Novel Autoantibody to Cu/Zn Superoxide Dismutase in Patients with Localized Scleroderma

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Abnormal production of reactive oxygen species (ROS) induces tissue damage and superoxide dismutase (SOD) that converts superoxide radicals to hydrogen peroxide functions as defense against ROS. Cu/Zn SOD administration has been shown to be effective for various fibrotic conditions by inhibiting the fibrogenic effects of ROS. We hypothesized that autoimmune background in localized scleroderma induced anti-Cu/Zn SOD autoantibodies that inhibited SOD activity and thereby contributed to fibrosis by increasing ROS. ELISA using human purified Cu/Zn SOD revealed that IgG or IgM anti-Cu/Zn SOD Ab was detected in the serum of 89% of localized scleroderma patients, especially 100% of patients with generalized morphea, the severest form of localized scleroderma, but was positive only in the serum of less than 15% of patients with other autoimmune disorders, including systemic sclerosis, systemic lupus erythematosus, dermatomyositis, and autoimmune bullous disorders. The immunoblotting analysis confirmed the presence of IgG anti-Cu/Zn SOD Ab in sera from localized scleroderma patients. Remarkably, anti-Cu/Zn SOD autoantibody could inhibit Cu/Zn SOD enzymatic activity. Collectively, these results indicate that anti-Cu/Zn SOD Ab is a novel, major autoantibody in localized scleroderma, and also suggest that the autoantibody may play a role in the development of fibrosis by directly inhibiting SOD activity.

Key words: autoimmunity/fibrosis/reactive oxygen species
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Scleroderma is a chronic connective tissue disorder with an autoimmune background, and is divided into two types: localized scleroderma and systemic sclerosis (SSc). In localized scleroderma, fibrosis is limited to the skin and the subcutaneous tissues beneath the cutaneous lesions. This disease differs from SSc in that it is not accompanied by Raynaud's phenomenon, acrosclerosis, and internal organ involvement (Jablonska and Rodnan, 1979). Therefore, the prognosis for patients with localized scleroderma is good; however, the disfigurement and deformities of the extremities and face resulting from deep fibrosis markedly impair the quality of life (Jablonska and Rodnan, 1979). Morphologically, localized scleroderma is classified into three subsets: morphea, linear scleroderma, and generalized morphea (Sato *et al*, 1994b). Morphea is usually characterized by one or a few circumscribed sclerotic plaques with an ivory-colored center and a surrounding violaceous halo. Linear scleroderma appears in a linear, band-like distribution, and often involves the muscle and bone underlying the skin lesions. Generalized morphea is a severe form of localized scleroderma characterized by widespread skin involvement with multiple lesions.

Both localized scleroderma and SSc exhibit autoimmunity as a central feature of the diseases. Localized scleroderma, especially generalized morphea, is accompanied by the presence of various autoantibodies, such as antinuclear antibody (Ab), antihistone Ab, anti-single-stranded DNA (ssDNA) Ab, anti-phospholipid Ab, rheumatoid factor, and lupus erythematosus cell phenomenon (Falanga *et al*, 1986, 1987; Sato *et al*, 1993, 1994a, 2003). Similarly, SSc exhibits the production of autoantibodies against various intracellular components, such as DNA topoisomerase I, centromere, RNA polymerases, and U3RNP (Okano, 1996). Although localized scleroderma and SSc share some autoantibody specificities such as antihistone Ab, anti-ssDNA Ab, and rheumatoid factor, autoantibodies specific to SSc, such as anti-DNA topoisomerase I and anticentromere Abs, are absent in localized scleroderma with few exceptions (Sato *et al*, 1993). Thus, although both SSc and localized scleroderma show cutaneous fibrosis, autoantibody specificities associated with these diseases are different.

Life in an aerobic environment is associated with the production of superoxide radicals (O_2^-) that can lead to the formation of various reactive oxygen species (ROS) (Noor *et al*, 2002; Zelko *et al*, 2002). While physiological levels of ROS are beneficial to prevent invading pathogens, an unbalanced, elevated level of ROS induces tissue damage,

Abbreviations: Ab, antibody; DM, dermatomyositis; PV/PF/BP, pemphigus vulgaris/pemphigus foliaceus/bullous pemphigoid; ROS, reactive oxygen species; SLE, systemic lupus erythematosus; SOD, superoxide dismutase; SSc, systemic sclerosis; ssDNA, single-stranded DNA; TGF-β, transforming growth factor-β; TIMP, tissue inhibitor of metalloproteinases

resulting in the development of various disorders, such as inflammation, arthritis, diabetes, atherosclerosis, hypertension, and premature aging. The first and most important line of defense against the damaging effects of ROS is the superoxide dismutase (SOD) that converts O_2^- to hydrogen peroxide. There are three forms of the enzyme in mammals: a cytosolic form (Cu/Zn SOD or SOD1), a mitochondrial form (Mn SOD or SOD2), and an extracellular form (EC SOD or SOD3). The Cu/Zn SOD is a ubiquitous and constitutively expressed enzyme that is dependent upon copper and zinc ions for activity and accounts for 75% of total SOD activity (Marklund, 1984; Noor *et al*, 2002; Zelko *et al*, 2002).

A recent study has shown that ROS production is increased in dermal fibroblasts by stimulation with cytokines and induces fibroblast proliferation and expression of type I collagen genes in SSc-derived fibroblasts (Sambo *et al*, 2001). Furthermore, cutaneous infiltration of inflammatory cells, especially macrophages, is a potential source of ROS in localized scleroderma and SSc (Speer *et al*, 1984). Thus, the increased ROS production may be related to skin fibrosis in localized scleroderma and SSc. On the other hand, exogenous administration of Cu/Zn SOD has been shown to be effective for various fibrotic conditions, including radiation-induced cutaneous fibrosis (Delanian *et al*, 1994; Lefaix *et al*, 1996), bleomycin-induced fibrosis of the lung and skin (Yamazaki *et al*, 1997; Yamamoto *et al*, 1999), and cholestasis-induced liver fibrosis (Zhong *et al*, 2002). Furthermore, autoantibodies against Mn SOD have been detected in patients with acute Epstein–Barr virus infection (Ritter *et al*, 1994; Semrau *et al*, 1998), while autoantibodies to Cu/Zn SOD are present in patients with autoimmune hepatitis (Miyata *et al*, 1995). Therefore, we hypothesized that autoimmune background in localized scleroderma and SSc induced anti-Cu/Zn SOD autoantibodies that inhibited Cu/Zn SOD activity and thereby contributed to fibrosis by increasing ROS levels. To test this possibility, the presence or levels of autoantibodies to Cu/Zn SOD, their clinical correlation, and their functional significance were investigated in the current study. In this study, autoantibodies against Cu/Zn SOD were significantly elevated in patients with localized scleroderma, but not in those with SSc, compared with normal controls. The results of this study suggest that anti-Cu/Zn SOD autoantibodies detected in patients with localized scleroderma play a role in the development of fibrosis by directly inhibiting Cu/Zn SOD activity.

Results

Anti-Cu/Zn SOD autoantibody levels by ELISA were elevated in localized scleroderma The presence and levels of anti-Cu/Zn SOD autoantibodies in serum samples from patients with autoimmune diseases and normal controls were assessed by ELISA. Patients with localized scleroderma exhibited significantly higher anti-Cu/Zn SOD Ab levels of both IgG (Fig 1*a*) and IgM (Fig 1*b*) than those found in normal controls ($p < 0.0001$). By contrast, patients with SSc, SLE, DM, or PV/PF/BP had normal IgG and IgM anti-Cu/Zn SOD Ab levels; therefore, both IgG and IgM levels of anti-Cu/Zn SOD Ab in patients with localized scleroderma were significantly increased compared with those with SSc, SLE, DM, or PV/PF/BP ($p < 0.0001$ for IgG isotype and $p < 0.0005$ for IgM isotype). There were no significant differences in IgG and IgM anti-Cu/Zn SOD Ab levels between patients with SSc, SLE, DM, or PV/PF/BP.

IgG anti-Cu/Zn SOD Ab levels in patients with generalized morphea, linear scleroderma, or morphea were significantly higher than those found in normal controls ($p < 0.0001$, Fig 2), but were comparable among the three subsets of localized scleroderma. Similarly, IgM anti-Cu/Zn SOD Ab levels were significantly elevated in patients with generalized morphea ($p < 0.0001$), linear scleroderma ($p < 0.001$), or morphea ($p < 0.01$) relative to normal controls. Patients with generalized morphea had significantly higher IgM anti-SOD levels than those with morphea ($p < 0.05$), while there was no significant difference in IgM anti-Cu/Zn SOD Ab levels between patients with generalized morphea and those with linear scleroderma. IgG anti-Cu/Zn SOD Ab levels did not significantly correlate with IgM anti-Cu/Zn

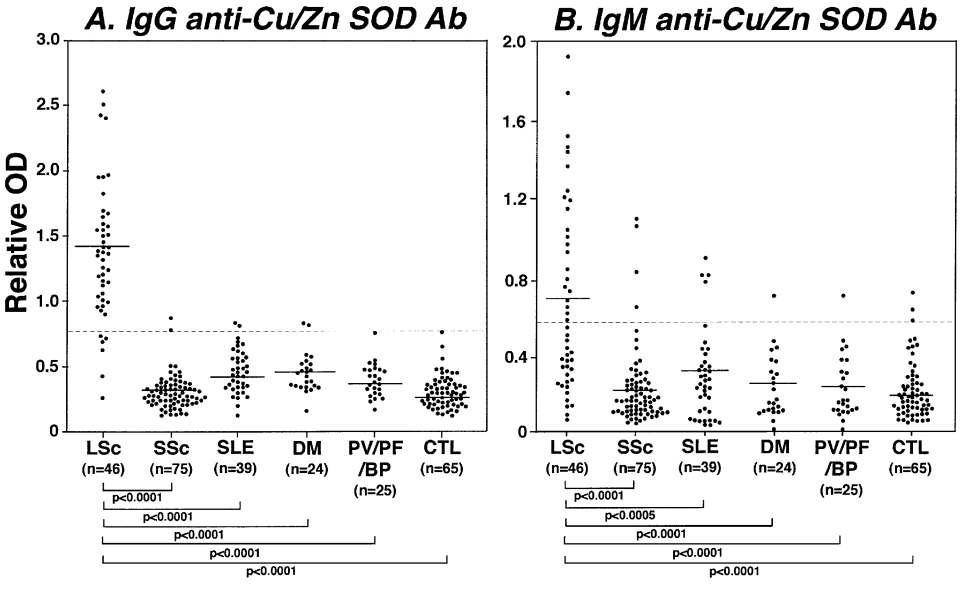

Figure 1
Anti-Cu/Zn SOD autoantibody levels were elevated in serum samples from patients with localized scleroderma (LSc). Anti-Cu/Zn SOD Ab levels were examined in serum samples from patients with LSc, SSc, SLE, DM, or PV/PF/BP and normal controls (CTL). Anti-Cu/Zn SOD Ab levels as the relative optical density (OD) were determined by ELISA using human purified Cu/Zn SOD. The short bars indicate the mean values in each group. Broken lines indicate the cut-off values (mean + 3SD of the control samples).

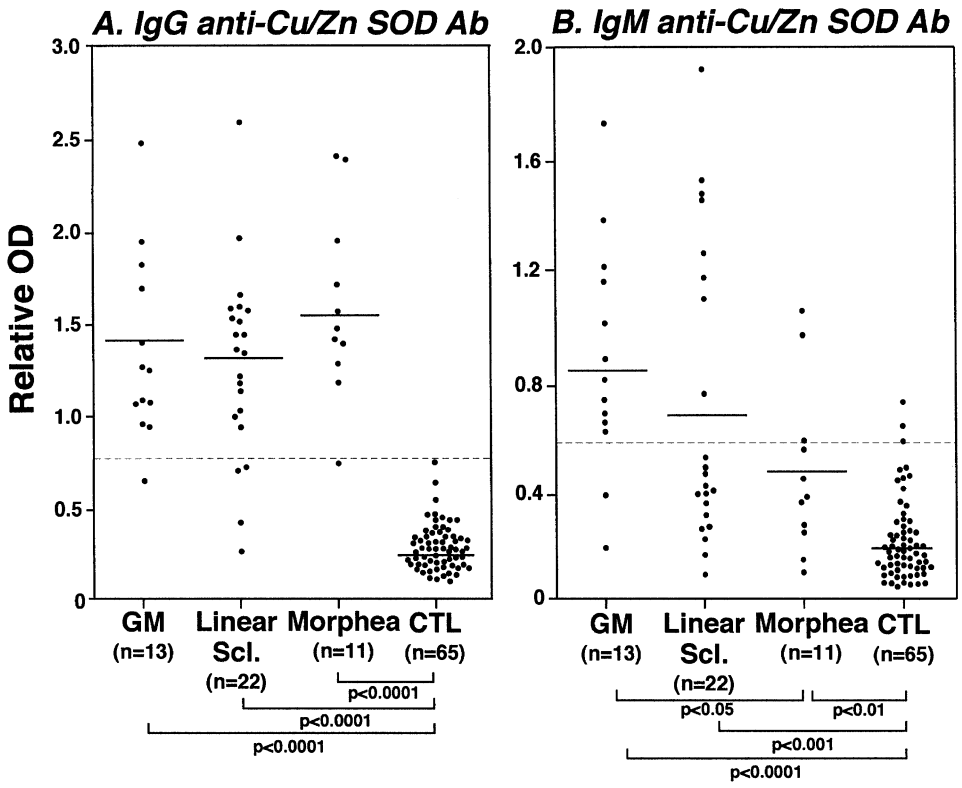

Figure 2
IgG anti-Cu/Zn SOD Ab levels were similar in the three subgroups of localized scleroderma while IgM anti-Cu/Zn SOD Ab levels were elevated in generalized morphea relative to morphea. Anti-Cu/Zn SOD Ab levels as the relative optical density (OD) were measured by ELISA in serum samples from patients with generalized morphea (GM), linear scleroderma (Linear Scl.), or morphea and normal controls (CTL). Broken lines indicate the cut-off values (mean + 3SD of the control samples) and the short bars indicate the mean values in each group.

SOD Ab levels in patients with localized scleroderma (data not shown). Furthermore, IgG and IgM anti-Cu/Zn SOD Ab levels did not correlate with serum total IgG and IgM levels, respectively (data not shown). Thus, IgG and IgM anti-Cu/Zn SOD autoantibody levels were increased in localized scleroderma, but not in other autoimmune diseases, including SSc, SLE, DM, and PV/PF/BP.

High prevalence of anti-Cu/Zn SOD Ab in localized scleroderma Absorbance values higher than the mean + 3SD (0.75 for IgG anti-Cu/Zn SOD Ab and 0.58 for IgM anti-Cu/Zn SOD Ab) of the control serum samples were considered positive in this study (Fig 1). In total patients with localized scleroderma, IgG or IgM anti-Cu/Zn SOD Ab was found in 89% (Table I). IgG isotype of this autoantibody (87%) was more frequently detected than IgM isotype (48%). By contrast, IgG or IgM anti-Cu/Zn SOD Ab was detected only in 8% of patients with SSc, 15% of those with SLE, 13% of those with DM, and 4% of those with PV/PF/BP. IgM anti-Cu/Zn SOD Ab was detected in 5% of healthy controls, while IgG anti-Cu/Zn SOD Ab was not detected in any healthy individuals. In the subgroups of localized scleroderma, IgG or IgM anti-Cu/Zn SOD Ab was present in 100% of patients with generalized morphea, 82% of those with linear scleroderma, and 91% of those with morphea. Thus, anti-Cu/Zn SOD Ab was detected in $\sim$ 90% of localized scleroderma patients with 100% of generalized morphea patients.

IgM anti-Cu/Zn SOD Ab correlated with other autoantibodies and disease severity in localized scleroderma Since there were only six localized scleroderma patients negative for IgG anti-Cu/Zn SOD Ab *versus* 40 patients positive for this autoantibody, the presence of IgG anti-Cu/Zn SOD Ab was not significantly associated with the presence or absence of any serological and clinical parameters, except for the presence of antinuclear Abs (27 of 40, 68% of patients with this Ab *vs* one of six, 17% of patients without this Ab, $p < 0.05$). Localized scleroderma patients with IgM anti-Cu/Zn SOD Ab correlated significantly with the presence of antinuclear Abs ($p < 0.05$) or rheumatoid factor ($p < 0.01$), IgG antihistone Ab levels ($p < 0.05$), IgM antihistone Ab levels ($p < 0.01$), or IgG anti-ssDNA levels ($p < 0.01$; Table II). The age at onset was

Table I. Frequency of anti-Cu/Zn SOD Ab in autoimmune diseases and normal controls[a]

	Anti-Cu/Zn SOD Ab		
	IgG	IgM	IgG or IgM
LSc[b] (n = 46)	40 (87)	22 (48)	41 (89)
GM (n = 13)	12 (92)	11 (85)	13 (100)
Linear Scl. (n = 22)	18 (82)	8 (36)	18 (82)
Morphea (n = 11)	10 (91)	3 (27)	10 (91)
SSc (n = 75)	2 (3)	4 (5)	6 (8)
SLE (n = 39)	2 (5)	4 (10)	6 (15)
DM (n = 24)	2 (8)	1 (4)	3 (13)
PV/PF/BP (n = 25)	0 (0)	1 (4)	1 (4)
Normal (n = 65)	0 (0)	3 (5)	3 (5)

[a]Values are the number (%) of patients with anti-Cu/Zn SOD Ab. IgG and IgM anti-Cu/Zn SOD Abs were determined by ELISA using anti-human IgG and IgM Abs as secondary Abs, respectively.
[b]LSc, localized scleroderma; GM, generalized morphea; Linear Scl., linear scleroderma.

Table II. Clinical and serological features of patients with localized scleroderma positive for IgM anti-Cu/Zn SOD Ab[a]

	IgM anti-Cu/Zn SOD Ab + (n = 22)	IgM anti-Cu/Zn SOD Ab − (n = 24)
Serological		
Antinuclear Ab	19 (86)*	9 (38)
IgG antihistone Ab (OD)[b]	0.85 ± 0.21*	0.66 ± 0.28
IgM antihistone Ab (OD)	0.76 ± 0.20**	0.42 ± 0.13
IgG anti-ssDNA Ab (OD)	0.63 ± 0.23**	0.43 ± 0.15
Rheumatoid factor	13 (59)**	1 (4)
Clinical		
Sex (male/female)	5/17	7/17
Age at onset (y)	16 ± 14*	27 ± 17
Disease duration (y)	3.7 ± 4.4	6.5 ± 9.3
No. of linear lesions	1.1 ± 1.1	0.8 ± 0.8
No. of plaque lesions	2.8 ± 2.3*	1.7 ± 3.0
Total no. of lesions	4.0 ± 2.8	2.5 ± 3.1
No. of involved body areas	2.3 ± 1.5*	1.8 ± 1.6
Muscle involvement	7 (32)	2 (8)

[a]Values are mean ± SD. Unless otherwise indicated, values are the number (%) of positive patients. IgM anti-Cu/Zn SOD Abs were determined by ELISA using anti-human IgM Abs as secondary Abs.
[b]Antihistone Ab and anti-ssDNA Ab levels as the relative optical density (OD) were determined by specific ELISA.
*$p < 0.05$ and **$p < 0.01$ *versus* patients without IgM anti-Cu/Zn SOD Ab.

significantly younger in patients with IgM anti-Cu/Zn SOD Ab than those without IgM anti-Cu/Zn SOD Ab ($p < 0.05$). The number of plaque lesions and involved body areas were significantly greater in patients with IgM anti-Cu/Zn SOD Ab than those without IgM anti-Cu/Zn SOD Ab ($p < 0.05$). Thus, IgM anti-Cu/Zn SOD Ab levels correlated with the disease severity as well as the presence or levels of other autoantibodies in patients with localized scleroderma.

The presence of anti-Cu/Zn SOD Ab in localized scleroderma was confirmed by immunoblotting The presence of anti-Cu/Zn SOD Ab was further evaluated by immunoblotting analysis using human purified Cu/Zn SOD. Serum samples from localized scleroderma patients positive for IgG anti-Cu/Zn SOD Ab by ELISA exhibited reactivity with SOD (32 kDa) by immunoblotting (Fig 3, *lanes 1–5*). By contrast, serum samples from SSc patients negative for IgG anti-Cu/Zn SOD Ab by ELISA did not react with Cu/Zn SOD (*lane 6*). Similarly, any reactivity with Cu/Zn SOD was not observed using serum samples from patients with either localized scleroderma or SLE negative for IgG anti-Cu/Zn SOD Ab by ELISA (data not shown). Furthermore, serum samples from healthy individuals did not react with Cu/Zn SOD (*lane 7*). Thus, the presence of anti-Cu/Zn SOD autoantibody in patients with localized scleroderma was confirmed by immunoblotting analysis.

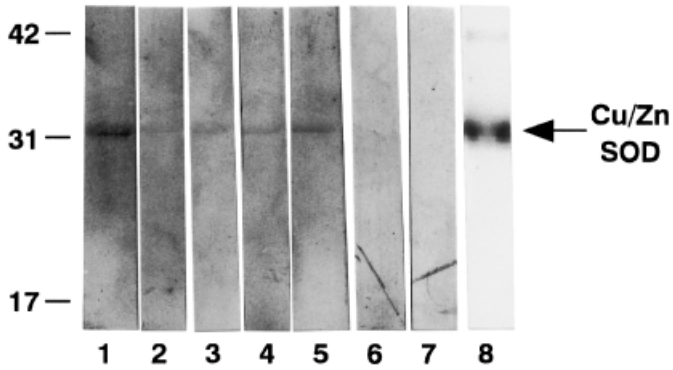

Figure 3
The presence of IgG anti-Cu/Zn SOD Ab in sera from patients with localized scleroderma was confirmed by immunoblotting. Representative immunoblotting of human purified Cu/Zn SOD with sera from patients or a normal control is shown. *Lanes 1–5*, serum samples from patients with localized scleroderma positive for IgG anti-Cu/Zn SOD Ab by ELISA; *lane 6*, a serum sample from an SSc patient negative for IgG anti-Cu/Zn SOD Ab by ELISA; *lane 7*, a normal human serum; *lane 8*, colloidal gold-stained Cu/Zn SOD (32 kDa). Makers for molecular weights (kDa) are shown to the left. The results represent those obtained with 10 serum samples of patients with localized scleroderma positive for IgG anti-Cu/Zn SOD Ab by ELISA, 15 serum samples of patients with either localized scleroderma, SSc, or SLE negative for IgG anti-Cu/Zn SOD Ab by ELISA, and 10 serum samples from healthy individuals.

Anti-Cu/Zn SOD Ab in localized scleroderma inhibited SOD activity To determine the functional relevance of anti-Cu/Zn SOD autoantibody, it was assessed whether anti-Cu/Zn SOD Ab was able to inhibit SOD activity. SOD activity was determined using a specific spectrophotometric assay, in which SOD activity was assessed by the SOD-induced increase in the autoxidation rates of 5,6,6*a*,11*b*-tetrahydro-3,9,10-trihydroxybenzo[*c*]fluorine. SOD activity was not inhibited by IgG isolated from healthy individuals (Fig 4). By contrast, IgG isolated from serum samples of localized scleroderma patients positive for IgG anti-Cu/Zn SOD Ab by ELISA significantly inhibited SOD activity by 59% compared with normal control ($p < 0.001$). This inhibitory activity was not due to the presence of autoantibodies other than anti-Cu/Zn SOD Ab since SOD activity was not inhibited by IgG isolated from serum samples that contained autoantibodies against ssDNA or histone, but not IgG anti-Cu/Zn SOD Ab. Similarly, the inhibition of SOD activity was not detected using serum samples from SSc or SLE patients without IgG anti-Cu/Zn SOD Ab by ELISA (data not shown). Thus, IgG anti-Cu/Zn SOD Ab from patients with localized scleroderma was able to inhibit SOD enzymatic activity.

Discussion

In the current study, IgG or IgM anti-Cu/Zn SOD Ab was detected in the serum of 89% of patients with localized scleroderma, especially 100% of patients with generalized morphea, the severest form of localized scleroderma (Figs 1 and 2 and Table I). By contrast, anti-Cu/Zn SOD Ab was positive at much lower frequency in the serum of SSc (8%) as well as SLE (15%), DM (13%), and autoimmune bullous disorders (4%). Although localized scleroderma and SSc

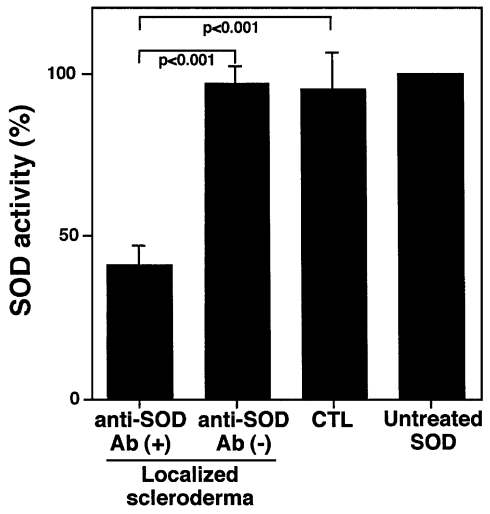

Figure 4
IgG anti-Cu/Zn SOD Ab from patients with localized scleroderma inhibited SOD activity. IgG was purified from serum samples of localized scleroderma patients positive for IgG anti-Cu/Zn SOD Ab by ELISA (anti-SOD Ab (+); n = 10), serum samples of localized scleroderma negative for IgG anti-Cu/Zn SOD Ab by ELISA (anti-SOD Ab (−); *n* = 5), and serum samples from healthy individuals (CTL; n = 10). Purified IgG was incubated with Cu/Zn SOD and SOD activity was determined using a specific spectrophotometric assay, in which SOD activity was assessed by the SOD-induced increase in the autoxidation rates of 5,6,6*a*,11*b*-tetrahydro-3,9,10-trihydroxybenzo[*c*]-fluorine. SOD activity incubated with purified IgG is shown as a percentage of untreated SOD that was defined as 100%. The histogram shows the mean ± SD.

share skin fibrosis and autoimmunity (Sato *et al*, 1993; Okano, 1996), the production of anti-Cu/Zn SOD autoantibody is highly specific to localized scleroderma, indicating that autoimmune reactions are distinct between these two diseases. The presence of IgG anti-Cu/Zn SOD Ab in sera from patients with localized scleroderma was further confirmed by the immunoblotting analysis (Fig 3). The presence of IgM anti-Cu/Zn SOD Ab was associated with the greater number of plaque-type sclerotic lesions, the wider distribution of lesions, and the production of other autoantibodies including antinuclear Ab, antihistone Ab, anti-ssDNA Ab, and rheumatoid factor in patients with localized scleroderma (Table II). Remarkably, the anti-Cu/Zn SOD autoantibodies were able to inhibit Cu/Zn SOD enzymatic activity (Fig 4). Collectively, the results of the present study indicate that the anti-Cu/Zn SOD Ab is a novel, major autoantibody with potential functional significance in localized scleroderma.

Accumulating evidence suggests that ROS may directly induce collagen gene expression via effects on lipid peroxidation (Chojkier *et al*, 1989; Ruiz *et al*, 2000). Furthermore, many recent reports have shown that Cu/Zn SOD reduces fibrosis by inhibiting the production of ROS. Lecithinized Cu/Zn SOD is effective for bleomycin-induced fibrosis of both lung and skin (Yamazaki *et al*, 1997; Yamamoto *et al*, 1999). Gene delivery of Cu/Zn SOD attenuates experimental cholestasis-induced liver fibrosis in the rat (Zhong *et al*, 2002). Furthermore, administration with liposomal Cu/Zn SOD significantly reduces radiation-induced skin fibrosis of human and pig (Delanian *et al*, 1994; Lefaix *et al*, 1996). *In vitro* analysis using cultured human skin fibroblasts from radiation-induced fibrosis has revealed that Cu/Zn SOD treatment significantly reduces the expression levels of tissue inhibitor of metalloproteinases (TIMP) and transforming growth factor (TGF)-β (Delanian *et al*, 2001). Similar results are obtained using the three-dimensional model of skin fibrosis that is reconstituted by primary fibroblasts and keratinocytes from pig skin of radiation-induced fibrosis (Vozenin-Brotons *et al*, 2001). TIMP can increase extracellular matrix deposition by inhibiting the activity of matrix metalloproteinases, a family of zinc-dependent endopeptidases that collectively can digest all extracellular matrix components (Gomez *et al*, 1997). In addition, TGF-β is a major fibrogenic growth factor since it not only stimulates matrix synthesis but also controls virtually all fibroblast function relevant to fibrosis, including proliferation, chemotaxis, and differentiation (Varga, 2002). These findings suggest that, in a model of radiation-induced skin fibrosis, Cu/Zn SOD diminishes fibrosis possibly by reducing the expression of TIMP and TGF-β. In the affected skin from patients with localized scleroderma, increased expression of TIMP, TGF-β, and TGF-β receptors on dermal fibroblasts has been reported (Higley *et al*, 1994; Mattila *et al*, 1998; Kubo *et al*, 2001). Furthermore, anti-Cu/Zn SOD Ab in sera from patients with localized scleroderma was able to inhibit Cu/Zn SOD activity (Fig 4), which may result in increased expression of TIMP and TGF-β. Collectively, the results of this study suggest that anti-Cu/Zn SOD autoantibodies can inhibit the antifibrogenic activity of Cu/Zn SOD in localized scleroderma.

Unlike SSc, localized scleroderma is characterized by the scattered sclerotic lesions that do not involve the whole body even if the lesions are multiple. Cu/Zn SOD, however, is a ubiquitous antioxidant enzyme that is expressed in all cell types (Noor *et al*, 2002; Zelko *et al*, 2002). Therefore, it remains unknown as to why systemic production of autoantibodies against Cu/Zn SOD in localized scleroderma affects only partial body areas, given that the autoantibodies can block the antifibrogenic activity of Cu/Zn SOD. It has been reported that some causes of familial amyotrophic lateral sclerosis are associated with mutations in the Cu/Zn SOD gene, many of which reduce the total activity and half-life of the enzyme (Rosen *et al*, 1993; Noor *et al*, 2002). The role of the mutated Cu/Zn SOD gene, however, as a cause of familial amyotrophic lateral sclerosis remains unknown since some lines of transgenic mice overexpressing mutant Cu/Zn SOD develop motor neuron disease, despite having elevated Cu/Zn SOD activity (Gurney *et al*, 1994; Noor *et al*, 2002). Furthermore, Cu/Zn SOD-deficient mice develop normally and show no overt motor deficits (Reaume *et al*, 1996). Interestingly, Cu/Zn SOD-deficient mice exhibit marked vulnerability to motor neuron loss only after axonal injury, indicating that Cu/Zn SOD is not necessary for normal motor neuron development and function, and that an important function of Cu/Zn SOD is the protection of various cell types from the oxidative burden imposed by injury (Reaume *et al*, 1996). It has been reported that various physical injury, including trauma, radiation, burn, local corticosteroid injection, surgical procedures, immunization, and varicella infection, is a provoking factor in localized scleroderma (Trattner *et al*, 1991; Komocsi *et al*, 2000). Therefore, it may be possible that physical skin injury

induces the local production of potentially fibrogenic ROS, the activity of which may be enhanced by anti-Cu/Zn SOD autoantibodies in localized scleroderma.

IgM autoantibodies to Mn SOD have been detected in patients with acute Epstein–Barr virus infection (Ritter *et al*, 1994; Semrau *et al*, 1998). This anti-Mn SOD Ab can inhibit Mn SOD enzymatic activity and their levels coincide with the clinical symptoms, suggesting that anti-Mn SOD Ab contributes to tissue injury (Ritter *et al*, 1994). Thus, anti-SOD Ab may be generated by environmental stimuli, such as viral infection. Although none of the localized scleroderma patients examined in this study had a recent history of infection, the observation that localized scleroderma patients with IgM anti-Cu/Zn SOD Ab exhibited significantly younger age at onset compared with those without this Ab may be related to virus-induced production of this autoantibody (Table II). Consistently, it has been suggested that autoimmunity may be environmentally driven in localized scleroderma since localized scleroderma, especially generalized morphea, and drug-induced lupus share specificities of autoantibodies (Sato *et al*, 1993, 2003). Although the mechanisms for the autoantibody production in localized scleroderma remain unknown, the high prevalence of anti-Cu/Zn SOD Abs and their functional inhibition of Cu/Zn SOD may be an important clue for understanding the pathogenesis of this disease.

Materials and Methods

Serum samples Serum samples were obtained from 46 Japanese patients with localized scleroderma (12 males and 34 females). Patients were classified into the following three subgroups as described previously (Sato *et al*, 1994b): 13 patients with generalized morphea (two males and 11 females), 22 patients with linear scleroderma (six males and 16 females), and 11 patients with morphea (four males and seven females). The age of patients with localized scleroderma (mean $\pm$ SD) was 27 ± 17 y (generalized morphea, 24 ± 19; linear scleroderma, 22 ± 13; morphea, 40 ± 17 y). The disease duration of patients with localized scleroderma was 5.1 ± 7.4 y (generalized morphea, 3.2 ± 2.8; linear scleroderma, 6.9 ± 9.4; morphea, 3.8 ± 6.0 y). None of the localized scleroderma patients were treated with steroids or immunosuppressive therapy. None of the patients had a recent history of infection and abnormal liver function. The number of sclerotic lesions more than 3 cm in diameter was counted in each patient with localized scleroderma when the serum samples were obtained. The sclerotic lesions were morphologically classified into plaque and linear lesions. We divided the whole body into the following seven areas: head and neck; right upper extremity; left upper extremity; anterior trunk; posterior trunk; right lower extremity; and left lower extremity. Then we counted the number of involved areas as described previously (Sato *et al*, 1994b).

Serum samples from 75 patients with SSc who fulfilled the criteria proposed by the American College of Rheumatology (Subcommittee for Scleroderma Criteria of the American Rheumatism Association Diagnostic and Therapeutic Criteria Committee, 1980) were also examined in this study. These patients were grouped according to LeRoy *et al* (1988): 40 patients (two males and 38 females) had limited cutaneous SSc and 35 patients (five males and 30 females) had diffuse cutaneous SSc. The age of patients with SSc was 50 ± 15 y (limited cutaneous SSc, 54 ± 11; diffuse cutaneous SSc, 45 ± 18 y). The disease duration of patients with limited cutaneous SSc and diffuse cutaneous SSc was 8.3 ± 8.0 and 5.9 ± 8.0 y, respectively. None of the SSc patients were treated with steroids, D-penicillamine, or immunosuppressive therapy. In this study, 39 patients with systemic lupus erythematosus (SLE; five males and 34 females; age 36 ± 12 y), 24 patients with dermatomyositis (DM; five males and 19 females; age 42 ± 25 y), and 25 patients with autoimmune bullous diseases (nine males and 16 females; age 43 ± 10 y) including pemphigus vulgaris (PV; $n = 11$)/pemphigus foliaceus (PF; $n = 5$)/bullous pemphigoid (BP; $n = 9$) were also examined as disease control. SLE and DM were diagnosed according to the criteria proposed by the American College of Rheumatology (Tan *et al*, 1982) and the criteria proposed by Bohan and Peter (1975a,b), respectively. Sixty-five healthy Japanese people (17 males and 48 females; age 29 ± 14 y) that were age- and sex-matched to patients with localized scleroderma were used as normal controls. Fresh venous blood samples were centrifuged shortly after clot formation. All samples were stored at -70°C prior to use. All investigations were performed after approval by the Kanazawa University Graduate School of Medical Science and according to the Declaration of Helsinki principles.

ELISA for anti-Cu/Zn SOD Ab ELISA for anti-Cu/Zn SOD Ab was performed as previously described (Sato *et al*, 1993). Briefly, 96-well plates (EIA/RIA plate, Coster, Cambridge, Massachussets) were coated with Cu/Zn SOD purified from human red blood cells (1 μg per mL; Sigma-Aldrich, St Louis, Missouri) at 4°C overnight. The wells were blocked with 2% bovine serum albumin and 1% gelatin in Tris-buffered saline for 1 h at 37°C. The serum samples (100 μL) diluted to 1:100 were added to triplicate wells for 90 min at 20°C. After washing four times, the bound antibodies were detected with alkaline phosphatase-conjugated goat anti-human IgG or IgM Abs (Cappel, Durham, North Carolina) using *p*-nitrophenyl phosphate (Sigma-Aldrich) as substrate. The optical density of the wells was subsequently determined. Absorbance values greater than the mean + 3SD of normal controls were considered positive in this study.

Immunoblotting analysis Cu/Zn SOD (5 μg per lane; Sigma-Aldrich) was subjected to electrophoresis on 10%–20% gradient sodium dodecyl sulfate-polyacrylamide slab gels. The proteins were electrotransferred from the gels to nitrocellulose sheets for immunoblotting analysis. The nitrocellulose sheets were cut into strips and incubated overnight with serum samples diluted to 1:50. The strips were then incubated for 1.5 h with alkaline phosphatase-conjugated goat anti-human IgG Ab (Cappel), and color was developed with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (Sigma-Aldrich). Ten localized scleroderma patients positive for IgG anti-Cu/Zn SOD Ab by ELISA, 15 patients with either localized scleroderma, SSc, or SLE negative for IgG anti-Cu/Zn SOD Ab by ELISA, and 10 healthy individuals were evaluated.

SOD activity assay IgG was purified from serum samples using magnetic beads coated with recombinant protein G covalently coupled to the surface (Dynal, Lake Success, New York). The final IgG concentration was measured by a spectrophotometer (Gene Quant II, Amersham Biosciences, Piscataway, New Jersey). SOD (5 μg) was incubated with purified IgG (40 μg) for 20 min at 25°C. SOD activity was determined by a specific spectrophotometric assay kit (Oxis Health Products, Portland, Oregon), according to the manufacturer's protocol. Briefly, SOD activity was assessed by the SOD-mediated increase in the rate of autoxidation of 5,6,6*a*,11*b*-tetrahydro-3,9,10-trihydroxybenzo[*c*]fluorene at pH 8.8 to yield a chromophore with maximum absorbance at 525 nm. The SOD activity was determined from the ratio of the autoxidation rates in the presence and absence of SOD. Ten localized scleroderma patients positive for IgG anti-Cu/Zn SOD Ab by ELISA, 15 patients with either localized scleroderma, SSc, or SLE negative for IgG anti-Cu/Zn SOD Ab by ELISA, and 10 healthy individuals were assessed.

Detection of antinuclear Ab, antihistone Ab, anti-ssDNA Ab, and rheumatoid factor Antinuclear Ab was assessed by indirect

immunofluorescence staining using HEp-2 substrate cells (MBL, Nagoya, Japan) as described (Sato *et al*, 1993). Acetone-fixed HEp-2 cells grown on the slide were incubated with serum samples diluted 1:64 for 30 min at 37°C and then with fluorescein isothiocyanate-conjugated goat anti-human γ-globulin Abs (MBL) for 30 min at 37°C. ELISA for antihistone and anti-ssDNA Ab was performed as described previously (Sato *et al*, 1993). Briefly, 96-well microtiter plates were coated with total histones (5 μg per mL; Sigma-Aldrich) at 4°C overnight. For anti-ssDNA Ab, wells were pre-treated with 0.1% protamine sulfate (grade X; Sigma-Aldrich) for 1 h at 20°C. After rinsing, the plates were coated with calf thymus ssDNA (1 μg per mL; Sigma-Aldrich) at 4°C overnight. ELISA was performed as described for anti-Cu/Zn SOD Ab ELISA. Absorbance values greater than the mean + 3SD of normal controls were considered positive in this study. Rheumatoid factor was measured using a latex agglutination slide test (Eiken, Tokyo, Japan), according to the manufacturer's protocol. Latex beads coated with human γ-globulin (50 μL) were incubated with 50 μL of serum samples for 1 min at 20°C. Rheumatoid factor was considered to be present when agglutination of latex beads was observed.

Statistical analysis Statistical analysis was performed using the Mann–Whitney *U* test for determining the level of significance of differences between sample means, Fisher's exact probability test for comparison of frequencies, and Bonferroni's test for multiple comparisons. Spearman's rank correlation coefficient was used to examine the relationship between two continuous variables. A p value less than 0.05 was considered statistically significant.

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