of human SSc (Maurer et al., 2009). In the skin of both, Fra-2 tg mice and SSc patients, but not in controls, Fra-2 protein was predominantly expressed in vascular structures, in particular in endothelial cells (EC) and vascular smooth muscle cells.

Whereas Fra-2 tg mice did not differ from wt mice at an age of 9 weeks, starting from week 12, a significant decrease in capillary density occurred (Fig. 1). Increased perivascular inflammatory infiltrates as another characteristic feature of early human SSc were also present in the skin of 9 week old Fra-2 tg mice compared to wt mice, but not in older mice. Of note, the rarefication of capillaries in Fra-2 tg mice paralleled the development of skin fibrosis. Starting from 12 weeks, Fra-2 tg mice showed a time-dependent increase of dermal thickness due to accumulation of extracellular matrix that became even more pronounced at an age of 16 weeks (Fig. 2).

The most remarkable finding was, however, that in Fra-2 tg mice the apoptosis of dermal EC at the age of 9 weeks clearly preceded the development of microangiopathy and skin fibrosis suggesting that similar to what has been proposed for human SSc, apoptosis of EC could be one of the initiating events for the microangiopathy in this model. By functional in vitro experiments, it could be shown that the expression of Fra-2 had impact mediated apoptosis, and inhibited proliferation, migration and tube formation capacity of human microvascular endothelial cells. Additional experiments indicated that the expression of Fra-2 in HUMECs was independent of major cytokines and growth factors operative in SSc such as TGFß, PDGF-B and VEGF. In summary, this study showed that in Fra-2 tg mice – as hypothesized in early human SSc – apoptosis of dermal EC was the initial pathogenic event, followed by the development of microangiopathy and fibrosis. Clinical manifestation of peripheral Microvasculopathy such as ulcers or tissue necrosis did not occur during the 16 week observation period.

2.1.2. Skin fibrosis

A recent study in our laboratories (Reich et al., 2010) elucidated the underlying mechanisms of skin fibrosis in Fra-2 tg mice. Previous data had indicated a role for c-Jun and Fra-2 in TGFβ signaling (Fichtner-Feigl et al., 2006). In this recent study, it could be demonstrated that Fra-2 protein is overexpressed not only in activated dermal fibroblasts of Fra-2 tg mice, but also in two other well established pro-fibrotic animal models, the bleomycin model and the TSK1-model, and most importantly in the skin fibroblasts of SSc patients. By in vitro experiments, it could be proven that in contrast to human microvascular EC (Maurer et al., 2009), stimulation with both, PDGF and TGFβ, increased the expression of Fra-2 in SSc skin fibroblasts, although the effects of PDGF were indirectly mediated through TGFβ signaling.

Further experiments provided evidence that in SSc skin fibroblasts, Fra-2 signaling was regulated by TGFβ-and ERK-, but not Smad-dependent pathways. As a downstream mediator of ERK-signaling, Fra-2 was shown to regulate the expression of Col1a1, Col1a2, and Col5a1, all of which are major components of fibrotic lesions in patients with SSc. Based on this and additional experiments it was suggested that Fra-2 might be the central mediator of the regulatory effects on collagen-synthesis described for AP-1. Underlining the pro-fibrotic role of Fra-2/AP-1 in vivo, a recent study demonstrated that inhibition of AP-1 prevented the development of skin fibrosis in different animal models of SSc (Avouac et al., 2012).

Table 1.
Microvascular features of human SSc.

	Microvascular changes	
	Destructive	Proliferative
Histopathological findings	Dilated, malformed, giant capillaries, microbleedings, finally loss of capillaries/small arteries	Intimal thickening, occlusion of small arteries and capillaries
Cellular key players	Apoptosis of endothelial cells	Proliferation of endothelial cells, smooth muscle cells
Molecular key players	Dysregulation of soluble pro- and anti-angiogenic mediators including soluble adhesion molecules, cytokines, chemokines and growth factors	
Transcriptional regulators	Overexpression of Fra-2 reduced expression of Fli1	Overexpression of Fra-2

2.2. Lung disease

2.2.1. Interstitial lung disease

Besides the peripheral disease manifestations, Fra-2 tg mice develop an involvement of internal organs, in particular of the lungs (Eferl et al., 2008). In the initial study, Fra-2 tg mice died at a median age of 17 weeks with clinical signs of respiratory distress such as tachypnea and hunched posture. Further examination revealed an increase of lung weight and size, accompanied by increased pulmonary stiffness due to tissue fibrosis. The expression of the transgene, visualized by EGFP fluorescence, was most prominent in pulmonary arteries, bronchi, and fibroblastic proliferations.

Whereas in the skin, apoptosis of EC occurred before the development of microangiopathy and fibrosis (Maurer et al., 2009), no increased apoptosis could be observed in the lungs. Of note, obliteration of pulmonary arteries, accompanied by perivascular inflammatory infiltrates, becoming apparent at an age of 12 weeks preceded the onset of fibrosis by 2–3 weeks. At later stages, interstitial inflammation and fibrosis became more prominent, and cellular structures reminding of fibroblastic foci occurred.

In end-stages, Fra-2 tg lungs displayed histological characteristics of human NSIP- and IPF-related usual interstitial pneumonia including fibrosis, dense lymphocytic infiltration, hyperplasia of bronchus-associated lymphoid tissue, and honeycombing. Interestingly, in our recent analysis (Maurer et al., 2012), the phenotype of ILD in Fra-2 tg mice resembled features of human NSIP, whereas fibroblastic foci and honeycombing, associated with UIP, were rarely detectable (Fig. 3). This might be explained by the different background of Fra-2 tg mice, as the mice in our study were backcrossed from a mixed (C57BL/6 × CBA) genetic background into a pure C57/Bl6 background. Interestingly, most pulmonary myofibroblasts expressed the epithelial marker cytokeratin 8/18 suggesting that epithelial-to-mesenchymal transition, an often discussed pathogenic mechanism, might be an important source of pulmonary myofibroblasts in Fra-2 tg mice.

In additional experiments, Eferl et al. showed that the increased collagen production of pulmonary fibroblasts did not occur in a cell-autonomous manner because 1) the deposition of extracellular matrix proteins was not elevated in primary pulmonary fibroblasts of Fra-2tg mice in vitro and 2) after the stimulation with pro-fibrotic cytokines such as TGFβ1 and IL-4 the collagen production of pulmonary myofibroblasts derived from Fra-2 tg mice did not differ from those of wt littermates.

Given the presence of perivascular and peribronchial inflammatory infiltrates, the role of inflammation in the pathogenesis of pulmonary fibrosis in Fra-2 tg mice was next assessed. In two different experimental settings, firstly by performing reciprocal bone marrow reconstitution experiments and secondly by assessing Fra-2 tg mice lacking functional B and T lymphocytes (fra-2tg/rag2$^{-/-}$), the authors

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PROVA ORALE DEL 22/3/2021

TRACCIA 3

Quesito tecnico:

Descrizione operativa di valutazione di espressione del transgene di una colonia murina transgenica.

Quesito su applicazioni informatiche:

In quale cella sarà visualizzato il risultato di questa formula "= B4*C4"?
[A] Nella cella che contiene la formula
[B] Nella cella C4
[C] Nella cella B4

Accertamento conoscenza lingua inglese:

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PROVA ORALE DEL 22/3/2021

TRACCIA 4

Quesito tecnico:

Fasi di preparazione di campioni di cute di topo per l'analisi di espressione di marker di fibrosi.

Quesito su applicazioni informatiche:

Quale tipo di dati è meglio rappresentato da un grafico a barre in excel?
[A] Mostrare valori percentuali di un insieme
[B] Mostrare il confronto tra più dati
[C] Mostrare dati che si modificano nel tempo

Accertamento conoscenza lingua inglese:

Lettura e traduzione del testo allegato.

most abundant in vascular structures suggesting a potential role for PDGF-BB signaling in the pulmonary pathophysiology of Fra-2 tg mice.

To address the potential use for interventional proof of concept studies, we additionally investigated the model's sensitivity to change over treatment by applying the tyrosine kinase inhibitor nilotinib to a subgroup of Fra-2 tg mice. As expected, the PDGF-BB/PDGFR pathway was inhibited by nilotinib, which was accompanied by a remarkable and almost complete prevention of vascular remodeling and also by a prevention of lung fibrosis in Fra-2 tg mice.

3. Conclusion and open questions

Given the fact that there are increasing data supporting the pathophysiologic interaction of vasculopathy and fibrosis in SSc, there is a shortage of animal models which do not only develop fibrosis, but also vascular features of SSc, and even less of these animal models display both loss of microvessels and a proliferative vasculopathy (Table 2). In addition, rat und mouse models of human IPAH lack SSc characteristic features (Stenmark et al., 2009; Gomez-Arroyo et al., 2012).

So far, Fra-2 tg mice are the only SSc model that mirrors the time-dependent development of both – destructive and proliferative – vasculopathy preceding the onset of peripheral and pulmonary fibrosis. As such, Fra-2 tg mice hold great promise to further delineate the pathophysiological links between vascular remodeling and fibrosis in peripheral and pulmonary SSc and to identify potential specific molecular and cellular targets for intervention. Furthermore, the studies also underline a prominent role of PDGF and TGFβ in the pathophysiology of pulmonary disease manifestations of SSc and suggest Fra-2 tg mice as a preclinical model for interventional proof of concept studies, especially in pulmonary SSc.

As a limitation, autoimmune phenomena do not occur in Fra-2 tg mice (Eferl et al., 2008), and it should therefore primarily be considered a model for microvascular and fibrotic disease manifestations of SSc. A couple of additional questions need to be addressed in future studies, e.g. 1) what are the molecular and cellular key players that potentially link both the peripheral destructive vasculopathy and the proliferative pulmonary vasculopathy to peripheral and pulmonary fibrosis in Fra-2 tg mice – and do they play a role in human SSc? 2) Does the vascular remodeling of pulmonary arteries in Fra-2 tg mice that closely resembles pulmonary SSc-P(A)H also result in changes of the pulmonary hemodynamics? 3) Do Fra-2 tg mice develop late-stage complications of PAH including the development of right heart hypertrophy/failure? 3) Can the histopathological changes of SSc-associated heart disease and maybe other organ manifestations also be detected in Fra-2 tg mice? These proposed experiments will provide further important insight into the pathophysiology of Fra-2 tg mice and also characterize for which specific aspects of vascular SSc (Table 2) and non-vascular SSc Fra-2 tg mice can be optimally used.

Table 2
Vascular features of human SSc that ought to be reflected in animal models.

Histopathology	
Microvascular changes	• Apoptosis of endothelial cells (Sgonc et al., 1996) • Dilatation and rarefication of dermal vessels resulting in ischemic lesions • Intimal proliferation of small arteries and capillaries of internal organs, esp. of kidneys and lungs leading to organ damage/failure • P(A)H: - Presence of: proliferation and obliteration of pulmonary arteries: intimal thickening with mainly concentric laminar lesions, medial hypertrophy; perivascular inflammatory infiltrates, adventitial fibrosis, lung fibrosis with interstitial inflammatory infiltrates, pulmonary occlusive venopathy - Absence of: complex lesions (e.g. plexiform and thrombotic lesions) (Dorfmuller et al., 2007; Overbeek et al., 2009) - Right heart disease with reduced ability of the right ventricle to adapt to pressure load; ambiguous findings on fibrosis and inflammation; impairment of microcirculation might play a role (Fernandes, Ramires et al. 2003; Allanore and Meune 2010; Overbeek, Mouchaers et al. 2010)
Macrovascular changes	Atherosclerosis, increased stiffness of arteries (Champion, 2008)
Echocardiography	
	Dilatation of the right heart chambers Hypertrophy of the right ventricle Pericardial effusion Decreased tricuspid annulus plane systolic excursion (<1.5 mm) RV systolic pressure >40 mm Hg
Right heart catheterization	
	Mean pulmonary artery pressure >25 mm Hg at rest, pulmonary wedge pressure <15 mm Hg (Simonneau et al., 2009)

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