Serum levels of soluble carbonic anhydrase IX are decreased in patients with diffuse cutaneous systemic sclerosis compared to those with limited cutaneous systemic sclerosis

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Summary
Hypoxia may play an important role in the pathogenesis of systemic sclerosis (SSc). Carbonic anhydrase IX (CA IX) is one of the hypoxia markers and its extracellular domain can be released into the serum. However, the clinical significance of serum CA IX levels in SSc is still unknown. The aim of this study is to evaluate the possibility that serum CA IX levels can be a specific disease marker of SSc. Serum samples were obtained from SSc patients and healthy controls. Patients diagnosed as scleroderma spectrum disorder (SSD), who did not fulfill the ACR criteria of SSc but were thought that they might develop SSc in the future, were also included in this study. Serum CA IX levels were measured with specific enzyme-linked immunosorbent assays. SSD patients had significantly lower CA IX levels than diffuse cutaneous SSc (dcSSc), limited cutaneous SSc (lcSSc) and healthy control groups. Also, we found a significant decrease in the values in dcSSc patients compared to those of lcSSc patients. Serum levels of CA IX may be useful for the differentiation of lcSSc from SSD. Decreased serum CA IX levels in spite of the presence of hypoxia in SSc may indicate an impaired response to hypoxia, which leads to the persistent hypoxic condition. Our results suggest that the abnormal response to hypoxia may already exist in SSD patients, and may be involved in its pathogenesis.

Keywords: Carbonic anhydrase IX, collagen disease, hypoxia, systemic sclerosis

1. Introduction

Systemic sclerosis (SSc) or scleroderma is an acquired disorder which typically results in fibrosis of the skin and internal organs. Although the pathogenesis of this disease is still unclear, it includes inflammation, autoimmune attack, and vascular damage, leading to activation of fibroblasts and abnormal accumulation of extracellular matrix, mainly collagen (1,2).

Microangiopathy is one of the primary pathologic components of SSc (3). Raynaud’s phenomenon or aberrant nailfold bleeding is known as an early vascular event of this disease. Telangiectasia, pitting scars, skin ulcers, impaired wound healing or pulmonary hypertension are frequently observed in the disease process, and they can severely affect the quality of life in these patients.

The microangiopathy causes a reduction of blood flow, which results in tissue hypoxia. The tissue ischemia leads usually to the expression of angiogenic growth factors, which act against the ischemic conditions. Hypoxia induced factor (HIF)-1α, one of the hypoxic markers, is a transcription factor which regulates cellular adaptation to low oxygen tension (4). Under normoxic conditions, the expression of HIF-1α is maintained at a low level by ubiquitination and degradation (5). In hypoxic conditions, HIF-1α is up-regulated and translocated to the nucleus where it induces transcription of target genes essential for survival and adaptation to hypoxic environments, such as vascular endothelial growth factor (VEGF), glucose transporter 1 (GLUT-1), and erythropoietin (6).
Cutaneous hypoxia in patients with SSc was reported (7). Distler O et al. describe that despite severely reduced oxygen levels, protein levels of HIF-1α in the skin of SSc patients were even below the levels seen in healthy control skin (8). Therefore, the impaired response to tissue hypoxia in SSc patients may lead to persistence of hypoxic conditions. Hypoxia contributes directly to progression of fibrosis by activation of fibroblasts in SSc. On the other hand, excess extracellular matrix deposition increases diffusion distances from blood vessels to cells, and induces further hypoxic conditions (9). This vicious circle is thought to be associated with the pathogenesis of SSc.

In this study, we focused on carbonic anhydrase IX (CA IX), another hypoxia marker. Carbonic anhydrases (CAs) are a family of zinc-containing enzymes, that catalyze a reversible conversion of carbon dioxide to bicarbonate and a proton in the reaction: CO₂+H₂O↔HCO₃⁻+H⁺ (10). These enzymes participate in a variety of biological processes, including respiration, ion transport, pH balance, and bone resorption (11). Human CAs exist in at least 15 isoforms (12). Among them, CA IX is a membrane-associated protein and is known as a biomarker of hypoxia or certain malignant tumors. The expression of CA IX can only be detected in a few normal tissues, whereas it is abnormally induced in hypoxic conditions or malignant tumors (13). The relationship of CA IX with hypoxia has been explained by the notion that the CA IX promoter contains a hypoxia response element (HRE) to which HIF-1 can bind (14). Because the extracellular domain of CA IX can be released into cell culture medium or into the body fluids (15), unlike HIF-1α, CA IX can be detected in serum. As described above, hypoxia plays an important role in the pathogenesis of SSc. Although the clinical significance of serum CA IX levels in SSc is still unknown, they can be correlated with disease activity. Thus, in this study, we try to evaluate the possibility that serum levels of CA IX can be a useful marker of SSc.

2. Materials and Methods

2.1. Clinical assessment and patient material

Serum samples were obtained from 43 patients with SSc (7 men and 36 women; age range, 7-85 years; mean, 57.4 years). All patients fulfilled the criteria proposed by the American College of Rheumatology, and were grouped according to the classification system proposed by LeRoy et al. (16): 20 patients had diffuse cutaneous SSc (dcSSc) and 23 patients had limited cutaneous SSc (lcSSc), as described previously (17). Control serum samples were also collected from healthy age- and sex-matched volunteers. Five patients diagnosed as scleroderma spectrum disorder (SSD), who did not fulfill the ACR criteria of SSc but were thought that they might develop SSc in the future based on the criteria proposed by Ihn et al., were also included in this study (18-20). Institutional review board approval and written informed consent were obtained before patients and healthy volunteers were entered into this study according to the Declaration of Helsinki. All serum samples were stored at –80°C prior to use.

2.2. Measurement of serum CA IX concentrations

Levels of serum CA IX were measured with a specific ELISA kit (R&D Systems, Minneapolis, MN, USA). Briefly, anti-CA IX monoclonal antibodies were precoated onto microtiter wells. Aliquots of serum were added to each well, followed by peroxidase-conjugated antibodies to CA IX. Color was developed with hydrogen peroxide and tetramethylbenzidine peroxidase and the absorbance at 450 nm was measured. Wavelength correction was performed by absorbance at 570 nm. The concentration of CA IX in each sample was determined by interpolation from a standard curve.

2.3. Statistical analysis

Statistical analysis was carried out with a Welch two sample t-test for the comparison of means, and Fisher's exact probability test for the analysis of frequency. p values less than 0.05 were considered significant.

3. Results and Discussion

The serum CA IX levels in patients with SSc and in healthy control subjects are shown in Figure 1. Serum samples were obtained from 43 patients with SSc. Twelve healthy control subjects and 5 SSD patients, who did not fulfill the criteria of SSc but were thought that they might develop SSc in the future based on the criteria proposed by Ihn et al. (20).

Although mean serum CA IX levels were higher in SSD patients (146 ± 198 pg/mL) than in healthy control subjects (118 ± 115 pg/mL), there was no statistically significant difference between the two groups. However, when SSc patients were classified into lcSSc and dcSSc as described in 'Patients and Methods', we found a significant decrease in the values of dcSSc patients than in those of lcSSc patients (82 ± 68 vs. 201 ± 254 pg/mL, p < 0.05). The mean serum levels were higher in lcSSc patients and lower in dcSSc patients than those in healthy controls, but there were no significant difference.

On the other hand, CA IX levels in all 5 SSD patients were decreased as compared to other groups;
SSD patients had significantly lower CA IX levels than healthy controls (42 ± 28 vs. 118 ± 114 pg/mL, p < 0.05) and SSC patients (42 ± 28 vs. 146 ± 198 pg/mL, p < 0.01). In addition, the difference between SSD patients and lcSSC patients (42 ± 28 vs. 201 ± 254 pg/mL, p < 0.01) was more significant than that between SSD patients and dcSSC patients (42 ± 28 vs. 82 ± 68 pg/mL, p < 0.05). Taken together, the serum CA IX levels were decreased in patients with SSD and dcSSC in that order, with statistical significance.

Table 1 shows the association of serum CA IX levels with clinical features in SSC patients. Considering that HIF-1α expression in the skin of SSC patients was previously reported to be below the levels seen in healthy control skin (8), and that the serum CA IX levels in SSD and dcSSC patients tended to be decreased in our study, we regarded reduction of CA IX levels as the meaningful change in SSC patients. As shown in Table 1, in SSC patients with reduced CA IX levels, the percentage of dcSSC was significantly increased compared to those with normal CA IX levels (dcSSC:lcSSC = 14:8 vs. 6:15, p = 0.022). There was no statistically significant difference between these groups in terms of sex, mean age at onset, duration of disease, and other clinical or laboratory features including therapy, smoking history, respiratory dysfunction or anemia.

In this study, although we expected that hypoxia marker CA IX was up-regulated in SSC sera due to its hypoxic conditions, there was no significant difference in serum CA IX between healthy controls and SSC patients. However, we found that SSD patients had significantly lower CA IX levels than control subjects, dcSSC or lcSSC patients. In addition, serum CA IX levels were significantly lower in dcSSC patients compared to lcSSC patients.

The concept of SSD was originally proposed by Ihn et al. to unify typical SSC, early forms of SSC and closely related disorders including mixed connective tissue disease (MCTD) (18). Thereafter, Ihn et al. defined SSD as patients who did not fulfill the criteria of SSC but were thought that they might develop SSC in the future, and established a new diagnostic method using a points system to distinguish patients with SSD from those with early SSC. A total score was obtained as the sum of the following five factors: (1) extent of skin sclerosis (maximum, 10 points); (2) pulmonary changes (maximum, 4 points); (3) antinuclear antibodies (maximum, 5 points); (4) pattern of Raynaud’s phenomenon (maximum, 3 points); and (5) nailfold bleeding (maximum, 2 points). The authors suggest the conditions with 9 or more points are consistent with SSD (20). Because progressive fibrosis of SSC is often irreversible, at least clinically, there is an urgent need to develop new strategies to diagnose patients as early as possible and follow them carefully. For that purpose, the concept of SSD should be further understood and characterized. Our study is the first to perform ELISA experiments using SSD sera.

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To note, there were significant difference between
SSD patients and lcSSc patients. The diagnosis of SSc presents little problem when the clinical features have fully developed. However, it may be difficult to distinguish lcSSc from SSD, because skin sclerosis is sometimes not apparent in lcSSc, especially in a very early stage. Serum levels of CA IX may be useful for the differentiation of lcSSc from SSD. Moreover, we frequently encounter SSD patients with an increased risk of future development of SSc. Serial time-course measurement of serum CA IX concentration in SSD patients may lead to early detection of developing SSc.

As described above, CA IX is thought to be a downstream target of HIF-1α (14). Thus, reduced CA IX levels seen in dcSSc patients in our study is consistent with down-regulation of HIF-1α in SSc skin in spite of the presence of hypoxia (8), which may result in the persistent hypoxic condition of the disease. Our results suggest that such dysregulation of HIF-1α as well as CA IX and subsequent persistent hypoxia may already occur in SSD patients. Considering that microangiopathy is one of the primary symptoms of SSc and tissue fibrosis is observed in the disease process, the impaired response to microangiopathy-induced hypoxic conditions may be involved in the pathogenesis of SSD. Furthermore, a sustained abnormal response may lead to the development of severe skin sclerosis in dcSSc patients, whereas the reduction of CA IX may be transient in lcSSc. Otherwise, the discrepancy between dcSSc and lcSSc in CA IX levels may indicate heterogeneity between the two clinical subtypes.

There are some limitations to our results. First, we could not collect large number of SSD patients because of the rarity of this condition. However, our approach may be effective to clarify the properties of SSD. Larger studies are needed in the future. In addition, recent studies show transforming growth factor (TGF)-β, one of the key cytokines in the pathogenesis of SSc, can also regulate CA IX gene expression (21). Thus, there may be another pathway other than HIF-1α in the regulation of CA IX. The regulatory mechanisms of CA IX in this disease should be clarified.

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References


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