Expression of transforming growth factor β receptor II in mesenchymal stem cells from systemic sclerosis patients

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ABSTRACT

Objectives: The present work aimed to evaluate the expression of transforming growth factor-β (TGF-β) receptors on bone marrow-derived multipotent mesenchymal stromal cells (MSCs) in patients with systemic sclerosis (SSc) and the consequences of TGF-β activation in these cells, since MSC have potential therapeutic interest for SSc patients and knowing that TGF-β plays a critical role during the development of fibrosis in SSc.

Design: This is a prospective research study using MSC samples obtained from SSc patients and compared with MSC from healthy donors.

Setting: One medical hospital involving collaboration between an internal medicine department for initial patient recruitment, a clinical biotherapeutic unit for MSC preparation and an academic laboratory for research.

Participants: 9 patients with diffuse SSc for which bone marrow (BM) aspiration was prescribed by sternum aspiration before haematopoietic stem cell transplantation, versus nine healthy donors for normal BM.

Primary and secondary outcome measures: TGF-β, TGF-β receptor types I (TBRI) and II (TBRII) mRNA and protein expression were assessed by quantitative PCR and flow cytometry, respectively, in MSC from both SSc patients and healthy donors. MSC were exposed to TGF-β and assessed for collagen 1α2 synthesis and Smad expression. As positive controls, primary cultures of dermal fibroblasts were also analysed.

Results: Compared with nine controls, MSC from nine SSc patients showed significant increase in mRNA levels (p<0.002) in membrane expression (p<0.0001) of TBRII. In response to TGF-β activation, increases in collagen 1α2 synthesis (p<0.05) and Smad-3 phosphorylation were upregulated in SSc MSC. Similar results were obtained on eight SSc-derived dermal fibroblasts compared to six healthy controls.

Conclusions: TBRII gene and protein expression defect in MSC derived from SSc patients may have pathological significance. These findings should be taken into account when considering the use of MSC-based therapies in an autologous setting.

ARTICLE SUMMARY

Article focus

- In systemic sclerosis (SSc), transforming growth factor-β (TGF-β) seems pivotal since the extent of TGF-β signalling activation in SSc skin fibroblasts correlates with disease severity and constitutes the best characterised therapeutic target in SSc fibrosis.
- Although multipotent progenitor mesenchymal stem cells (MSCs) from SSc patients exhibited similar differentiation potentials and immunosuppressive properties to that compared with their healthy counterparts, their response to TGF-β has not been studied.
- We wished to determine if TGF-β signalling in MSC was altered in SSc.

Key messages

- The results evidence an increase in TGF-β receptor type II (TBRII) gene and protein expression in MSC derived from SSc patients compared to MSC derived from healthy donors.
- Regarding that MSC may differentiate into tissue fibroblasts, these results suggest that the TBRII alteration observed in peripheral dermal fibroblasts from SSc patients might derive from a defect in their parental MSCs.
- The present results question the use of autologous MSC from SSc patients, alone or as adjuvant therapeutic product.

Strengths and limitations of this study

- Although limited by the small numbers of SSc patients, this is the first study revealing a defect in MSC derived from SSc patients and these data may have potent pathological significance in SSc pathophysiology.
- If these findings should be taken into account when discussing considering the use of MSC-based therapies in an autologous setting, functional experiments must be designed to validate the consequences of the defect of patients' MSC described in this in vitro study.

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INTRODUCTION

Systemic sclerosis (SSc) is a chronic systemic disease characterised by autoimmunity, vascular lesions and progressive fibrosis, with heterogeneous clinical presentation. As shown in vitro, SSc skin fibroblasts are characterised by abnormal growth and are responsible for abnormal extracellular matrix (ECM) accumulation. Multiple factors and signalling pathways are involved in the development or persistence of the SSc fibroblast phenotype. Although its exact relevance and interplay are still to be determined, transforming growth factor-β (TGF-β), which is mainly produced by fibroblasts, as well as by T helper type 2 lymphocytes, is the major cytokine involved in collagen production leading to fibrosis. TGF-β binds to transmembrane receptors that have intrinsic serine/threonine kinase activity. TGF-β receptor type II (TBRII) links TGF-β, and the TBRI is then recruited into a heteromeric complex. TBRII transphosphorylates the glycine/serine rich domain of TBRI kinase. Following the phosphorylation of Smad-2 or Smad-3 by the activated TBRI, a heteromeric complex is formed with Smad-4, resulting in translocation of the complex into the nucleus. This complex acts directly as a transcription activator or indirectly regulates gene transcription by interacting with other transcription factors. TGF-β receptors expression level is a critical mechanism for regulating the cellular response to cytokines. In particular, upregulation of TBR expression has been demonstrated in fibrotic diseases such as SSc, resulting in the deposition of ECM components. TGF-β signaling activation in SSc skin fibroblasts correlated with disease severity. Moreover, increased circulating TGF-β levels have been observed in patients with SSc.

Mesenchymal stem cells (MSCs) are multipotent progenitor cells, which display inhibitory function on T, B, natural killer and dendritic cells and represent promising therapeutic tool. These immunosuppressive properties, in addition to their potential usefulness for tissue repair have led to use autologous MSC for treating graft versus host disease and several types of autoimmune diseases including SSc. We previously demonstrated that in vitro MSC from SSc patients exhibit similar differentiation potentials and immunosuppressive properties compared with their healthy counterparts. However, it is still unknown as to whether MSC from SSc patients displayed similar abnormalities as those observed SSc patient-derived fibroblasts, which are of particular interest since MSC may differentiate into fibroblasts. We therefore designed the present prospective research study to analyse TGF-β signaling in MSC and in skin fibroblasts from SSc patients.

MATERIALS AND METHODS

Cell cultures and characterisation

Mesenchymal stem cells

Bone marrow (BM) samples were obtained by sternum aspiration in SSc patients (n=9), which characteristics are in table 1, prior to autologous haematopoietic stem cell transplantation for rapidly progressive disease. BM samples from healthy donors (n=9) were obtained from healthy donors. BM-derived MSCs were isolated and expanded as previously described, in minimum essential medium-a (Invitrogen, Cergy Pontoise, France), supplemented with 10% foetal calf serum (HyClone, Logan, UK), t-glutamine (2 mM; Invitrogen), basic fibroblast growth factor (1 ng/ml; R&D Systems, Lille, France) and antibiotic/antimycotic (Invitrogen).

Monoclonal antibodies conjugated with either fluorescein isothiocyanate or phycoerythrin and directed to CD34, CD45, CD73, CD29, CD90, CD105, CD44, TBRII or matched isotype control (Becton Dickinson, Le Pont de Claix, France, except TBRII, Sigma, St Louis, Missouri, USA) were used for immunophenotyping, according to the manufacturer’s protocol. Data from patients and controls were acquired and analysed on a four parameter flow cytometer FACSCalibur using ProCellQuest software (BD Biosciences, San Jose, California, USA).

For activation experiments, MSC were cultured in the presence of 5 ng/ml TGF-β1 (Peprotec, Lille, France).

Table 1 Systemic sclerosis (SSc) patients’ clinical characteristics: total or cutaneous biopsy site modified Rodnan Skin Score (mRSS)

| SSc patients | Age (years) at bone marrow aspiration | Total mRSS | Cutaneous mRSS |
|--------------|--------------------------------------|-------------|---------------|
| 1            | 60                                   | 30          | 1 (Abdomen)  |
| 2            | 54                                   | 37          | 2 (Abdomen)  |
| 3            | 31                                   | 2           | 1 (Thigh)    |
| 4            | 37                                   | 25          | 1 (Thigh)    |
| 5            | 29                                   | 24          | 2 (Abdomen)  |
| 6            | 56                                   | 24          | 2 (Abdomen)  |
| 7            | 25                                   | 15          | 1 (Thigh)    |
| 8            | 25                                   | 16          | 2 (Thigh)    |
| 9            | 55                                   | 4           | 1 (Thigh)    |
| Mean         | 41                                   | 20          |               |

Fibroblasts

Primary cultures of dermal fibroblasts derived from skin biopsies obtained during mammoplasty surgery (controls) or from SSc patients, at site with modified Rodnan Skin Score (mRSS) ≥2 (n=9). Approval from ethics committee from Hôpital Saint-Louis and informed signed consent were obtained for both donors and patients. Cells were cultured in Dulbecco’s Modified Eagle Medium containing 10% fetal bovine serum, 100 U/ml penicillin and 100mg/ml streptomycin in a humidified environment.
incubator at 37°C with 5% CO₂ atmosphere. Fibroblasts were used between the third and fifth passage.

**RNA preparation and real-time PCR**

**Reverse transcription-PCR analysis**

Total RNA was extracted using an RNeasy mini-kit (Qiagen, Hilden, Germany), according to the manufacturer’s instructions. DNase I treatment (25 units, 15 min) of total RNA was performed to eliminate genomic contamination of the RNA samples. One microgram of total RNA was used for first strand cDNA synthesis using a RT-for-PCR kit (Invitrogen, Carlsbad, California, USA). Real-time PCR was performed with an ABI PRISM 7300 instrument (Applied Biosystems, Foster City, California, USA) using SYBRGreen PCR Core Reagents (Applied Biosystems). Gene expression was corrected against GAPDH and GUS mRNA level in each sample. The sequences of the forward and reverse primers for TGF-β, TBRI, TBRII, Smad-2, Smad-3, Smad-4, Smad-7, collagen (col)1α2, GAPDH and GUS mRNA are available under request.

**ELISA**

TGF-β1, metalloproteinase 2 and 9 levels in MSC culture supernatants were quantified by Quantikine ELISA according to the manufacturer’s instruction (R&D Systems).

**Statistical analyses**

Statistical significance for all experiments was assessed using the two-sided Student’s t test. Differences were considered as significant at p<0.05.

**RESULTS**

**Increased expression of TBRII in MSC from SSc patients**

All SSc MSC highly expressed CD29, CD73, CD90, CD105 and adhesion molecules (CD44). The absence of contaminating haematopoietic stem cells was confirmed by the negative expression of CD34 and CD45 antigens. No phenotypic differences were observed between BM-MSCs from SSc patients and controls.

As compared with healthy donors, SSc MSC displayed significantly higher expression of TBRII mRNA, as measured by qRT-PCR (p<0.002; figure 1A) and the percentage of TBRII receptors at the cell membrane was significantly increased (p<0.01) as the index of TBRII expression (TBRII%×mean fluorescence intensity) (p<0.0001), as shown in figure 1B.

No significant difference in TBRII mRNA levels was observed between controls and SSc MSC, nor in the expression of Smad-2, Smad-3, Smad-4 and Smad-7 mRNA (figure 1A).

Although TGF-β1 mRNA levels were higher in MSC from SSc patients than in controls (figure 1A), the increase was not significant. The levels of metalloproteinase 2, 9 and TGF-β1 in culture supernatants did not differ significantly between donors and SSc MSC (data not shown).

**TGF-β-induced Smad pathway and collagen 1α2 synthesis are altered in MSC from SSc patients**

In order to determine whether the increased TBRII expression was linked to Smad-pathway signalling, MSC were cultured in the presence of TGF-β. Target gene expression, including collagen 1α2, and Smad-3 phosphorylation were assessed. In response to TGF-β activation, MSC from SSc patients expressed significantly more col1α2 mRNA than MSC from healthy donors during the first 12 h (p<0.05) as shown in figure 2A. In addition, mRNA synthesis of plasminogen activator inhibitor (PAI-1) was increased by TGF-β activation in MSC from SSc patients as compared with healthy MSC (data not shown).

By western blot analysis, a 10-fold increase in Smad-3 phosphorylation level was observed in MSC from SSc patients compared with healthy donors (figure 2B).

In accordance with data obtained on MSC, a significant increase in TBRII and col1α2 mRNA expression was also detected in dermal fibroblasts from SSc patients (n=8) as compared with fibroblasts from normal skin (n=6; figure 2C).

We previously reported that the increase of Smad-3 phosphorylation and of col1α2 mRNA levels parallels the mRSS values and therefore relates to the histological grade of fibrosis. In the present study, MSC were obtained from patients with a mean mRSS Rodnan score of 19.7±3.8 (n=9; table 1). This mean value revealed that cutaneous fibrosis was quite intense in the studied patients.

**DISCUSSION**

Our present analysis of TGF-β signalling in MSC and in skin fibroblasts from SSc patients allowed us to demonstrate a significantly increased expression of TBRII in both MSC and skin fibroblasts from SSc patients that lead to alteration of the TGF-β-induced Smad pathway and collagen 1α2 synthesis in these cells. These data are in agreement with our previous results showing that TGF-β-dependent Smad pathway was increased in dermal SSc fibroblasts, and with previous reports showing that mRNA steady state levels of TGF-β receptors, col1α2 and PAI-1 were increased in SSc fibroblasts as compared to their normal counterparts.

As TGF-β receptors were shown to be up-regulated by several growth factors including TGF-β itself, the overexpression of TBRII observed in MSCs from SSc patients as well as in dermal SSc fibroblasts could be due to exposure to their autocrine TGF-β synthesis. Enhanced expression of TGF-β receptors might favour SSc fibrosis since the overexpression of TGF-β receptors was shown to induce collagen transcription in cultured dermal fibroblasts, and of dominant negative TBRII to inhibit ECM production in SSc fibroblasts.
In accordance with the recent demonstration that MSC could be differentiated into fibroblasts, our results suggest that the TBRII alteration observed in peripheral dermal fibroblasts from SSc patients might derive from a defect in their parental mesenchymal stem cells. It can be suggested that the TGF-β hypersensitivity of MSC from SSc patients, as shown by the increase in collagen mRNA expression in response to the mediator, might favour their own differentiation into fibroblasts and/or organ fibrosis. The relation we observed between the increase of TGF-β–signalling pathway in both MSC and fibroblasts with the fibrosis histological grade, as measured by mRSS score, favours the link between central mesenchymal stem cell defect and peripheral fibrosis in SSc patients. However, further studies with larger numbers of SSc patients will be helpful for refined analysis of TBR expression in SSc MSC and SSc fibroblasts and better understanding of their potent impact on cell lineage.

The present results question the use of autologous MSC from SSc patients, alone or as adjuvant therapeutic product. While in vitro SSc-MSC immunomodulatory properties have been found to be similar to that of healthy donors, other studies revealed that SSc-MSC displayed impaired endothelial cell differentiation capacities, and overexpressed and secreted proangiogenic factors such as VEGF and SDF-1. These points should be carefully considered when choosing the source of MSC, allogeneic or autologous, for clinical use in SSc patients.

Figure 1 Basal mRNA and TBRII membrane expression in MSC. MSC were derived from bone marrow aspirates of normal donors (normal MSC) and SSc patients (SSc MSC). (A) Basal mRNA levels of TBRII, TBRI, TGF-β, Smad-2, Smad-3, Smad-4 and Smad-7 were measured by real-time PCR in normal MSC (n=6) and SSc MSC (n=9). (B) TBRII membrane expression was quantified by mean fluorescence intensity (MFI) and percentage of positive cells (%) using flow cytometry analysis. An index of membrane expression (TBRII%MFI) was calculated and expressed as mean±SD for normal MSC (n=9) and SSc MSC (n=9, left panel). TBRII expression on MSC from one representative normal donor (medium panel) and one representative SSc patient (right panel) is shown in grey, with overlaying of respective isotype controls. MSC, mesenchymal stromal cell; SSc, systemic sclerosis; TBRII, transforming growth factor-β receptor type II.
CONCLUSION

Our study reveals that the expression of TBRII is significantly increased in SSc MSC, with a consequent defect in the production of collagen type 1α2 in response to TGF-β activation. This defect in the MSC upstream may have a pathological significance in SSc. The present results question the use of autologous MSC from SSc patients, alone or as adjuvant therapeutic product.

Figure 2  Activation of the TGF-β-induced Smad pathway and increased synthesis of collagen 1α in MSC from SSc patients. (A) Normal (n=6) and SSc MSC (n=6) were cultured in the presence of TGF-β during 1, 3, 12 and 24 h. Collagen 1α2 mRNA (Coll1α2) was measured by real-time PCR. *p<0.05. (B) SSc MSC (left panels) and control MSC (right panels) were cultured with 5 ng/ml TGF-β during 15, 30 and 60 min. Phosphorylated Smad-3 and respective β-actin protein expressions were analysed by western blotting. (C) Primary cultures of dermal fibroblasts were derived from skin of healthy donors (n=6) or SSc patients (n=8). Basal TBRII (left) and coll1α2 (right) mRNA levels were measured by real-time PCR. MSC, mesenchymal stromal cell; SSc, systemic sclerosis; TGF-β, transforming growth factor-β.

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TGFβ RII in MSC from patients with scleroderma

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