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Autoimmune mechanisms of scleroderma and a role of oxidative stress

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Scleroderma is a fibrotic condition characterized by immunological abnormalities, vascular injury and increased accumulation of extracellular matrix proteins in the skin. Although the etiology of scleroderma has not yet been fully elucidated, a growing body of evidence suggests that extracellular matrix overproduction by activated fibroblasts results from complex interactions among endothelial cells, lymphocytes, macrophages and fibroblasts via a number of mediators, such as cytokines, chemokines and growth factors. Recent investigations have further suggested that reactive oxygen species (ROS) are involved and play a role of autoimmunology in scleroderma. In this review, current findings on the autoimmune mechanisms in the pathophysiology of scleroderma are described.

Introduction

Scleroderma is a connective tissue disease involving fibrosis of the skin, which is characterized by the excessive accumulation of extracellular matrix (ECM) proteins, vascular injury and immunological abnormalities.[1] In early stages of scleroderma, activated fibroblasts in the affected areas produce high amounts of collagen. Histological analysis of the initial stage of scleroderma reveals perivascular infiltrates of mononuclear cells in the dermis, which is associated with increased collagen synthesis in the surrounding fibroblasts. Although the pathogenesis of systemic sclerosis (SSc) has not been fully elucidated as yet, a number of studies have demonstrated the crucial role of several fibrogenic cytokines released from immunocytes in initiating the sequence of events leading to fibrosis. Additionally, other mechanisms such as vascular injury and apoptosis are also participated in the induction of fibrotic conditions. In this review, current findings on the autoimmune mechanisms and a role of oxidative stress in the pathophysiology of scleroderma are discussed.

Autoimmune Mechanisms

T cells, macrophages and mast cells are present in increased numbers or in an activated state in the lesional scleroderma skin, which play an active role in the pathogenesis of the disease by releasing a number of mediators, cytokines/chemokines and growth factors. In addition, activated peripheral B cells are found in abnormally large numbers in patients with SSc.[2] B cells contribute not only to antibody production, but also to T cell activation and differentiation and the production of various cytokines.

Pathogenic autoantibodies in scleroderma. Circulating antibodies are present in most patients with SSc. Although their role in the pathogenesis of scleroderma remains unclear, the symptomology of SSc can be classified to some extent by the presence of specific antibodies. Many patients with limited SSc have antibodies against centromeres, whereas anti-topoisomerase-1 (Scl-70) antibodies are often detected in patients with diffuse SSc. Anti-RNA polymerase III antibodies are associated with scleroderma renal crisis and anti-Th/To antibodies are associated with pulmonary fibrosis. Anti-PM-Scl and anti-U1-RNP antibodies are associated with myositis and overlap syndrome.

Recently, circulating antibodies to platelet-derived growth factor (PDGF) receptors, which stimulate reactive oxygen species (ROS) and collagen,[3] have been identified in patients with SSc. The ROS-Ras-ERK1/2 cascade results in fibroblast activation and the formation of a myofibroblastic phenotype.

Cytokines and chemokines in scleroderma. *TGF*β. Transforming growth factorβ (TGFβ), which occurs abundantly in platelets and is released by activated macrophages or lymphocytes, is a strong chemoattractant for fibroblasts. TGFβ increases the synthesis of ECM, such as collagen type I and type III, or fibronectin by fibroblasts, modulates cell-matrix adhesion protein receptors, and regulates the production of proteins such as plasminogen activator, an inhibitor of plasminogen, or procollagenase, which can modify the ECM by proteolytic action. In addition, TGFβ is capable of stimulating its own synthesis by fibroblasts through autoinduction, and also increases TGFβ receptor (TGFβR) levels in fibroblasts.[4] TGFβ mRNA levels are elevated in the lesional skin of SSc, and shown to co-localize with type I collagen. Overexpression of TGFβR, which is regulated at the transcriptional level,[5] is recognized in fibroblasts in the skin of scleroderma patients.[6] Blocking endogenous TGFβ signaling eradicates the scleroderma phenotype.[7] Thus, TGFβ plays a key role via autocrine signaling in the pathogenesis of scleroderma.

Signaling by TGFβ elicits potent profibrotic responses in fibroblasts. TGFβ binds to the type II receptor, thereby activating the type I receptor. Signaling occurs predominantly by phosphorylation of cytoplasmic mediators belonging to the Smad family. In

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scleroderma fibroblasts, phosphorylation and nuclear translocation of Smad2/3 are increased, suggesting activation of the Smad pathway.[8] Smad7 is shown to act as an intracellular antagonist of TGFβ signaling, and an inhibitor of TGFβ-induced transcriptional responses. In scleroderma skin and cultured scleroderma fibroblasts, the basal level and the TGFβ-inducible expression of Smad7 are selectively decreased, whereas Smad3 expression is increased.[9] On the other hand, Smad7 expression levels in scleroderma fibroblasts are uncertain. Smad7-Smurf-mediated negative regulation of TGFβ signaling is impaired in scleroderma fibroblasts.[10] Other signaling pathways besides the Smad proteins, such as the p38 mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3K), c-Myb, Ets and Egr pathways, have also been shown to mediate TGFβ signaling in scleroderma fibroblasts.

CTGF. CTGF is selectively induced in fibroblasts after activation by the active form of TGFβ. Recombinant CTGF protein stimulates DNA synthesis and upregulate collagen, fibronectin and integrin expression in fibroblasts. CTGF functions as a downstream mediator of TGFβ, and may coordinate the action of TGFβ, such as fibroblast proliferation, adhesion, and ECM production.[11] Overexpression of CTGF is known to occur in cultured scleroderma fibroblasts.[12,13] Selective overexpression of CTGF in fibroblasts led to a fibrotic phenotype.[14] The constitutive overexpression of CTGF in scleroderma fibroblasts is independent of TGFβ signaling but dependent on Sp1.[15] Moreover, serum levels of CTGF are elevated in patients with SSc.[16] The CTGF silencing by small interfering RNA (siRNA) increased the production of matrix metalloproteinase-1 (MMP-1), while decreased tissue inhibitor of metalloproteinase-1 (TIMP-1) in scleroderma fibroblasts.[17] Dermal fibroblasts exposed to hypoxia (1% O_2) or $CoCl_2$ (1–100 μM) enhance expression of CTGF mRNA.[18] Skin fibroblasts transfected with hypoxia-inducible factor (HIF)-1α show increased levels of CTGF protein and mRNA, as well as nuclear staining of HIF-1α, which was enhanced further by treatment with $CoCl_2$. This data may suggest that hypoxia, caused possibly by microvascular alterations, upregulates CTGF expression through the activation of HIF-1α in dermal fibroblasts of SSc patients, and thereby contributes to the progression of skin fibrosis.

A recent study has demonstrated that variations in the promoter region of the CTGF gene (G-945C polymorphism) are linked to susceptibility to SSc.[19]

IL-13. An imbalance exists between the type 1 and type 2 cytokine response in the pathogenesis of scleroderma. IL-13 is a pleiotropic cytokine, elaborated in significant quantities by appropriately stimulated type 2 cells. IL-13 has the ability to suppress proinflammatory cytokine production in monocytes/macrophages, and is known to enhance the growth and differentiation of B cells and to promote immunoglobulin synthesis. In addition, in vitro studies demonstrate that IL-13 is a potent stimulator of fibroblast proliferation and collagen production. The profibrotic effect of IL-13 is thought to involve irreversible fibroblast activation, triggered either directly or indirectly through TGFβ.[20,21] Serum levels of IL-13 are elevated in patients with SSc, correlated with the number of plaque lesions[22] or nailfold capillaroscopic features.[23]

IL-33 is a member of IL-1 cytokine family, which is released from cells undergoing necrotic cell death and thus functions as damage-associated molecular pattern (DAMP). Repeated administration of IL-33 induced skin fibrosis and inflammation in mice.[24] IL-13 is a critical down-stream mediator of IL-33-induced cutaneous fibrosis.

Chemokines. Recent studies have suggested that chemokines and their receptors may be important mediators of inflammation and fibrosis in scleroderma.[25] CCL2/monocyte chemoattractant protein-1 (MCP-1) belongs to a C-C chemokine superfamily and numerous types of cells are capable of expressing CCL2 in the presence of serum or specific stimuli. A growing body of evidence has demonstrated that CCL2 gene expression is upregulated in human fibrosis, as well as in animal models of fibrosis. In vitro studies show that CCL2 upregulates type I collagen mRNA expression in rat fibroblasts, which is indirectly mediated by endogenous upregulation of TGFβ gene expression.[26] CCL2 enhances expression of MMP-1, MMP-2 as well as TIMP-1 in cultured skin fibroblasts.[27] Recent studies have demonstrated increased expression of CCL2 in patients with SSc. Serum levels and spontaneous production levels of CCL2 by peripheral blood mononuclear cells are elevated in patients with SSc, compared with normal controls, and are correlated with pulmonary fibrosis.[28] Increased expression of CCL2 is demonstrated in scleroderma skin,[29-32] and scleroderma fibroblasts express increased levels of CCL2 mRNA and protein.[30,31] Stimulation with PDGF results in a significant increase in CCL2 mRNA and protein.[29] Furthermore, the autoinduction of CCL2 is observed in scleroderma fibroblasts, but not in normal fibroblasts.[31] CCL2 levels may also be increased by IL-13, a potent stimulator of CCL2.[33] These in vivo and in vitro results suggest an important involvement of CCL2 in the pathogenesis of scleroderma.

Increased numbers of mast cells are noted in scleroderma skin. CCL2 also recruits mast cells, in addition to monocytes. Human mast cells are shown to be a rich source of chemokines, including CCL2, CCL3/macrophage inflammatory protein-1α (MIP-1α), CCL4/MIP-1β and CCL5/RANTES,[34] as well as a number of cytokines/growth factors and mediators capable of activating fibroblasts or endothelial cells. Expression of SCF is upregulated in scleroderma fibroblasts,[35] and is thought to contribute to the increase of mast cells in scleroderma. SCF enhances CCL2 expression in human mast cells.[36] Because CCL2 enhances type I collagen mRNA expression in skin fibroblasts, the interaction between mast cells and fibroblasts via SCF/CCL2 may play an important role in the development of fibrosis.

CCR2 is a major CCL2 receptor. CCR2 upregulation in vascular structures, perivascular inflammatory infiltrates, and fibroblasts has recently been demonstrated in SSc.[37] In particular, CCR2-positive fibroblasts in early-stage dSSc showed a profibrotic phenotype, with overexpression of α-smooth muscle actin (α-SMA), CTGF and CCL2.[38] Their results suggest potential autocrine regulation of key fibrotic properties via the CCL2/CCR2 loop in the early phases of scleroderma.

A novel protein, MCPIP (MCP-induced protein), upregulates members of the apoptotic gene family involved in the induction of cell death,[39] and may provide a novel molecular pathway by which CCL2/CCR2 signal transduction is linked to transcriptional gene regulation leading to apoptosis. CCL2 promoter polymorphism is associated with SSc.[40] CCL2 may contribute to the induction of dermal sclerosis directly, via its upregulation of mRNA expression of ECM on fibroblasts, as well as indirectly through the mediation of a number of cytokines released from immunocytes recruited into the lesional skin.

Others. PDGF has mitogenic activity for mesenchymal cells, regulates matrix metabolism, has chemotactic and vasoactive properties, and produces inflammatory cytokines. Overexpression of PDGF has been reported in a number of fibrotic diseases. Elevated levels of PDGF-A chain are demonstrated in scleroderma skin.[41] In addition, TGFβ upregulates PDGF-α mRNA and protein levels in scleroderma fibroblasts, in comparison with the control.[42] On the other hand, increased expression of the PDGF B-chain and β-receptor in scleroderma skin has also been reported.[43-45]

IL-4 is known to promote fibroblast proliferation, gene expression, and synthesis of ECM proteins such as collagen and tenascin. IL-4 has been shown to upregulate TIMP-2 in dermal fibroblasts via the MAPK pathway[46] as well as to upregulate TGFβ production. Increased IL-4 production is detected in the sera or in activated peripheral blood mononuclear cells of patients with SSc.[47] Scleroderma fibroblasts express more IL-4 receptor α and produce more collagen after IL-4 stimulation.[48]

TGFβ can contribute to the differentiation of both regulatory T cells (Tregs) and inflammatory Th17 cells. IL-17 is a T cell-derived cytokine, and functions to secret various cytokines and chemokines by different cell types. Elevated levels of IL-17 have been observed in patients with SSc, especially in the early stages,[49] and limited SSc.[50] IL-17 has been reported to induce fibroblast proliferation, but not collagen production in SSc fibroblasts.[49] Also, Th17 promotes inflammation in SSc. IL-23 is associated with the activation and proliferation of Th17 cells. Increased serum IL-23 levels are shown in patients with SSc, in association with the disease duration and prevalence of pulmonary fibrosis.[51]

IL-21/IL-21R signaling has recently been shown to promote fibrosis by facilitating the development of the $CD4^+$ Th2 response.[52] IL-21 increases IL-4 and IL-13 receptor expression in macrophages,[53] thereby possibly enhancing fibrosis, and is abundantly expressed in the epidermis in SSc.[54]

Innate immunity. Recent studies suggest that toll-like receptors (TLRs), which play an important role in the regulation of innate and adaptive immune responses, are involved in the regulation of inflammatory responses. Along with the recognition of microbial components, TLR signaling also plays an important role in the activation of the adaptive immune system by inducing proinflammatory cytokines and upregulating costimulatory molecules of antigen presenting cells. Thus, the dysregulation of TLR signaling may cause autoimmunity by priming autoreactive T cells. Also, stimulation of dendritic cells from patients with SSc TLR ligands resulted in enhanced secretion of IL-6 and TNFα.[55] Production of CCL2 by fibroblasts is partially upregulated by TLR4 recognition.[56] Mast cells are in increased numbers in the lesional skin of human as well as animal models of SSc. Mast cells can express TLRs, and may play a role in the induction of fibrosis by releasing various mediators upon stimulation via TLRs.

Regulatory T cells (Treg). $CD4^+$ T cells are now devided into two distinct lineages; Tregs and T helper (Th) cells. Tregs are critical in maintaining self tolerance and preventing autoimmunity. Recent data suggest a defective Treg function may underlie the immune dysfunction in SSc.[57] TGFβ induces expression of Foxp3, a Treg marker. The numbers of Tregs in the peripheral blood were increased, and correlated significantly with disease activity and severity.[58] By contrast, the number of $FoxP3^+$ Tregs was fewer in the skin of patients with scleroderma.[59] The number of Tregs is increased, which however had a diminished capacity to control $CD4^+$ effector T cells. Further, the defective function correlated with altered CD69 and TGFβ expression.

Vascular Injury

Vascular injury causes endothelial cell activation, dysfunction and altered capillary permeability as a primary event. These are followed by increased expression of adhesion molecules leading to mononuclear cell infiltrates in the skin. Microvascular injury may be the result of direct or indirect injury by anti-endothelial cell antibodies (AECAs), which are frequently detected in sera of patients with SSc.[60] AECAs can activate endothelial cells to express cell adhesion molecules which alter leukocyte attachment, and can lead to endothelial cell damage and apoptosis. Kuwana et al.[61] however, proposed that insufficient vascular repair machinery due to defective vasculogenesis contributes to the microvascular abnormality in SSc. Although circulating concentrations of angiogenic factors are high in SSc, the levels of bone marrow-derived circulating endothelial precursors (CEP) are low, suggesting a dysregulation of vasculogenesis in SSc.

Endothelin-1 (ET-1) is a prototypical endothelial cell-derived product. Since ET-1 is a vasoconstrictive agent, loss of normal vessel compliance and vasorelaxation may be induced by increased levels of ET-1. ET-1 is induced by TGFβ, and a downstream mediator of TGFβ.[62] ET-1 promotes fibroblast synthesis of collagen,[63] and thus provides the link between vasculopathy and fibrosis. ET-1 can induce CTGF, and may mediate the induction of collagen synthesis by activation of CTGF.[64] Further, ET-1 can also induce myofibroblast differentiation in fibroblasts.[65] Circulating ET-1 levels have been observed in patients with diffuse SSc and those with limited SSc and hypertensive disease,[66] suggesting that soluble ET-1 levels may be a marker of fibrosis and vascular damage. These facts underscore the importance ET-1 in scleroderma.

Nitric oxide (NO) is a vasodilator substance produced by endothelium, and dysregulated control of NO may be involved in SSc. Although the results of serum levels of NO in patients with SSc is controversy, NO synthase is overexpressed in scleroderma skin.[67] NO may be involved in the process of vascular damage associated with SSc.

Scleroderma Fibroblasts

Fibroblasts are stimulated by inflammatory cells, such as activated T cells, monocytes/macrophages, mast cells and eosinophils. Additionally, fibroblasts themselves are not only structural elements but also part of the immune system, and can be activated to perform new functions important for controlling ECM synthesis and for producing various cytokines, growth factors, chemokines, growth factor receptors, integrins and oxidants. The phenotype and activation of fibroblasts is dependent on both soluble factors and ECM-generated signals. Fibroblasts interact with the surrounding collagens via integrins. Aberrant signaling by ECM may disturb this interaction, thereby contributing to the persistent modulation of fibroblasts which results in fibrosis, as seen in the autocrine loops of cytokine production and excessive deposition of ECM proteins in the skin.[68] It is widely[68] accepted that human skin fibroblasts are heterogeneous with regard to their synthesis of collagen, proliferative responses and response to growth factors. Enhanced collagen synthesis is regulated at the transcriptional level. Some researchers think that scleroderma fibroblasts are the result of phenotypic changes in dermal fibroblasts caused by soluble factors; others contend that scleroderma fibroblasts are recruited from circulating or resting mesenchymal precursor cells as fibrocytes. Alternatively, they may be generated by clonal selection of high-collagen-producing fibroblasts. Scleroderma fibroblasts possess high Rac activity and a Rac inhibitor suppressed fibrotic phenotype of scleroderma fibroblasts.[69]

Myofibroblasts represent activated and contractile phenotypes which exist in fibrotic lesions. Myofibroblasts express α-SMA, and can produce various cytokines, growth factors and chemokines. TGFβ1 is a central regulator of the phenotypic changes of fibroblasts into myofibroblasts; the modulators are mechanical tension and fibronectin involving the ED-A domain. The differentiation into myofibroblasts is regulated by mast cell mediators, i.e., tryptase.[70]

Fibrocytes are derived from circulating monocytes ($CD34^+$ bone marrow-derived progenitors) and enter into the tissues. Fibrocytes produce matrix proteins such as collagens I and III, and participate in the remodeling process by secreting matrix metalloproteinases.[71] TGFβ signaling in fibrocytes activates both Smad2/3 and MAP kinases, specifically the ERK1/2 and SAPK/JNK pathways.[72] Fibrocytes are also a source of inflammatory cytokines, growth factors and chemokines, and involved in scleroderma.

Oxidative Stress

Oxidative stress is an imbalance between oxidants [reactive oxygen and nitrogen species (ROS/RNS)] and antioxidants which affect lipids, DNA, carbohydrates and proteins. ROS generated during various metabolic and biochemical reactions have multifarious effects that include oxidative damage to DNA. ROS can cause several abnormalities such as endothelial cell damage or enhanced platelet activation, leading to upregulation of the expression of adhesion molecules or secretion of inflammatory or fibrogenic cytokines including PDGF and TGFβ. Scleroderma fibroblasts produce ROS constitutively.[73] Other effects of oxygen radicals include the stimulation of skin fibroblast proliferation at low concentrations[74] and the production of increased amounts of collagen[75] suggesting that low oxygen tension may contribute to the increased fibrogenic properties of scleroderma fibroblasts. Treatment of dermal fibroblasts from scleroderma skin with antioxidant epigallocatechin-3-gallate (EGCG) reduced the expression of ECM proteins and CTGF.[76] Also, EGCG suppressed intracellular ROS, ERK1/2 kinase signaling and NFκB activity.[76]

Furthermore, several of the autoantigens targeted by scleroderma autoantibodies fragment in the presence of ROS and specific metals such as iron or copper.[77] The authors suggest that tissue ischemia generates ROS, which in turn induces the fragmentation of specific autoantigens. On the other hand, oxidative stress transiently induces CCL2 mRNA and protein expression in cultured skin fibroblasts,[78] suggesting that ROS may play a regulatory role in inflammation by modulating monocyte chemotactic activity. Thus, excessive oxidative stress has been implicated in the pathogenesis of scleroderma.[79] Reduced levels of micronutrient antioxidants and increased susceptibility of serum lipoproteins to oxidation have been reported in patients with SSc.[80]

Free radicals are produced by several mechanisms such as hypoxanthine-xanthine oxidase system and activation of polymorphonuclear leukocytes. Several markers which reflect free radical formation, i.e., 8-isoprostane and N(epsilon)-(hexanoyl) lysine, are elevated in the serum of patients with SSc.[81,82] Also, autoantibodies against antioxidant enzymes such as peroxiredoxin I and methionine sulfoxide reductase A (MSRA) are elevated in the serum of patients with SSc.[83,84]

Role of Apoptosis

Excessive ROS/RNS induce cell death. Autoreactive clones that survive the apoptotic process may lead to increased susceptibility to autoimmune disorders. Apoptosis causes typical cellular morphological changes including cell shrinkage, nuclear condensation, DNA fragmentation and membrane alterations. This may in turn cause apoptotic cells to become a possible source of autoantigens.[85] Scleroderma fibroblasts are thought to escape apoptosis because cultured scleroderma fibroblasts are resistant to Fas-induced apoptosis,[86,87] and apoptosis of fibroblasts in SSc skin lesions has not been observed.[87] TGFβ protects myofibroblasts from undergoing apoptosis. Serum-starved rat lung fibroblasts treated with IL-1 result in apoptosis which can be reduced by concomitant treatment with TGFβ.[88] Also, α-SMA-positive myofibroblasts increase in number following stimulation by TGFβ, which protects these myofibroblasts against apoptosis induction. Other studies have shown that pretreatment with TGFβ significantly reduced apoptosis caused by serum starvation in myofibroblasts, whereas this was not the case with non-myofibroblasts.[87] Thus TGFβ1 may play a role in inducing apoptosis-resistant fibroblast populations in SSc. In scleroderma fibroblasts, the Bcl-2 level is significantly higher, whereas the Bax level significantly lower.[87]

On the other hand, endothelial cell apoptosis is thought to occur early in the pathogenesis of scleroderma. Endothelial cell

apoptosis was first noted in the UCD-200/206 chickens, which develop hereditary systemic connective tissue disease resembling human SSc.[89] This phenomenon occurs before perivascular mononuclear cell infiltration. Also, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end-labeling (TUNEL) is shown to be positive on the endothelial cells in human scleroderma skin.[89] A recent study showed that sera of patients with SSc induced apoptosis of endothelial progenitor cells, which is mediated by Akt-FOXO3a-Bim pathway.[90] On the other hand, apoptosis of endothelial cells induces resistance to apoptosis in fibroblasts largely through PI3K-dependent mechanisms.[91] Furthermore, fibroblasts exposed to a medium conditioned by apoptotic endothelial cells present myofibroblast changes.[91]

The serum soluble Fas (sFas) levels are higher in patients with SSc.[92-94] Untreated SSc patients have significantly higher serum sFas levels than the treated SSc patients and healthy controls.[95] It has been suggested that increased sFas levels in the serum of SSc patients can protect autoreactive T cells from FasL-induced apoptosis.[93] Spontaneous apoptosis of $CD8^+$ T cells in the peripheral blood is significantly higher in patients with SSc compared with normal controls, while spontaneous apoptosis in $CD4^+$ T cells occur at similar rates in both SSc and controls.[96] Enhanced helper T cell function, resulting in the reduction $CD8^+$ T cells, may lead to autoimmunity by modifying the immune balance.

Akt is one of the key enzymes inhibiting both spontaneous and stress-induced apoptosis. 3'-phosphorylated phosphoinositides bind to the pleckstrin domain of Akt. Akt activity may result in the inhibition of pro-apoptotic Bad, Bax, Bik and caspase-9 by phosphorylation. It has recently been reported that Akt is active in scleroderma fibroblasts. Cultured scleroderma fibroblasts exhibited high levels of p-Akt, in comparison to control fibroblasts.[97] TGFβ can activate Akt in fibroblasts, and by doing so, may also induce apoptosis resistance in scleroderma fibroblasts. These findings point to a potential role for Akt in the resistance of scleroderma fibroblasts to apoptosis.

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