Role of the interferon-inducible gene IFI16 in the etiopathogenesis of systemic autoimmune disorders

Role of the Interferon-Inducible Gene IFI16 in the Etiopathogenesis of Systemic Autoimmune Disorders

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ABSTRACT: Interferons (IFNs) are now known to exert a multitude of immunological functions on both the innate and adaptive immunity. Given their pleiotropic effects on the immune system, it is conceivable that excess type I IFN or aberrant regulation of its signaling could contribute to autoimmunity. Several lines of evidence link IFNs to autoimmune disorders, in particular to systemic lupus erythematosus (SLE) and systemic sclerosis (SSc). Expression of a spectrum of genes that constitutes an "IFN signature" is the most significant observation indicating that IFNs may be dominant among the pathogenic mediators involved in some autoimmune diseases. A family of IFN-inducible genes, designated HIN-200 in the human and IFI-200 in the murine species, encodes evolutionary related human (IFI16, MNDA, AIM2, IFIX) and murine proteins (Ifi202 a, Ifi202b, Ifi203, Ifi204, Ifi205/D3). Physiological IFI16 expression was found in cells of the immune system, in endothelial cells, and in stratified squamous epithelia, such as skin. The presence of anti-IFI16 antibodies was reported in SLE and primary/secondary Sjögren's syndrome. More recently, we reported that anti-IFI16 autoantibodies differentiate limited cutaneous systemic sclerosis (lcSSc) from diffuse systemic sclerosis

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Ann. N.Y. Acad. Sci. 1110: 47–56 (2007).
doi: 10.1196/annals.1423.006

(dcSSc). Molecular studies performed in primary endothelial cells overexpressing IFI16 demonstrated that it may be involved in the early steps of inflammation by modulating endothelial cell function, such as expression of adhesion molecules and chemokine production, cell growth, and apoptosis. Moreover, here we report that IFI16 expression is induced by proinflammatory cytokines. In this article the role of the IFI16 protein and its corresponding autoantibodies in the etiopathogenesis of systemic autoimmune diseases, in which chronic inflammation is involved, are discussed.

KEYWORDS: autoimmunity; systemic sclerosis; SSc; systemic lupus erythematosus; SLE; interferon-inducible gene IFI16; inflammation

INTRODUCTION

Systemic sclerosis (SSc, scleroderma) is a generalized connective tissue disorder of unknown etiology, characterized by a wide spectrum of microvascular and immunological abnormalities, leading to progressive thickening and fibrosis of the skin and visceral organs, including the lung, heart, gastrointestinal tract, and kidney. Patients with SSc express a variety of autoantibodies that have their own clinical association. Disease heterogeneity and difficulties separating SSc from SSc-like conditions make classification an important issue. According to the criteria of LeRoy *et al.*,[1] established in 1988, SSc can be classified into two major subsets, on the basis of the extent of skin thickening: one characterized by diffuse skin fibrosis with more severe internal organ involvement (dcSSc), the other with limited cutaneous involvement (lcSSc). lcSSc and dcSSc, with different severity and survival, have been recognized for several years as distinct subsets. This issue remains unsettled, but transition from one to the other is seldom seen, which could favor the former interpretation. Importantly, the two classical SSc-selective autoantibodies segregate clearly between the subsets, lcSSc being associated with anticentromere (ACA), and dcSSc with antitopoisomerase I (Scl70) antibodies. The lcSSc/dcSSc classification has now been widely accepted and used in numerous clinical studies and therapeutic trials.

Several lines of evidence link the interferons (IFNs) to autoimmune disorders, in particular to SSc and systemic lupus erythematosus (SLE). Serum levels of IFN-α are elevated in active SLE, and therapeutic IFN-α has occasionally been noted to induce lupus autoantibodies and even clinical SLE. Finally, the prominent role of IFNs in the hierarchy of immune system mediators involved in SSc and SLE has been demonstrated by data from analyses of gene expression in peripheral blood mononuclear cells (PBMCs) from patients with active SLE and SSc and in healthy subjects.[2,3] Expression of a spectrum of genes that constitutes an "IFN signature" is the most significant result from these studies and indicates that either type I or type II IFNs may be dominant among the pathogenic mediators involved in lupus. The link between IFNs

and SSc pathogenesis is also supported by observations of several cases of IFN-induced severe Raynaud's phenomenon[4] and of some cases of progression to a full-blown picture of SSc.[5] Moreover, a clinical trial performed to evaluate the possible antifibrotic action of α-IFN in patients with early and diffuse SSc failed to show any benefit from treatment. On the contrary, in patients receiving α-IFN, there was a significant worsening of lung function test compared to placebo-treated patients, and more withdrawals due to lack of efficacy and deaths.[6] These observations suggest that IFN may have a deleterious effect on the onset and the evolution of SSc.

THE IFI-200/HIN-200 GENE FAMILY

One family of IFN-inducible genes is the IFI-200/HIN-200 gene family, which encodes evolutionarily related human (IFI16, MNDA, AIM2, and IFIX) and murine proteins (Ifi202 a, Ifi202b, Ifi203, Ifi204, Ifi205/D3).[7,8] The human and murine gene clusters are located on syntenic genomic regions of chromosome 1 and probably stem from repeated gene duplications of one ancestral gene. The IFI16, Ifi202b, and Ifi204 nuclear phosphoproteins are relatively well characterized with respect to their role in IFN action: these proteins are demonstrated to participate in the inhibition of cell cycle progression, modulation of differentiation, and cell survival. Generally, IFI-200 proteins are thought to act as scaffolds to assemble large protein complexes involved in the regulation of transcription. The HIN-200 proteins contain at least a 200-amino acid repeat that constitutes the HIN domain, which is always near the C terminus. The common domain architecture PYD-HIN of these protein families basically consists of one or two copies of the HIN domain and an N-terminal PYD domain, also named PAAD, DAPIN, or PYRIN domain after the protein pyrin/marenostrin, the product of the familial Mediterranean fever gene.[9] The PYD domain is frequently found in regulators of inflammatory immune responses and apoptosis.

In the human IFI16 protein the PYD domain is followed by two copies of a HIN domain. These are separated by a serine–threonine–proline (S/T/P)-rich spacer region. The size of the spacer region in IFI16 is regulated by mRNA splicing and can contain one, two, or three copies of the highly conserved 56-aa S/T/P/ domain. Three IFI16 isoforms (designated A, B, and C) arise due to alternative RNA splicing in the exons encoding the S/T/P domain.[10] The 200-amino-acid regions are unique to the HIN-200 proteins and contain no known functional motifs that could provide some clue to their physiological relevance. However, within the HIN-200 domains, there are stretches of amino acids, such as MFHATVAT, which exhibit almost complete identity across the A and B domains of all family members, and have been implicated in mediating protein–protein interactions and dimerization.

MOLECULAR MECHANISMS INVOLVED IN IFI16-INDUCED IMMUNE DYSREGULATION

IFI16 overexpression can result in decreased cell proliferation and a block in the cell cycle progression at the G1-S phase transition. A large body of evidence indicates that p53 and pRb are indeed involved in IFI16-mediated growth arrest. Overexpression of human papillomavirus 16 E6 and E7 oncoproteins inactivating p53 and pRb, respectively, inhibited the growth-suppressive effects of IFI16.[11] Prostate cancer cell lines expressing functional p53 and pRb were significantly more sensitive to the antiproliferative activity of IFI16.[12] The interaction of IFI16 with p53 in the nucleoplasm results in its stabilization and transcriptional activation of the p53-responsive bax promoter and expression of p53 target genes, such as p21 and HDM2.[13] Consistent with these observations, siRNA-mediated knockdown of IFI16 in human fibroblasts suppressed p53-mediated transcription of $p21^{WAF/CIP11}$ and Gadd45 promoters and resulted in downregulated $p21^{WAF1/CIP1}$ protein levels.[12] IFI16 has been identified as an essential growth-specific effector of the cell extrinsic growth–inhibitory pathway of Ras/Raf signaling in medullary thyroid carcinoma cells.[14] Finally, IFI16 expression has been found deregulated in several forms of human cancer.[12,13,15] Evaluation of some features of *in vitro* angiogenesis, namely chemotaxis, Matrigel invasion, tube morphogenesis, and cell cycle progression, has demonstrated that IFI16 overexpression impairs tube morphogenesis and proliferation of human endothelial cells.[11] Altogether these results point to a role for IFI16 in the regulation of cell growth, differentiation, and angiogenesis.

IFI16 is expressed in CD34+ myeloid precursor cells and remains strongly expressed within monocyte precursors, peripheral blood monocytes, and throughout lymphoid development.[16] However, IFI16 is not expressed in mature macrophages, nor is it found in cells of the erythroid and polymorphonuclear lineages. Following IFN-treatment, IFI16 localizes within the nucleolus and the nucleoplasm of human cells as shown by both confocal microscopy and immunoblotting of nuclear proteins. This nuclear import is mediated by a bipartite nuclear localization sequence (NLS) located at the N terminus of the protein. It has recently become evident that some HIN-200 proteins are also present in nonhemopoietic tissues. Immunohistochemical analysis of IFI16 expression in normal human tissues revealed that it is expressed in a highly restricted pattern in selected cells within certain organs.[17,18] IFI16 was found in epithelial cells of the skin, gastrointestinal tract, urogenital tract, and glands and ducts of breast tissues. Prominent IFI16 expression was seen in stratified squamous epithelia, particularly intense in basal cells in the proliferating compartments, whereas it gradually decreases in a more differentiated suprabasal compartment. In the underlying dermis, staining of connective tissue was restricted to scattered fibroblasts. In addition, all vascular endothelial cells from both blood and lymph vessels strongly expressed IFI16. This

physiological expression of IFI16 in endothelial cells and in stratified squamous epithelia, such as skin, both of which are targets for the main clinical manifestations of autoimmune diseases, indicated that IFI16 may be involved in the early steps of inflammation by modulating endothelial and keratinocyte cell function. Consistent with this hypothesis, transduction of IFI16 into human umbilical vein endothelial cells (HUVECs) with a herpes simplex virus-derived replication-defective vector efficiently suppressed formation of capillary-like structures *in vitro*, and cell cycle progression associated with cell death.[11] Moreover, recent data obtained in our laboratory revealed that IFI16-overexpressing HUVECs displayed an increase in expression of genes involved in immunomodulation, cell growth, and apoptosis (unpublished data). IFI16 triggered the expression of genes encoding adhesion molecules, such as intracellular adhesion molecule-1 (ICAM-1) and E-selectin, or chemokines, such as interleukin-8 (IL-8) and monocyte chemoattractant protein-1 (MCP-1). Finally, treatment of cells with small interfering RNA (siRNA) targeting IFI16 significantly inhibited ICAM-1 induction upon IFN-α treatment, demonstrating that IFI16 is required for proinflammatory gene stimulation.

Transcriptional regulation by type I and II IFN is a classic feature of the HIN-200 gene cluster, but it is also evident that these genes can be induced by an array of cytokines and differentiating agents. Several reports describe the induction of HIN-200 proteins by other factors; Ifi202b was upregulated by platelet-derived growth factor (PDGF), IL-6, and under reduced serum conditions.[19–21] Ifi205 gene expression was induced by proinflammatory agents, such as LPS, tumor necrosis factor-α (TNF-α), and PDGF.[22,23] In HL-60 cells, IFI16 was induced by dimetylsulfoxide, retinoic acid, and 1,25-dihydroxy vitamin D3.[24] According to these findings, we reported that IFI16 is stimulated in HUVECs by oxidative stress.[25] We found that H_2O_2 produced rapid accumulation of IFI16, indicating that IFI16 may be involved in the response of endothelial cells to oxidative stress. Regulation of IFI16 accumulation by H_2O_2 appears to be the result of its redox activity, as other compounds known to generate ROS intracellularly caused an IFI16 increase as well. Moreover, we analyzed whether its expression could be modulated by proinflammatory cytokines as well. To this end, HUVECs were treated with a panel of either proinflammatory cytokines including IL-1β, and TNF-α or anti-inflammatory cytokines, such as IL-4, IL-10, IL-13, and IL-17. Total cell extracts were prepared at 24 h after treatment and assessed for IFI16 expression by Western blotting. As expected, IFI16 expression was strongly induced by the known inducers, such as IFN-α, -β, and -γ (FIG. 1, lanes 6–8). Treatment with both proinflammatory molecules resulted in a significant induction of IFI16 protein, already present at the lowest cytokine concentrations used (lanes 2–5). In contrast, upon treatment with anti-inflammatory cytokines, no significant variation of the IFI16 expression levels in comparison to the basal level of untreated cells was observed (lanes 9–12). This finding, together with the observation that IFI16 triggers expression of

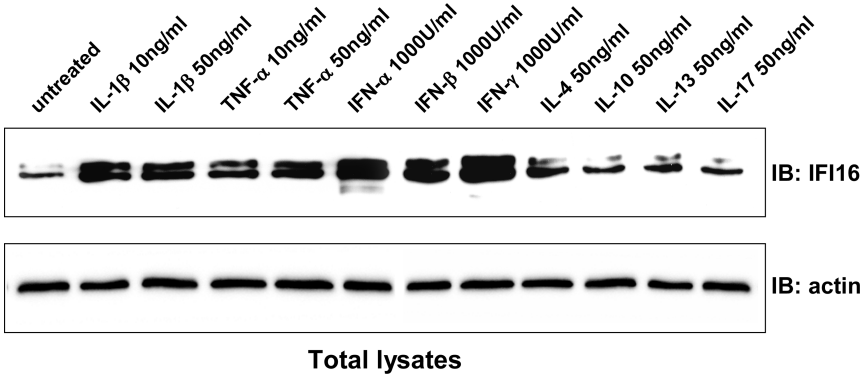

FIGURE 1. Modulation of IFI16 protein levels by cytokines in primary human endothelial cells. HUVECs (2.5×10^4 cells/cm^2), cultured in endothelial growth medium (EGM-2, Clonetics, San Diego, CA, USA) containing 2% fetal bovine serum (FBS), were left untreated or incubated with human recombinant IL-1β, TNF-α (from Chemicon International, Temecula, CA, USA), IFN-α and IFN-β (kindly provided by Antonio Ponzetto, Turin, Italy), IFN-γ (kindly provided by Gianni Garotta, Geneva), IL-4, IL-10, IL-13 (Chemicon), and IL-17 (R&D Systems, Minneapolis, MN, USA) at the indicated concentrations for 24 h. Total cell lysates were collected and analyzed by immunoblotting with a rabbit polyclonal antibody to IFI16[17] or a mouse monoclonal antibody to actin (Chemicon) as loading control.

proinflammatory molecules, strongly indicates that it may play a crucial role as inducer of the first steps of inflammation.

ANTI-IFI16 AUTOANTIBODIES IN SYSTEMIC AUTOIMMUNE DISEASES

Vascular involvement in autoimmune connective tissue diseases is a common complication. SSc is associated with reactive angiogenesis, but in spite of this, the disease finally leads to irreversible loss of capillaries.[26] The reduction in the number of capillaries is associated with endothelial swelling, basement membrane thickening, and hyperplasia of the intima, with infiltration of inflammatory cells in the skin. It is therefore conceivable to hypothesize that the release of nuclear proteins by endothelial cells from the cutaneous lesions, undergoing apoptosis and necrosis, triggers the immune system, leading to the production of autoantibodies. With this scenario in mind, one can hypothesize a role for IFI16 in systemic autoimmune diseases, in which chronic inflammation is involved.[27] Accordingly, generation of mice congenic for the Nba2 locus (derived from the NZB strain of mice) interval on C57 BL/6 background, coupled with gene expression analyses, has identified Ifi202, which belongs to the murine IFI-200 gene family, as a candidate for lupus susceptibility.[28] These studies also showed that increased expression of Ifi202 in splenic cells (both

B and T cells) correlated with splenomegaly and autoantibody production in female mice.

Several groups have evaluated the presence of anti-IFI16 antibodies in autoimmune disease patients (TABLE 1).[29] In 1994 Seelig *et al.*[30] first detected anti-IFI16 antibodies in a serum positive for antinuclear antibodies (ANAs), anti-SSA/Ro, and anti-SSB/La autoantibodies. By immunoblotting analysis on recombinant IFI16 expressed as MS2-polymerase fusion protein, these investigators also reported the presence of anti-IFI16 antibodies in 29% of sera obtained from 374 SLE patients. With a different technique, such as serological analysis of antigens by recombinant cDNA expression cloning (SEREX), Uchida *et al.*[31] detected anti-IFI16 antibodies in 70% of patients suffering from both primary and secondary Sjögren's syndrome (SjS). Moreover, in a recent paper, we identified anti-IFI16 antibodies in 21% of SSc patients by solid-phase enzyme-linked immunosorbent assay (ELISA), with a recombinant purified His-tagged IFI16 protein as antigen.[32] By using anti-IFI16-positive sera from SSc patients, we also found that the immunoblot technique appeared to be as specific as ELISA, but differed in sensitivity, very likely because immunoblot detects antibodies directed against linear epitopes, while ELISA detects either linear or conformational epitopes. No correlation between anti-IFI16 autoantibody levels by ELISA and the intensity of immunoreactive bands was observed. Finally, anti-IFI16-positive sera seen by immunoblotting, tested with both N-terminal and C-terminal fragments of IFI16 as antigens, reacted with either the N-terminal and C-terminal fragment or both of them, suggesting that there was a polyclonal immune response against IFI16 in patients with SSc. A lower prevalence was found in other systemic autoimmune diseases, such as rheumatoid arthritis (RA) (0–13%) and scleroderma/polymyositis overlap syndrome (4%). Seelig *et al.*[30] found that the presence of anti-IFI16 autoantibodies was more frequent in anti-dsDNA-positive sera from SLE patients, but no correlation was found between the titer of anti-IFI16 and the presence of anti-dsDNA, other autoantibodies, such as anti-RNP, anti-Sm, anti-SSA/Ro, and anti-SSB/La or with any particular clinical manifestation. In contrast, in our report, by analyzing SSc sera we demonstrated a strict association between anti-IFI16 reactivity and the cutaneous form of the disease, with patients in the limited cutaneous scleroderma (lcSSc) category having higher anti-IFI16 IgG titers than patients with the diffuse form (dcSSc).[32] Surprisingly, anti-IFI16 antibody did not associate with the presence of other serological markers of SSc, such as anti-Scl70 antibodies or ACA antibodies. For the differential diagnosis of the cutaneous form of SSc, anti-IFI16 reactivity displayed lower sensitivity (28%) and higher specificity (96%) than those found with either anti-Scl70 (95% and 67%, respectively) or anticentromere (65% and 92%, respectively). We found no correlation between anti-IFI16 autoreactivity and either disease duration, disease severity, or disability and no association was found with any particular clinical manifestation.

TABLE 1. Prevalence of anti-IFI16 autoantibodies in autoimmune diseases

Study group	Method of detection		
	Immunoblotting[30]	SEREX[31]	ELISA[32]
SLE	29% (374)	33% (15)	26% (100)
RA	0 (30)	13% (15)	4% (50)
SSc	NT	NT	21% (82)
PM/Scl	4% (26)	NT	NT
Primary SjS	NT	82% (11)	50% (20)
Secondary SjS	36% (74)	63% (19)	NT
Controls	0 (188)	0 (12)	5% (80)

NOTE: Percentage of positivity for anti-IFI16 autoantibodies in patients and healthy controls. The numbers in parentheses indicate the number of cases tested.

SEREX = serological analysis of antigens by recombinant cDNA expression cloning; ELISA = solid-phase enzyme-linked immunosorbent assay. SLE = systemic lupus erythematosus; RA = rheumatoid arthritis; SSc = scleroderma; PM/Scl = polymyositis/scleroderma overlap; SjS = Sjögren's syndrome. NT = not tested.

CONCLUSIONS

Although further studies are needed to confirm the diagnostic value of anti-IFI16 antibodies, there is compelling evidence that they could work as a supplementary marker of SSc. Moreover, our data suggest that combined use of anti-IFI16 and ACA markers improves the sensitivity and specificity score to discriminate lcSSc from dcSSc. In particular, the finding that anti-IFI16 positivity allowed us to detect lcSSc patients among the subgroup negative for both ACA and anti-Scl70 reactivity strongly indicates that it can be a valuable tool for the differential diagnosis of lcSSc in the double-negative patients.

Additionally, our observations provide a mechanism by which IFI16 upregulation by both IFN and inflammatory cytokines in endothelial cells may contribute to the development of autoimmunity. Increased levels of IFI16 are indeed correlated with triggering of proinflammatory molecules and cell death by apoptosis, providing support for the idea that IFI16 could be an important mediator of the chronic inflammation and vascular involvement in systemic autoimmune diseases.

Altogether, these studies are likely to advance our knowledge of IFI16 pathogenetic role in systemic autoimmune diseases, which will be helpful in considering new strategies for the diagnosis and treatment of these diseases.

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