Scleroderma Autoantigens Are Uniquely Fragmented by Metal-catalyzed Oxidation Reactions: Implications for Pathogenesis

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Summary

The observation that revelation of immunocryptic epitopes in self antigens may initiate the autoimmune response has prompted the search for processes which induce novel fragmentation of autoantigens as potential initiators of autoimmunity. The reversible ischemia reperfusion which characterizes scleroderma has focused attention on reactive oxygen species as molecules which might induce autoantigen fragmentation. We demonstrate that several of the autoantigens targeted in diffuse scleroderma are uniquely susceptible to cleavage by reactive oxygen species, in a metal-dependent manner. Multiple features of the fragmentation reaction and its inhibition indicate that these autoantigens possess metal-binding sites, which focus metal-catalyzed oxidation reactions (and consequent fragmentation) to specific regions of the antigens. These data suggest that the autoantibody response in scleroderma is the immune marker of unique protein fragmentation, induced by ischemia reperfusion in the presence of appropriate metals, and focus attention on abnormal metal status as a potential pathogenic principle in this disease.

The highly specific humoral immune response to autoantigens in many autoimmune diseases is antigen driven and T cell dependent (1, 2), but the initial mechanisms for breaking T cell tolerance to these molecules remain unclear. Several recent studies demonstrate that a potential for T cell autoreactivity resides in the immunological non-equivalency of different areas of self-molecules, since self-tolerance is only induced to efficiently presented, dominant epitopes, but not to cryptic ones (for reviews see references 3 and 4). Thus, potentially autoreactive T cells that have not previously encountered the cryptic self still exist (5). As determinant dominance is influenced by protein structure, circumstances that change the molecular context of epitopes (e.g., novel cleavage, altered conformation, or tertiary structure) may permit the efficient presentation of previously cryptic determinants, thereby breaking T cell tolerance (6–10). The unique autoantibody response observed in different autoimmune diseases may therefore be viewed as the long-lived immunologic memory of the altered circumstances that revealed this cryptic structure. Thus, these antibodies are useful probes with which to search for the initial perturbed state. For example, the autoantibodies elaborated in systemic lupus erythematosus (SLE)† have focused attention on apoptosis as a possible setting in which cryptic structure is revealed. During apoptosis, the lupus autoantigens cluster and become concentrated in the surface blebs of apoptotic cells (11) where several of these molecules are specifically cleaved by proteases of the interleukin 1β converting enzyme (ICE) family (12–14). The fact that specific proteolytic cleavage unifies these lupus autoantigens has suggested that fragmentation might define molecules as autoantigens in other autoimmune diseases (13).

Scleroderma is a disease of unknown etiology which is characterized by increased vasoactivity, widespread tissue fibrosis, and the elaboration of unique autoantibodies. Since the autoantigens recognized are not substrates for the ICE-like enzymes during apoptosis, it is likely that other mechanisms are responsible for revealing cryptic structure in this disease (13). One potential mechanism that might result in the specific fragmentation of scleroderma autoantigens is suggested by the striking reversible ischemia-reperfusion that occurs in patients with scleroderma (for review see reference 15). This vascular phenomenon, the result of dysfunction of small arteries and arterioles of the extremities and internal organs, has been proposed to underlie the exuberant tissue fibrosis in this disease (16). The injury associated with reperfusion of ischemic tissues results in part from the production of free radical species (17–19), and indirect evidence exists for increased production of reactive oxygen species (ROS) in patients with scleroderma (20, 21). Since ROS can induce the oxidative modification of proteins

†Abbreviations used in this paper: EDAC, 1-ethyl 3-(3-dimethylaminopropyl) carbodiimide; Fe, ferrous sulfate; ICE, interleukin 1β converting enzyme; NuMA, nuclear mitotic apparatus protein; OH, hydroxyl radical; ROS, reactive oxygen species; SLE, systemic lupus erythematosus.
(including fragmentation; 22 and 23), we used the autoantibodies from sclerodema patients to address whether ROS-mediated fragmentation might unify the autoantigens in this disease.

Materials and Methods

Sera. After obtaining informed consent, human autoimmune sera were collected from 60 patients with diffuse scleroderma, and their reactivity with saline soluble extracts of rabbit thymus was determined by Ouchterlony immunodiffusion using standard reference serum to topoisomerase I. The sera were further screened by immunoblotting against control HeLa cell lysates, using reference antibodies to NOR-90/UBF, U1-70kDa, topoisomerase I, and RNA polymerase I, II, and III as standards. Of the RNA polymerases, only the large subunit of RNA polymerase II was recognized by immunoblotting. Other autoimmune sera recognizing lupus autoantigens have been described previously (11–13).

Metal-catalyzed Oxidation Reactions. Confluent HeLa monolayers were washed twice with KRB (20 mM Hepes, pH 7.4, 127 mM NaCl, 5.5 mM KCl, 10 mM dextrose, 1 mM CaCl₂, and 2 mM MgSO₄), and then lysed in a buffer containing 20 mM Hepes, pH 7.4, 1% Nonidet P40, 150 mM NaCl, and the following protease inhibitors: leupeptin, pepstatin A, chymostatin, antipain, and PMSF. Equal lysate aliquots were pipetted into microfuge tubes before adding metal, H₂O₂, and/or ascorbate. Samples were incubated at 25°C for 30 min, with vortexing every 5 min. Reactions were terminated by adding 10 mM EDTA, SDS gel buffer, and boiling for 3 min. Equal protein amounts of each sample were electrophoresed on 10% SDS-polyacrylamide gels. Proteins were then transferred to nitrocellulose and immunoblotted with patient sera monospecific for topoisomerase I, RNA polymerase II large subunit, UBF/NOR-90, or U1-70kDa. Blotted proteins were visualized using the Supersignal CL-HRP substrate system (Pierce, Rockford, IL) according to the manufacturer’s directions. Primary antibodies were used at dilutions of 1:5,000–1:20,000, and autoradiogram exposure times ranged from 2 s–1 min. Experiments were always performed using freshly made lysate, and fresh stock solutions of Fe, Cu, H₂O₂, and ascorbate. Oxygen depletion was accomplished by vigorously blowing O₂-free argon through the lysate for 5 min before adding Fe and ascorbate. For these experiments, reactions on both control and O₂-depleted lysates were performed in sealed tubes (without vortexing) at 25°C for 30 min.

Metal Chelator, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, and Zinc Competition Experiments. Metal-catalyzed oxidation reactions were performed on HeLa lysates essentially as described above, with the following modifications. Experiments performed in the presence of 1 mM desferoxamine, 200 µM bathocuproine disulfonate or 1 mM D-penicillamine were carried out by adding the chelators immediately before addition of the Fe or Cu. ZnCl₂ competition reactions, and those performed in the presence of 5 mM 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC), were executed by adding these reagents to HeLa lysates 5 min before adding Fe/ascorbate.

Intact Cell Experiments. Confluent monolayers (secondary passage) of human foreskin keratinocytes were cultured as described (11). Experiments were initiated by adding keratinocyte growth medium supplemented with 20 µM CuSO₄ to the cells for 2 or 18 h; 2 mM H₂O₂ was subsequently added to some of the dishes, and after a further 30 min incubation, the cells were harvested and immunoblotted.

Morphologic Studies. Microscopy was performed on HeLa cells grown on No. 1 glass coverslips. For immunofluorescence, cells were fixed in 4% paraformaldehyde (5 min, 4°C), permeabilized in acetone (30 s, 4°C), and stained using a standard monospecific patient serum recognizing topoisomerase I (diluted 1:160 in PBS). Bound antibodies were visualized with FITC-conjugated goat anti-human F(ab′)₂ (Organon Teknika Corp./Cappel, Durham, NC). Coverslips were mounted with Permafluor (Lipshaw, Pittsburgh, PA), and viewed on a scanning confocal microscope system (MRC 600; Bio-Rad Laboratories, Richmond, CA). For metal staining, cells were fixed in filtered 1% ammonium sulfide (5 min, 25°C). After extensive washing with H₂O₂, coverslips were mounted, viewed, and photographed under bright field on a Zeiss Axioshot microscope.

Results

Scleroderma Autoantigens Are Fragmented by Metal-catalyzed Oxidation Reactions. Substantial evidence exists for the in vitro production of reactive oxygen species (including hydroxyl radical [OH·]) via the metal-catalyzed Fenton (H₂O₂ + Fe²⁺ → Fe³⁺ + H₂O₂• + OH·) and Haber-Weiss reactions (O₂⁻ + H₂O₂ → O₂ + OH· + OH²⁻) (see reviews in references 24 and 25). In initial studies, we added ferrous sulfate (Fe) to NP-40 lysates of HeLa cells to generate free radical species by Fenton chemistry (26–29), and addressed whether several of the major autoantigens in diffuse scleroderma were fragmented (30–33). After incubating in vitro for 30 min in the absence of added metal, topoisomerase I remained stable, and migrated as a single 100-kD species (Fig. 1, lane 1). In these metal-free conditions, addition of either ascorbate or H₂O₂ failed to generate any fragments (Fig. 1, lanes 2 and 3). Addition of 100 µM Fe induced the specific fragmentation of topoisomerase I, generating a major fragment of 95 kD, and several minor, discrete species migrating between 65 and 90 kD (Fig. 1, lane 4). In the presence of 1.7 mM ascorbate, generation of these fragments was enhanced (Fig. 1, lane 5); the reaction went to completion (that is, total loss of the intact protein) when 17 mM ascorbate was added (data not shown). In contrast, 1 mM H₂O₂ resulted in a slight, but reproducible, decrease in fragmentation when compared to Fe alone (Fig. 1, lane 6 versus lane 4). Fe also induced specific fragmentation of three additional scleroderma autoantigens: the large subunit of RNA polymerase II, upstream binding factor (UBF/NOR90), and the 70 kD protein component of the U1 small nuclear ribonucleoprotein (U1-70kDa). Although the sensitivity of the different proteins to metal-induced fragmentation varied (e.g., the large subunit of RNA polymerase II was efficiently fragmented using <10 µM Fe), cleavage was enhanced by the addition of ascorbate in all cases: (a) the large subunit of RNA polymerase II (a protein doublet of 220 and 240 kD) was specifically fragmented, generating major species of 190, 160, and 140 kD, as well as several minor species of 200, 180, 170, and 130 kD (Fig. 1, lane 5); (b) Fragmentation of UBF/NOR90 (a doublet of 90 and 100 kD in control lysates) yielded a poorly resolved smear of fragments between 70 and 90 kD (Fig. 1, lane 5); (c) U1-70kDa fragmentation in response to Fe generated a
and those generated by Fe/ascorbate were noted. (a) Addition of Cu alone did not result in autoantigen fragmentation, which was dependent on the addition of H₂O₂. (b) The fragments observed in response to Cu/H₂O₂ were distinct from those generated by Fe/ascorbate; Cu-induced fragmentation of topoisomerase I produced a major species of 95 kD, but did not generate the other fragments detected in the Fe system (Fig. 1, lanes 5 and 9). Cu-induced breakage of U1-70kDa generated a single product of 65 kD (not seen in the Fe system), and did not give rise to the 33–38kD fragments typical of Fe-induced fragmentation (Fig. 1, lanes 5 and 9). Incubation with Cu/H₂O₂ induced aggregation of the large subunit of RNA polymerase II (detected as immunoreactive protein species migrating >250 kD), and also gave rise to several distinct cleavage fragments migrating between 170 and 210 kD, which differed from the predominant Fe-induced fragments (Fig. 1, lanes 5 and 9). (c) Topoisomerase I, the large subunit of RNA polymerase II, and UBF/NOR-90 were more efficiently fragmented by Fe/ascorbate than Cu/H₂O₂. In contrast, fragmentation of U1-70kDa was more effectively induced with the latter oxidation system.

Thus, several of the antigens frequently targeted in patients with diffuse scleroderma are specifically fragmented in both of the metal-catalyzed oxidation systems we have examined. The specificity of these fragmentation reactions was confirmed in several different ways. (a) Except for the decreased staining of a protein of ~85 kD (Fig. 2 A, arrow), the coomassie blue staining of cell lysates incubated with Fe/ascorbate was not significantly different from control lysates (Fig. 2 A, lanes 1 and 2). The coomassie blue staining profile of control lysates, and those treated with Cu/H₂O₂ were identical (data not shown). These data are consistent with previous observations showing that although modification of proteins by metal-catalyzed reactions is frequent (22, 23, 34), fragmentation of these proteins is an extremely uncommon event (23). (b) Fragmentation reactions were absolutely dependent on metals capable of supporting Fen- ton chemistry (Fe, Cu); no autoantigen cleavages were observed upon addition of 100 μM zinc, cobalt, mercury, magnesium, manganese, nickel, cadmium, or silver, either alone, in the presence of H₂O₂, or ascorbate (data not shown). (c) None of the SLE autoantigens tested (including 52- and 60-kD Ro, La, and the Sm proteins), nor several other proteins proteolytically cleaved during apoptosis (including poly(ADP-ribose) polymerase, actin, and the nuclear mitotic apparatus protein, NuMA) were fragmented in oxidation reactions containing Fe/ascorbate or Cu/ H₂O₂ (Fig. 2 B). (d) Identical fragments were produced in >20 separate experiments, immunoblotted with 7 different topoisomerase I sera, and 3 different RNA polymerase II large subunits, U1-70kDa, and UBF/NOR-90 sera (data not shown). Identical fragments were also generated in lysates of several different cell types, including human keratinocytes and human umbilical vein endothelial cells (data not shown). (e) Fragmentation was inhibited under O₂-depleted conditions (Fig. 2 C), confirming that the fragmentation was free-radical mediated.

Figure 1. Several scleroderma autoantigens are uniquely fragmented by Fe/ascorbate or Cu/H₂O₂ oxidation reactions. HeLa lysates were prepared as described in the Materials and Methods section, and metal-catalyzed oxidation reactions were performed by adding the following: no additions (lane 1), 1.7 mM ascorbate (lane 2), 1 mM H₂O₂ (lane 3), 100 μM Fe(II)SO₄ (lane 4), 100 μM Fe(II)SO₄ + 1.7 mM ascorbate (lane 5), 100 μM Fe(II)SO₄ + 1 mM H₂O₂ (lane 6), 100 μM Cu(II)SO₄ (lane 7), 100 μM Cu(II)SO₄ + 1.7 mM ascorbate (lane 8) and 100 μM Cu(II)SO₄ + 1 mM H₂O₂ (lane 8). Samples were immunoblotted with the sera denoted on the left side of each panel. Equal amounts of protein were electrophoresed in the lanes of each panel. Migration positions of molecular weight standards are indicated on the right.

Few discrete fragments that migrated between 33 and 38 kD (Fig. 1, lane 5); this Fe-induced fragmentation was inefficient compared to that of the other autoantigens. Identical U1-70kDa fragments were also observed when [³⁵S]methionine-labeled U1-70kDa was generated by coupled in vitro transcription/translation, and then subjected to Fe/ascorbate treatment (data not shown). In contrast to the autoantigens described above, fibrillarin, the major scleroderma ribonucleoprotein autoantigen, was not fragmented under identical conditions (data not shown).

Some of the autoantigens that fragmented in response to Fe/ascorbate were also fragmented in a Cu-catalyzed oxidation system. Thus, specific fragments of topoisomerase I, RNA polymerase II large subunit, and U1-70kDa were observed when 100 μM Cu and 1 mM H₂O₂ were used to initiate oxygen radical production (Fig. 1, lanes 7–9). In contrast, no fragmentation of UBF/NOR-90 was detected under these conditions (Fig. 1, lanes 7–9). Three striking differences between the fragments generated by Cu/H₂O₂
Metal Chelators, Zinc, or EDAC Inhibit the Fragmentation of Scleroderma Autoantigens. The site-specific nature of metal-catalyzed oxidation reactions has previously indicated that these reactions are “caged” processes in which amino acid residues at metal-binding sites are specific targets of highly reactive free radical species generated at that site during a Fenton reaction (25, 35, 36). Sequestration of metals by chelators in the bulk solution, although only minimally influencing the capacity of the metal to catalyze a Fenton reaction, prevents the metal from doing so at the metal-binding site, thus inhibiting highly localized protein oxidation (25, 35). We therefore evaluated the effects of metal chelators on the fragmentation reactions. Fe-induced cleavages were markedly inhibited by 1 mM desferoxamine (Fig. 3 A, lanes 2 and 4), 1 mM D-penicillamine (Fig. 3 A, lanes 2 and 8), or 1 mM EDTA (data not shown). Cu-induced fragmentation was entirely abolished by 200 μM bathocuproine disulfonate (a Cu(I)-specific chelator; Fig. 3 A, lanes 3 and 7), and 1 mM EDTA (data not shown), and was diminished by 1 mM desferoxamine or 1 mM D-penicillamine (Fig. 3 A, lanes 3, 5, and 9). These data strongly indicate that metal binding to the autoantigen itself is required for the fragmentation reactions to occur. Since fragmentation requires exogenous metals capable of supporting a Fenton reaction, we addressed whether zinc, a metal that potentially binds to the same site, but is unable to support Fenton chemistry, might influence the ability of Fe or Cu to induce oxidative fragmentation. Increasing concentrations of zinc were added to cell lysates before the addition of Fe/ascorbate, and the effects on autoantigen fragmentation were assessed (Fig. 3 B). Cleavage of topoisomerase I and RNA polymerase II large subunit was inhibited by zinc in a dose-dependent manner, with fragmentation entirely abolished by 300 μM zinc (IC₅₀ of 30–50 μM) (Fig. 3 B). A similar IC₅₀ for inhibition by zinc was observed for Cu/H₂O₂-induced fragmentation of these autoantigens; in contrast, the specific aggregation of RNA polymerase II large subunit induced by Cu/H₂O₂ was not prevented (data not shown).

Interestingly, a striking structural feature common to these fragmented autoantigens is extended charged tracts, containing regions rich in acidic residues, or regions of mixed charge (37). For example, topoisomerase I contains a single highly charged (mixed) tract containing 72 residues, NOR/UBF contains two highly negatively charged tracts of 21 and 28 residues, U1-70kDa contains two highly charged (mixed) tracts containing 89 and 48 mixed residues, and the large subunit of RNA polymerase II contains three highly charged (mixed) tracts of 25, 17, and 12 residues. Since the carboxylate side-chains of acidic residues constitute excellent sites for metal chelation, we addressed whether chemical modification of the accessible carboxylates by 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC) might alter the metal-catalyzed fragmentation of these proteins. Lysates of HeLa cells were incubated with 5 mM EDAC for 5 min before initiation of a metal-catalyzed oxidation reaction by addition of Fe and ascorbate. EDAC significantly decreased the specific cleavage of topoisomerase I (Fig. 3 C) and RNA polymerase II large subunit (data not shown), indicating that negatively charged amino acid side chains are involved in the fragmentation reaction. In contrast, treatment with either diethyl pyrocarbonate (1 mM) or iodoacetamide (1 mM) (which modify histidine and cysteine residues, respectively), failed to decrease metal-catalyzed
autoantigen fragmentation (data not shown). Taken together, the specific cleavage sites, inhibition by chelators and EDAC, and competition by zinc strongly suggest that the cleaved scleroderma autoantigens possess metal-binding sites which focus metal-catalyzed oxidation and resulting fragmentation at specific places. Since neither γ-irradiation (which generates OH• by radiolysis of H2O), nor addition of H2O2 or ascorbate alone to control lysates results in autoantigen fragmentation (Fig. 1 and data not shown), it is likely that the physiologic state of these sites is either unoccupied, or occupied by a metal incapable of supporting Fenton chemistry.

Specific Autoantigen Fragmentation Is Induced in Intact Cells Chronically Exposed to Supraphysiologic Concentrations of Copper. The absolute dependence of autoantigen fragmentation on exogenous Fe or Cu in cell lysates prompted us to address whether similar autoantigen fragmentation could be induced in intact cells chronically exposed to supraphysiologic concentrations of free Cu. Human foreskin keratinocytes were cultured for 18 h in vitro in defined, serum- and albumin-free growth medium supplemented with 20 μM CuSO4. Oxidation reactions were initiated by adding 2 mM H2O2 to some cultures for an additional 30 min. This choice of cell type, metal ion and concentration, and H2O2 dose were based on preliminary studies which demonstrated that (a) loading cells with Fe was not feasible, (b) serum-free medium greatly facilitates the reproducible uptake of Cu, and (c) 20 μM Cu (with H2O2) induces very little fragmentation in the in vitro lysate system (data not shown). Only intact topoisomerase I was detected in control keratinocytes (Fig. 4, lane 1), and H2O2 treatment alone did not result in autoantigen fragmentation (Fig. 4, lane 2). Incubation with 20 μM Cu overnight did not affect cell morphology or viability (data not shown), and did not produce any fragmentation of topoisomerase I in the absence of added H2O2 (Fig. 4, lane 4). In contrast, addition of H2O2 after overnight Cu loading resulted in the marked fragmentation of topoisomerase I into the predominant Cu-characteristic band of 95 kD (Fig. 4, lane 4). Incubation of cells in medium containing 20 μM Cu for 2 h generated no topoisomerase I fragments in the presence or absence of H2O2 (Fig. 4, lanes 5 and 6), clearly demonstrating that overnight preincubation of cells with Cu sensitizes cells for H2O2-induced fragmentation of topoisomerase I. Washing away the Cu-containing medium before adding...
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H<sub>2</sub>O<sub>2</sub> had little effect on the extent or characteristics of fragmentation (data not shown), indicating that the Cu effect was cell associated. Since addition of 20 μM Cu and H<sub>2</sub>O<sub>2</sub> fails to induce autoantigen fragmentation in cell lysates, the fragmentation observed in the intact cells pre-incubated with this Cu concentration strongly implies that higher localized concentrations of Cu are generated in these cells, which facilitate binding of Cu to the relevant autoantigens.

The most likely subcellular site in which these increased metal concentrations occur is the nucleolus, since the scleroderma autoantigens are enriched in nucleoli (reviewed in reference 33), and several histochemical studies have demonstrated that the nucleolus has the unusual capacity to concentrate metal ions, including silver, zinc, cobalt, and lead (38–41). Morphologic studies were performed to evaluate whether the patterns of staining of scleroderma autoantigens and metals in the nucleolus were similar (Fig. 5). Exclusive staining of punctate intranucleolar structures (nucleolini) was observed when HeLa cells were fixed with lead acetate and stained with ammonium sulfide (Fig. 5 A), or when ethanol-fixed HeLa cells were incubated with a solution containing zinc ions, before staining with di-thizone (40, and data not shown). Identical punctate intranucleolar structures were observed when confocal immunofluorescence microscopy was performed with antibodies to topoisomerase I (Fig. 5 B) and other scleroderma autoantigens (data not shown).

Discussion

Although the mechanisms underlying the initiation and propagation of scleroderma remain unclear, the constellation of severe ischemia reperfusion (associated with tissue injury), widespread fibrosis, and specific autoantibody response are unique to this disease, and provide essential clues to the initiating process. The great specificity of the autoantibody response for a limited number of molecules in scleroderma strongly suggests that these molecules share some feature, hitherto unrecognized, that renders them susceptible to forces which reveal previously cryptic structure. The studies presented here demonstrate a striking feature that unifies several of the scleroderma autoantigens: these molecules are cleaved at highly specific sites in a reaction that requires metal binding and the generation of ROS. Cleavage by this process is unique to this group of autoantigens; numerous other proteins, including several lupus autoantigens, are resistant to fragmentation by this method. The sensitivity of the scleroderma antigens to ROS-induced fragmentation focuses attention on episodic ischemia reperfusion (a striking clinical feature of the disease) as the potential circumstance under which such fragmentation might occur in vivo, and provides a novel framework within which to view the pathophysiology of scleroderma.

Although several modifications of proteins (including loss of catalytic activity [34], amino acid modification [22], increase in proteolytic susceptibility [26], and increase in acidity [22]) are commonly observed during metal-catalyzed oxidation reactions, highly specific fragmentation of protein molecules is an extremely unusual consequence (23). Specific fragmentation can, however, be generated by covalently linking redox-active coordination complexes (e.g., ferrous EDTA or 1,10-phenanthroline–copper) to specific sites on the protein, generating a highly selective scission reagent at that site (36, 42, 43). The discrete autoantigen fragments described here, coupled with the inhibition of fragmentation by chelators and the competition of fragmentation by zinc, strongly indicate that these autoantigens are unified by their capacity to assemble a highly focused scission reagent, likely by binding a redox active metal at specific sites. The observation that the U1-70kDa

Figure 5. Metal ions and topoisomerase I are concentrated in punctate intranucleolar structures. (A) HeLa cells were fixed in 1% lead acetate, and subsequently stained with 1% ammonium sulfide. Bright field microscopy demonstrates intense staining of punctate intranucleolar structures (nucleolini). (B) HeLa cells were stained with a monospecific human serum recognizing topoisomerase I and FITC-goat anti-human IgG, and were examined by confocal fluorescence microscopy. Diffuse nuclear staining, as well as punctate intranucleolar structures (nucleolini), are seen. Similar results were obtained with six other monospecific topoisomerase I sera. Nucleolini were never stained when similar experiments were performed using sera obtained from healthy individuals, or from Ro/La-positive lupus patients. Bar, 10 μM.
fragments generated from protein translated in vitro are identical to those observed in cell lysates further demonstrates that specific fragmentation is a property of the protein itself, rather than merely its subcellular environment (see below). Furthermore, there is direct evidence that an aspartic acid-rich region in the large subunit of bacterial RNA polymerase (which chelates the active center Mg\textsuperscript{2+} ion; 44) can also bind Fe(II); under these circumstances, fragmentation of the protein occurs in the region of the metal-binding site in a reaction that requires generation of hydroxyl radicals (44). Both metal binding and cleavage were dependent upon the presence of an aspartic acid-rich motif (DFDGD) (44), which is absolutely conserved in eukaryotic RNA polymerases I, II, and III (44). It is of great interest that all three of the human RNA polymerases are autoantigens in scleroderma (32); the sizes of the fragments produced by metal-catalyzed oxidation of human RNA polymerase II large subunit (Fig. 1) correspond well with those predicted to arise if cleavage occurred at the equivalent DFDGD site in this molecule.

Interestingly, effective autoantigen fragmentation results after prolonged incubation of intact cells with concentrations of extracellular Cu that are unable to induce protein fragmentation in cell lysates. The preincubation required suggests several possibilities, including the development of localized increases in the Cu concentration within the cell over time, and/or the slow exchange of Cu for a metal already bound to the autoantigen. The observation that scleroderma autoantigens are enriched in nucleoli (reviewed in reference 33; Fig. 5 B), where they colocalize with sites of high-affinity metal binding (40, 45; and Fig. 5 A), is therefore of great interest. It suggests that these antigens are unified both by their capacity to bind metals and their location at sites containing potentially increased local concentrations of redox active metals (see below).

The importance of autoantigen fragments as potential initiators of the autoimmune process has been underscored (3, 4). A recent study demonstrating the existence of autoreactive T cells which recognize several fragments of topoisomerase I (46) indicates that this proposed mechanism is feasible in scleroderma. Clearly, it will be critical to determine whether the novel scleroderma autoantigen fragments that we have demonstrated in this study are indeed able to break tolerance to the intact self molecules. The observation that fibrillarin, a major ribonucleoprotein autoantigen in scleroderma, is not cleaved by metal-catalyzed oxidation reactions is reminiscent of several ribonucleoprotein autoantigens targeted in SLE, which are not cleaved by ICE-like proteases during apoptosis (13). Since metal-catalyzed oxidation may also have effects on the structure of the nucleic acid components of these complex autoantigens, it is possible that other mechanisms might be responsible for revealing previously cryptic epitopes in these circumstances. It is also possible that exotic metal binding may alter the structure of these complexes. In this regard, it is of interest that a specific autoantibody response to fibrillarin is observed in metal-overloaded mice (47, 48).

We propose that the autoantibody response in scleroderma is the immune marker of unique protein structure, induced and driven by cycles of ischemia reperfusion. Since unique protein fragmentation is only observed when Fe or Cu is present, and can be induced in intact cells chronically exposed to supraphysiologic concentrations of Cu, we further suggest that abnormal cellular metal accumulation might be of critical pathogenic importance in scleroderma. In this regard, it is of great interest that D-penicillamine, a metal chelator that prevents autoantigen fragmentation in vitro, appears to improve the outcome in patients with diffuse scleroderma, when used for extended periods (49, 50). The observation that zinc decreases the fragmentation of these autoantigens is intriguing, and may have therapeutic implications. Whether a metal abnormality might also have some influence on vascular reactivity, lowering the threshold for developing vasospasm, and initiating a cycle of damage, remains to be determined. The characteristic autoantigen footprints produced by metal-catalyzed oxidation reactions will be important tools with which to study the existence, sites, mechanisms, and significance of autoantigen fragmentation in perturbed tissues of patients with scleroderma.

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References

1. Radic, M.Z., and M. Weigert. 1994. Genetic and structural evidence for antigen selection of anti-DNA antibodies. *Annu. Rev. Immunol.* 12:487–520.

2. Diamond, B., J.B. Katz, E. Paul, C. Aranow, D. Lustgarten, and M.D. Schaff. 1992. The role of somatic mutation in the pathogenic anti-DNA response. *Annu. Rev. Immunol.* 10:731–757.

3. Sercarz, E.E., P.V. Lehmann, A. Ametani, G. Benichou, A. Miller, and K. Moudgil. 1993. Dominance and crypticity of T cell antigenic determinants. *Annu. Rev. Immunol.* 11:729–766.

4. Lanzavecchia, A. 1995. How can cryptic epitopes trigger autoimmunity? *J. Exp. Med.* 181:1945–1948.

5. Gammon, G., and E.E. Sercarz. 1989. How some T cells escape tolerance induction. *Nature (Lond.)*. 342:183–185.

6. Mamula, M.J. 1993. The inability to process a self-peptide allows autoreactive T cells to escape tolerance. *J. Exp. Med.* 177:567–571.

7. Bockenstedt, L.K., D.G. Campbell, A. Lanzavecchia, N. Fairweather, and C. Watts. 1995. Modulation of antigen processing by bound antibodies can boost or suppress class II major histocompatibility complex presentation of different T cell determinants. *J. Exp. Med.* 181:1957–1963.

8. Saling, S., A.P. Caporossi, L. Boiffa, M.G. Longobardi, and V. Barnaba. 1995. HIVgp120 activates autoreactive CD4 specific T cell responses by unveiling of hidden CD4 peptides during processing. *J. Exp. Med.* 181:2253–2257.

9. Watts, C., and A. Lanzavecchia. 1993. Suppressive effect of an antibody on processing of T cell epitopes. *J. Exp. Med.* 178:1459–1463.

10. Smitteck, P.D., D.G. Campbell, A. Lanzavecchia, N. Fairweather, and C. Watts. 1995. Modulation of antigen processing by bound antibodies can boost or suppress class II major histocompatibility complex presentation of different T cell determinants. *J. Exp. Med.* 181:1957–1963.

11. Casciola-Rosen, L.A., G. Anhalt, and A. Rosen. 1994. Autoantigens targeted in systemic lupus erythematosus are clustered in two populations of surface structures on apoptotic keratinocytes. *J. Exp. Med.* 179:1317–1330.

12. Casciola-Rosen, L.A., D.K. Miller, G.J. Anhalt, and A. Rosen. 1994. Specific cleavage of the 70-kDa protein component of the U1 small nuclear ribonucleoprotein is a characteristic biochemical feature of apoptotic cell death. *J. Biol. Chem.* 269:30757–30760.

13. Casciola-Rosen, L.A., G.J. Anhalt, and A. Rosen. 1995. DNA-dependent protein kinase is one of a subset of autoantigens specifically cleaved early during apoptosis. *J. Exp. Med.* 182:1625–1634.

14. Casciola-Rosen, L.A., D.W. Nicholson, T. Chong, K.R. Rowan, N.A. Thomberry, D.K. Miller, and A. Rosen. 1996. Apopain/CPP32 cleaves proteins that are essential for cellular repair: a fundamental principle of apoptotic death. *J. Exp. Med.* 183:1957–1964.

15. Belch, J.J.F. 1991. Raynaud’s phenomenon: its relevance to scleroderma. *Ann. Rheum. Dis.* 50:839–845.

16. Norton, W.L., and J.M. Nardo. 1970. Vascular disease in progressive systemic sclerosis (scleroderma). *Ann. Intern. Med.* 73:317–324.

17. McCord, J.M. 1985. Oxygen-derived free radicals in postischemic tissue injury. *N. Engl. J. Med.* 312:159–163.

18. Zweir, J.L. 1988. Measurement of superoxide–derived free radicals in the reperfused heart. *J. Biol. Chem.* 263:1353–1357.

19. Oliver, C.N., P.E. Starke–Reed, E.R. Stadtman, G.J. Liu, J.M. Carney, and R.A. Floyd. 1990. Oxidative damage to brain proteins, loss of glutamine synthetase activity, and production of free radicals during ischemia/reperfusion–induced injury to gerbil brain. *Proc. Natl. Acad. Sci. USA.* 87:5144–5147.

20. Herrick, A.L., F. Rieley, D. Schofield, S. Hollis, J.M. Braganza, and M.I.V. Jayson. 1994. Micronutrient antioxidant status in patients with primary Raynaud’s phenomenon and systemic sclerosis. *J. Rheumatol.* 21:1477–1483.

21. Stein, C.M., S.B. Tanner, J.A. Awad, L.J.I. Roberts, and J.D. Morrow. 1996. Evidence for free radical-mediated injury (isoprostane overproduction) in scleroderma. *Arthritis Rheum.* 37:1146–1150.

22. Davies, K.J.A. 1987. Protein damage and degradation by oxygen radicals. I. General aspects. *J. Biol. Chem.* 262:9895–9901.

23. Stadtman, E.R. 1990. Metal ion–catalyzed oxidation of proteins: biochemical mechanism and biological consequences. *Free Radical Biol. Med.* 9:315–325.

24. Youngman, R.J. 1984. Oxygen activation: is the hydroxyl radical always biologically relevant? *TIBS (Trends Biochem. Sci.)*. 280–283.

25. Halliwell, B., and J.M.C. Gutteridge. 1986. Oxygen free radicals and iron in relation to biology and medicine: some problems and concepts. *Arch. Biochem. Biophys.* 246:501–514.

26. Levine, R.L., C.N. Oliver, R.M. Fults, and E.R. Stadtman. 1981. Turnover of bacterial glutamine synthetase: oxidative inactivation precedes proteolysis. *Proc. Natl. Acad. Sci. USA.* 78:2120–2124.

27. Levine, R.L. 1983. Oxidative modification of glutamine synthetase. II. Characterization of the ascorbate model system. *J. Biol. Chem.* 258:11828–11833.

28. Creeth, J.M., B. Cooper, A.S. Donald, and J.R. Clamp. 1983. Studies of the limited degradation of mucus glycoproteins. The effect of dilute hydrogen peroxide. *Biochem. J.* 211:323–332.

29. Cooper, B., J.M. Creeth, and A.S. Donald. 1985. Studies on the limited degradation of mucus glycoproteins. The mechanism of the peroxide reaction. *Biochem. J.* 228:615–626.

30. Shero, J.H., B. Bordwell, N.F. Rothfield, and W.C. Earnshaw. 1986. High titers of autoantibodies to topoisomerase I (ScI-70) in sera from scleroderma patients. *Science (Wash. DC).* 231:737–740.

31. Rodriguez-Sanchez, J.L., C. Gelpi, C. Juarez, and J.A. Hardin. 1987. Anti-NOR 90: a new autoantibody in scleroderma that recognizes a 90-kDa component of the nucleolus-organizing region of chromatin. *J. Immunol.* 139:2579–2584.

32. Hirakata, M., Y. Okano, A. Suwa, T.A. Medsger, Jr., J.A. Hardin, and J. Craft. 1993. Identification of autoantibodies to RNA polymerase II. Occurrence in systemic sclerosis and association with autoantibodies to RNA polymerases I and III. *J. Clin. Invest.* 91:2665–2672.

33. Tan, E.M. 1991. Autoantibodies in pathology and cell biology. *Cell.* 67:841–842.

34. Fucci, L., C.N. Oliver, M.J. Coon, and E.R. Stadtman. 1983. Inactivation of key metabolic enzymes by mixed function oxidation reactions: possible implications in protein turnover and ageing. *Proc. Natl. Acad. Sci. USA.* 80:1521–1525.

35. Stadtman, E.R. 1993. Oxidation of free amino acids and amino acid residues in proteins by radiolysis and by metal-catalyzed reactions. *Annu. Rev. Biochem.* 62:797–821.
protein by an attached iron chelate. *J. Am. Chem. Soc.* 112: 2457–2458.

37. Brendel, V., J. Dohlman, B.E. Blaisdell, and S. Karlin. 1991. Very long charge runs in systemic lupus erythematosus-associated autoantigens. *Proc. Natl. Acad. Sci. USA.* 88:1536–1540.

38. Tandler, C.J. 1956. An acid-soluble component of the nucleolus: the cytochemical specificity of the “lead-acetate reaction”. *J. Histochem. Cytochem.* 4:331–340.

39. Pihl, E. 1968. Recent improvements of the sulfide-silver procedure for ultrastructural localization of heavy metals. *J. Microsc.* (Paris). 7:509–520.

40. Studzinski, G.P. 1965. Selective binding of zinc by basic proteins of the HeLa cell nucleolus. *J. Histochem. Cytochem.* 13: 365–375.

41. Tandler, C.J., and A.J. Solari. 1969. Nucleolar orthophosphate ions: electron microscope and diffraction studies. *J. Cell Biol.* 41:91–108.

42. Wu, J., D.M. Perrin, D.S. Sigman, and H.R. Kaback. 1995. Helix packing of lactose permease in *Escherichia coli* studies by site-directed chemical cleavage. *Proc. Natl. Acad. Sci. USA.* 92:9186–9190.

43. Rana, T.M., and C.F. Meares. 1991. Transfer of oxygen from an artificial protease to peptide carbon during proteolysis. *Proc. Natl. Acad. Sci. USA.* 88:10578–10582.

44. Zaychikov, E., E. Martin, L. Denissova, M. Kozlov, V. Markovtsov, M. Kashlev, H. Heumann, V. Nikiforov, A. Goldfarb, and A. Mustaev. 1996. Mapping of catalytic residues in the RNA polymerase active center. *Science (Wash. DC).* 273:107–109.

45. Studzinski, G.P., and R. Love. 1964. Nucleolar organelles shown by lead precipitation in unfixed cultured cells. *Stain Technol.* 39:397–401.

46. Kuwana, M., T.A. Medsger, Jr., and T.M. Wright. 1995. T cell proliferative response induced by DNA topoisomerase I in patients with systemic sclerosis and healthy donors. *J. Clin. Invest.* 96:586–596.

47. Hultman, P., L.J. Bell, S. Enestrom, and K.M. Pollard. 1992. Murine susceptibility to mercury. I. Autoantibody profiles and systemic immune deposits in inbred, congenic, and intra-H-2 recombinant strains. *Clin. Immunol. Immunopathol.* 65: 98–109.

48. Hultman, P., S. Enestrom, S.J. Turley, and K.M. Pollard. 1994. Selective induction of anti-fibrillarin autoantibodies by silver nitrate in mice. *Clin. Exp. Immunol.* 96:285–291.

49. Steen, V.D., T.A.J. Medsger, and G.P. Rodnan. 1982. D-Penicillamine therapy in progressive systemic sclerosis (scleroderma): a retrospective analysis. *Ann. Intern. Med.* 97:652–659.

50. Jimenez, S.A., and S.H. Sigal. 1991. A 15 year prospective study of treatment of rapidly progressive systemic sclerosis with D-penicillamine. *J. Rheumatol.* 18:1496–1503.