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Identification of CD146 as a novel molecular actor involved in systemic sclerosis

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METHODS

Subjects

Sera from 50 SSc patients admitted to internal medicine departments in Marseille (South of France) were analyzed. All patients fulfilled the 2013ACR/EULAR Classification Criteria for

Scleroderma⁵ and were then sub classified according to LeRoy et al criteria.⁶ For 15 patients, sera were available 12 months later. As controls, we studied the sera of 50 age and sex matched blood donors. All samples came from a declared Biobank (DC 2012-1704) with respect of ethical directives and were stored to -80°C prior to use.

Soluble CD146 determination

A capture enzyme-linked immunosorbent assay (CY-QUANT ELISA) was used to determine the levels of sCD146 (Biocytex, Marseille, France), according to the manufacturer's protocol. Coefficients of variation of repeatability, reproducibility lot to lot, and reproducibility within batch were respectively 8.2%, 2.9%, and 3.5%.

***In vitro* tests**

Bio-activity of seric sCD146 in SSc patients was determined using cell proliferation experiments performed on endothelial progenitor cells (EPC) previously incubated with sera of SSc with high ($>734\text{ng/ml}$; $n=24$) or low ($<734\text{ng/ml}$; $n=17$) sCD146 levels, or with sCD146-immunodepleted samples ($n=21$).

sCD146 serum immunodepletion: In three independent experiments, sCD146 was removed from the plasma ($n=21$). First, sera containing high levels of sCD146 ($>734\text{ng/ml}$) were incubated with S-Endo-1 antibody, and tubes were loaded on a test tube rotator and agitated overnight at $+4^{\circ}\text{C}$. Then, immune complex were covalently immobilized on protein-A Sepharose beads.

The unabsorbed fraction was recovered by centrifugation at 1500rpm for 10 min, and was used in the following tests.

Isolation of circulating progenitor endothelial cells (EPC) and cell culture: Human umbilical cord blood samples from donors were collected, in compliance with French legislation, in a heparinized tube. Mononuclear cells (MNC) were isolated by density gradient centrifugation as previously described.^{E1}

Cell proliferation assay: EPC were seeded on 96-well plates ($7.10^3/\text{well}$) and starved in RPMI-10%FCS overnight. Cells were then incubated in EBM2 medium with 1% of serum for 24 hours. Cell proliferation was assayed by 5-bromo-2'-deoxy-uridine (BrdU) incorporation into cellular DNA using the BrdU Labeling and Detection Kit III (Roche Diagnostics, Mannheim, Germany). In brief, cells were incubated 15 hours with BrdU labeling solution in EBM-2 medium. Cellular DNA was partially digested by nuclease treatment and incorporated

BrdU was detected with a peroxidase-conjugated anti-BrdU primary antibody. The absorbance was measured at 450 nm using an Opsys MR micro-plate reader (Dynex technologies, Chantilly, USA). Results were expressed as arbitrary units.

Animal models of SSc

CD146KO mice^{E2} aged between 11-18 weeks were treated with subcutaneous bleomycin and were compared to littermates of the same age and gender wild type (WT) mice.

Skin fibrosis was induced, according to protocol modified from Ould-Ali et al.^{E3} by local subcutaneous injections of 100µL of bleomycin dissolved in 0.9% NaCl, at a concentration of 10µg/mL^{E4}, every other day for 28 days. sCD146, dissolved in PBS, was also injected at a concentration of 100µg/mL, every other day, jointly with bleomycin. Bleomycin with or without sCD146 was injected in defined areas on the right upper back of CD146 KO (10 and 3 mice respectively) and WT (11 and 3 mice respectively) mice. Vehicle (NaCl±PBS) was injected in defined areas on the left upper back of CD146 KO and WT mice and was used as controls for the bleomycin±sCD146 experiment, respectively.

After approval of the protocol by the Institution's Animal Care and Use Committee, the experiments were performed in an authorized laboratory and under the supervision of an authorized researcher (GUILLET 13-328).

Histological analysis

Murine skin sections were fixed in 4% formalin, embedded into paraffin. Several 7µm sections were stained with Haematoxylin-Eosin stain (HE).

Dermal and epidermal thickness was quantitatively evaluated using a stereological method, using a Merz grid^{E5,E6} (Fig E2). The grid is composed of a wavy array of lines (108 tests points, 40mm spacing, total length of test lines: 6785.8mm).^{E5}

Pictures of murine skin sections were taken, using Leica microscope (magnification x10) and the grid was surimposed on each pictures. The volume density of derm or epiderm was estimated by counting the points of the Merz grid located on the derm or epiderm. The surface area was estimated by counting the number of intersections of the sinous line with the border of each tissue. The dermal and epidermal thickness was thus quantitatively calculated using the volume density/surface area ratio.

Impact of bleomycin (1µg/mouse) and/or sCD146 injection (10µg/mouse) on dermal and epidermal thickness was evaluated after normalization with corresponding control condition

(vehicle).

Statistical Analysis

Statistical analysis was performed with the Prism software (GraphPad Software Inc., San Diego, CA). Significant differences were determined using non-parametric Mann Whitney test. A P-value < 0.05 was considered as significant.

REFERENCES

- E1. Bompais H, Chagraoui J, Canron X, Crisan M, Liu XH, Anjo A, et al. Human endothelial cells derived from circulating progenitors display specific functional properties compared with mature vessel wall endothelial cells. *Blood* 2004;103:2577–84. doi: 10.1182/blood-2003-08-2770
- E2. Jouve N, Bachelier R, Despoix N, Blin MG, Matinzadeh MK, Poitevin S, et al. CD146 mediates VEGF-induced melanoma cell extravasation through FAK activation. *Int J Cancer* 2015;137:50–60. doi: 10.1002/ijc.29370
- E3. Ould-Ali D, Hautier A, Andrac-Meyer L, Bardin N, Magalon G, Granel B. Bleomycin-induced Scleroderma in Nude Mice can be Reversed by Injection of Adipose Tissue: Evidence for a Novel Therapeutic Intervention in Systemic Sclerosis. *J Clin Exp Dermatol Res* [Internet] 2013 [cited 2016 Jun 27];03. Available from: <http://www.omicsonline.org/2155-9554/2155-9554-3-164.digital/2155-9554-3-164.html>. doi: 10.4172/2155-9554.1000164
- E4. Yamamoto T, Nishioka K. Cellular and molecular mechanisms of bleomycin-induced murine scleroderma: current update and future perspective. *Exp Dermatol* 2005;14(2):81–95. doi: 10.1111/j.0906-6705.2005.00280.x
- E5. Massacrier A, Nègre-Aminou P, Couraud F, et al. Quantitative analysis of fetal rat brain neurons developing in primary cultures. I. Stereological study of the neuronal differentiation. *Brain Res*. 1988;468(2):161-70.
- E6. Weibel, E.R., Stereological Methods. Vol. 1: Practical Methods for Biological Morphometry, Academic, London, 1979, 415 pp.

FIGURE LEGENDS (Online Repository Figures E1 and E2)

FIG E1. Demonstration of sCD146 bio-activity in serum of patients with SSc

A: sCD146 significantly correlated with proliferative index (n=41; p=0.021, Pearson test).

B: Sera containing highest sCD146 levels were immunodepleted. Proliferation was significantly reduced after depletion ($102.8\% \pm 8.7$ versus $72.14\% \pm 6.9$, p<0.0001, Wilcoxon test).

C: Efficiency of immunodepletion was checked by ELISA determination of sCD146, showing a reduction of 80% of sCD146 levels ($1029\text{ng/ml} \pm 41.7$ versus $228.1\text{ng/ml} \pm 4.4$; n=21; p<0.0001, Wilcoxon test).

FIG E2. Merz grid.

The grid consists of a square that delimits a test area containing a system of 108 points marked on a sinuous line formed by the succession of 9 chained semicircles. The thickness was thus calculated using the volume density/surface area ratio.