Concise report

A possible contribution of visfatin to the resolution of skin sclerosis in patients with diffuse cutaneous systemic sclerosis via a direct anti-fibrotic effect on dermal fibroblasts and Th1 polarization of the immune response

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Abstract

Objective. Visfatin is a member of the adipocytokines with pro-fibrotic, pro-inflammatory and immunomodulating properties potentially implicated in the pathogenesis of certain fibrotic and inflammatory autoimmune diseases. In this study, we investigated the clinical significance of serum visfatin levels and its contribution to the developmental process in SSc.

Methods. Serum visfatin levels were determined by a specific ELISA in 57 SSc patients and 19 healthy controls. The mRNA levels of target genes were determined in normal and SSc fibroblasts by real-time RT-PCR. The levels of IL-12p70 produced by THP-1 cells were measured by a specific ELISA.

Results. Serum visfatin levels were comparable among total SSc, diffuse cutaneous SSc (dcSSc), limited cutaneous SSc and healthy controls. The only finding in a series of analyses regarding the correlation of serum visfatin levels with clinical symptoms and laboratory data was the significantly longer disease duration in dcSSc with elevated serum visfatin levels than in those with normal levels. Consistently, serum visfatin levels were significantly elevated in late-stage dcSSc (disease duration >6 years), but not in early and mid-stage dcSSc compared with healthy controls. In vitro experiments, visfatin reversed the pro-fibrotic phenotype of SSc dermal fibroblasts and induced the expression of IL-12p70 in THP-1 cells treated with IFN-γ plus lipopolysaccharide.

Conclusion. Visfatin may contribute to the resolution of skin sclerosis in late-stage dcSSc via a direct anti-fibrotic effect on dermal fibroblasts and Th1 polarization of the immune response.

Key words: systemic sclerosis, visfatin, collagen, matrix metalloproteinase 1, interleukin-12.

Introduction

SSc is a multisystem autoimmune disease characterized by vascular injuries and fibrosis of skin and certain internal organs. Although the pathogenesis of SSc remains unknown, our latest studies disclosed a possible contribution of adipocytokines to the pathological process of SSc [1].

Visfatin is a member of the adipocytokines, which are key regulators of metabolism and insulin resistance [2]. Emerging evidence has demonstrated that some adipocytokines have pro- or anti-fibrotic, pro- or anti-inflammatory and immunomodulating properties [3–6]. Visfatin is ubiquitously expressed in different cell types, including adipocytes, macrophages and lymphocytes. Reflecting
its pro-inflammatory properties, visfatin is up-regulated in adequate inflammatory conditions, including models of acute lung injury and clinical and experimental sepsis [7]. On the other hand, visfatin appears to be involved in the pathological process of inflammatory autoimmune diseases, including RA, Behçet’s disease (BD) and IBD [8, 9]. Regarding SSc, despite the potential role of visfatin in cardiac and hepatic fibrosis [4–6], a previous report revealed that serum visfatin levels are comparable between patients and healthy controls [9], suggesting a small role of visfatin in the pathological process of SSc. However, since SSc is a heterogeneous disease consisting of certain disease subsets, including diffuse cutaneous SSc (dcSSc) and limited cutaneous SSc (lcSSc), this study was conducted to precisely evaluate the significance of visfatin in the pathological process of SSc.

Materials and methods

Patients

Serum samples, frozen at −80°C until assayed, were obtained from 57 SSc patients (55 women, 2 men) and 19 healthy individuals (18 women, 1 man). Patients treated with corticosteroids or other immunosuppressants prior to their first visits were excluded. Patients were grouped by the LeRoy classification system [10]: 28 patients with lcSSc and 29 with dcSSc. All dcSSc and 23 lcSSc patients fulfilled the criteria proposed by the ACR [11]. Five lcSSc patients not meeting these criteria had sclerodactyly and at least two of the following features: calcinosis, RP, esophageal dysmotility and telangiectasia. The study was performed according to the Declaration of Helsinki and approved by the ethical committee of the University of Tokyo Graduate School of Medicine.

Measurement of serum visfatin levels

Specific ELISA kits (Phoenix Pharmaceuticals, Belmont, CA, USA) were used to measure serum visfatin levels (analytical range 0.1–1000 ng/ml) according to the manufacturer’s instructions without any modification. Polystyrene 96-well plates coated with anti-visfatin antibodies were incubated with diluted sera, then washed and incubated with horseradish peroxidase-conjugated anti-visfatin antibodies. The wells were washed again, tetramethylbenzidine was added and then the wells were incubated. Finally, sulphuric acid was added to terminate the reaction and the absorbance at 450 nm was measured. Serum visfatin levels were calculated using a standard curve.

Clinical assessments

The clinical and laboratory data were obtained when the blood samples were drawn. Disease duration was defined as the interval between the onset, defined as the first clinical event of SSc other than RP, and the time the blood samples were drawn. The details of assessments for clinical symptoms are briefly summarized in the legends of supplementary Table S1, available at Rheumatology Online.

Cell cultures

Dermal fibroblasts were obtained by skin biopsy from the affected areas (dorsal forearm) of five dcSSc patients with <2 years of skin thickening (skin score of biopsy site: 3 for two patients, 2 for one patient and 1 for the others) and from the corresponding areas of five closely matched healthy donors. THP-1 cells were purchased from American Tissue Culture Collection (Rockville, MA, USA).

RNA isolation and real-time RT-PCR

RNA isolation and real-time RT-PCR were carried out as described previously [12]. The sequences of human α2(I) collagen (COL1A2), MMP-1 and 18S rRNA primers are summarized in supplementary Table S2, available at Rheumatology Online.

Measurement of IL-12p70 production by THP-1 cells

The production of IL-12p70 from THP-1 cells was analysed by a specific ELISA kit (BD Pharmingen, San Diego, CA, USA) using the cell culture media following the manufacturer’s specifications with minor modification. We diluted standard samples and confirmed the complete correlation of IL-12p70 concentrations with the OD 595 nm light absorption values between 0.488 pg/ml and 125 ng/ml ($R^2 = 0.9998$).

Statistical analysis

The statistical analysis was carried out with a Kruskal–Wallis test and a Steel–Dwass test for multiple comparison and with Mann–Whitney U test for two-group comparison. Correlations with clinical data were assessed by Spearman’s rank correlation coefficient. Statistical significance was defined as a P-value <0.05.

Results

Serum visfatin levels and their clinical correlation in SSc

There was no difference in serum visfatin levels among total SSc, dcSSc, lcSSc and healthy controls (Fig. 1A). Given that there was a subset of SSc patients with increased serum visfatin levels (above dotted line in Fig. 1A), especially in dcSSc, serum visfatin levels potentially reflect some aspect of disease process in SSc.

We next evaluated the correlation of serum visfatin levels with parameters reflecting dermal and pulmonary fibrosis, such as modified Rodnan total skin thickness score, the percentage of predicted vital capacity and the percentage of predicted diffusion lung capacity for carbon monoxide, in dcSSc, lcSSc and dcSSc with disease duration <6 years in which fibrotic response is extensively active. No significant correlation was detected (data not shown). For further analyses, SSc patients were classified into two groups according to cut-off value [mean + 2 s.d. (10.60 ng/ml) of controls, which is normally distributed]. Clinical features, other than skin sclerosis and interstitial lung disease, of SSc patients with elevated or normal serum visfatin levels were evaluated in dcSSc and lcSSc (supplementary Table S1, available at Rheumatology Online).
Online). There was no significant difference between these groups in terms of sex or age of onset, while disease duration was significantly longer in patients with elevated serum visfatin levels than in those with normal levels among dcSSc [7 years (2.3–14.5) vs 2 years (1.1–3)], but not among lcSSc. Regarding other symptoms, the prevalence of each was comparable between the two groups in dcSSc and lcSSc. Although visfatin is a pro-inflammatory cytokine [3], the levels of high-sensitivity CRP and ESR did not correlate with serum visfatin levels in dcSSc, dcSSc with disease duration <6 years and lcSSc (data not shown). Collectively, serum visfatin levels are

Fig. 1 Concentrations of visfatin in sera from patients with SSc and healthy individuals.
barely associated with any clinical features, except for disease duration, in SSC.

Increase in serum visfatin levels along with disease duration in dcSSc

We further classified dcSSc patients into three subgroups according to their disease duration: early dcSSc (disease duration <1 year), mid-stage dcSSc (disease duration 1–6 years) and late-stage dcSSc (disease duration >6 years), and evaluated the correlation of serum visfatin levels with disease stage. As shown in Fig. 1B, the multiple comparison analysis showed the significant difference between late-stage dcSSc and healthy controls, suggesting that visfatin contributes to certain pathological processes in late-stage dcSSc.

Visfatin exerted an anti-fibrotic effect on SSc dermal fibroblasts and increased IL-12p70 production in THP-1 cells

Late-stage dcSSc is generally characterized by the spontaneous resolution of skin sclerosis, suggesting that visfatin has an anti-fibrotic effect on SSc dermal fibroblasts. Therefore we investigated the effect of visfatin on the COL1A2 and MMP-1 mRNA levels in normal and SSc dermal fibroblasts. Consistent with previous reports [13, 14], COL1A2 mRNA levels significantly increased in SSc dermal fibroblasts compared with normal dermal fibroblasts, while MMP-1 mRNA levels were comparable between them (Fig. 2A), indicating that dermal fibroblasts from patients maintain SSc phenotype. Therefore the following experiments were carried out by using these cells. In SSc dermal fibroblasts, expectedly, visfatin suppressed COL1A2 mRNA expression, while increasing MMP-1 mRNA levels, in a dose-dependent manner. In contrast, visfatin did not affect the COL1A2 and MMP-1 mRNA levels in normal dermal fibroblasts. These results were also confirmed at protein levels by immunoblotting (Fig. 2B and C). Importantly, visfatin also reversed the pro-fibrotic phenotype of normal dermal fibroblasts stimulated with transforming growth factor β1 (supplementary Fig. S1, available at Rheumatology Online). Collectively visfatin exerts an anti-fibrotic effect on activated dermal fibroblasts with the pro-fibrotic phenotype.

Given that immune polarization in dcSSc generally shifts from T helper 2 (Th2) to T helper 1 (Th1) along with disease course [15], the elevation of visfatin in late-stage dcSSc suggests that visfatin promotes Th1 polarization of the immune response. Supporting this, visfatin induced the up-regulated expression of IL-12p70 in THP-1 cells differentiated with IFN-γ and lipopolysaccharide (LPS) (Fig. 2D). Thus visfatin-dependent Th1 immune polarization may contribute to the skin resolution in late-stage dcSSc.

Discussion

The present study provided the following new findings: (i) disease duration is significantly longer in dcSSc with elevated serum visfatin levels than in those with normal levels; (ii) serum visfatin levels are comparable to normal levels during early- and mid-stage dcSSc, but are significantly increased in late-stage dcSSc and (iii) visfatin exerts an anti-fibrotic effect on SSc dermal fibroblasts and is likely to skew toward Th1 immune responses. These suggest that the elevation of visfatin is involved in the mechanism explaining the resolution of skin sclerosis in late-stage dcSSc.

The immunomodulating property of visfatin has been well studied in RA and its animal models. In addition to high visfatin levels in plasma, serum and SF in RA [16, 17], visfatin gene expression is increased in synovial tissue from RA [17, 18] and in inflamed synovial tissue of mice with collagen-induced arthritis [18]. Importantly, pharmacological inhibition of visfatin significantly reduces inflammation, cartilage damage and the severity of arthritis with comparable activity to TNF inhibitors in a collagen-induced arthritis model [19]. Supporting this, an inhibitor of visfatin decreases the production of TNF-α and IL-6 by murine peritoneal exudate inflammatory cells and human monocytes in vitro [19]. Similar to RA, circulating visfatin levels are elevated and positively correlate with disease activity in BD [3, 9]. Given that RA and BD are characterized by Th1 polarization of Th immune response, visfatin may contribute to Th1 immune polarization in the active stage of these diseases. Since immune polarization in SSc generally shifts from Th2 to Th1 in parallel with disease duration [15], the present serum data, together with previous data in RA and BD, suggest that visfatin contributes to the resolution of skin sclerosis by promoting Th1 immune polarization in concert with various cytokines in SSc. Consistent with our hypothesis, visfatin increased the production of IL-12p70 in THP-1 cells differentiated with IFN-γ and LPS. Collectively visfatin may contribute to the resolution of skin sclerosis at least partially by promoting the Th1 immune polarization in late-stage dcSSc.

Another novel finding in this study was that visfatin elicits an anti-fibrotic effect on SSc dermal fibroblasts. The effect of visfatin on fibrosis has been well studied in a couple of fibrotic disorders. Serum visfatin levels correlate with fibrosis scores in patients with chronic hepatitis C [4] and visfatin expression is positively associated with fibrosis stage in non-alcoholic fatty liver disease [5]. Supporting this, visfatin induces the expression of basic fibroblast growth factor in rat hepatic stellate cells [20]. Furthermore, visfatin promotes the proliferation and the synthesis of type I and type III collagen in rat cardiac fibroblasts [6]. Thus visfatin is potentially associated with the development and progression of fibrotic condition in cardiac and liver fibrosis. Taken together with these previous data, our present observation suggests that visfatin has a dual effect on fibrosis in a context-dependent manner. Although the detailed mechanism by which visfatin reverses the pro-fibrotic phenotype in SSc fibroblasts remains unknown, visfatin coordinately promotes the resolution of skin sclerosis in late-stage dcSSc by inhibiting the pro-fibrotic phenotype of SSc dermal fibroblasts and driving Th1 polarization.
The role of visfatin in SSc

Subconfluent normal and SSc dermal fibroblasts were serum starved for 24 h, then the cell culture medium was refreshed and cells were stimulated with the indicated concentration of visfatin (PeprTech, Rocky Hill, NJ, USA) for another 24 h. mRNA levels of human \( \alpha 2(I) \) collagen (COL1A2) and MMP-1 genes were determined by real-time RT-PCR under a quiescent condition (A) or after the treatment with the indicated concentration of visfatin (B, C). The levels of type I collagen [\( \alpha 1(I) \) collagen (\( \alpha 1(I) \)) and \( \alpha 2(I) \) collagen (\( \alpha 2(I) \))] and MMP-1 proteins in the culture media were also determined by immunoblotting using anti-type I collagen antibody (Southern Biotech, Birmingham, AL, USA) and anti-MMP-1 antibody (Millipore, Billerica, MA, USA). The volume of each medium sample analysed was normalized based on cell number. THP-1 cells in the log phase of growth were primed with 100 ng/ml IFN-\( \gamma \) (R&D Systems, Minneapolis, MN, USA) for 6 h and stimulated with 100 ng/ml LPS (InvivoGen, San Diego, CA, USA) in the presence or absence of visfatin. Sixteen hours after treatment, the levels of IL-12p70 in each supernatant were analysed by a specific ELISA kit (D). The statistical analysis was carried out with Mann–Whitney U test for two-group comparison and with a Kruskal–Wallis test and a Steel–Dwass test for multiple comparison. *\( P < 0.05 \) versus normal dermal fibroblasts. **\( P < 0.05 \) versus SSc dermal fibroblasts without visfatin stimulation. ***\( P < 0.05 \) versus THP-1 cells without any stimulation and those treated with IFN-\( \gamma \) and LPS.
In summary, this study is the first regarding the potential role of visfatin in the disease process of dcSSc. Visfatin may exert a direct anti-fibrotic effect on dermal fibroblasts and an indirect anti-fibrotic effect by promoting Th1 immune polarization in dcSSc. The present data further support an emerging idea that adipocytokines play an important role in the pathogenesis of autoimmune diseases, including SSc.

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**Supplementary data**

Supplementary data are available at Rheumatology Online.

**References**


