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The proapoptotic activity of the Interferon-inducible gene IFI16 provides new insights into its etiopathogenetic role in autoimmunity

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ABSTRACT

Several lines of evidence link Interferons (IFNs) with autoimmune disorders. Autoantibodies against the Interferon-inducible IFI16 protein, a member of the HIN-200 family constitutively expressed in endothelial cells and keratinocytes, have been identified in patients affected by autoimmune diseases including Systemic Lupus Erythematosus (SLE), Sjogren Syndrome (SjS), and Scleroderma (SSc). These findings point to a role for IFI16 in the etiopathogenesis of autoimmune diseases, but the exact mechanisms involved in the development of autoimmunity remain obscure. In this study, we report for the first time that endothelial cells overexpressing IFI16 undergo apoptosis via the activation of caspase 2 and caspase 3, and that a positive feedback loop appears to link these two caspases. The relevance of IFI16-mediated apoptosis is highlighted by the observation that IFI16 knock down by RNA interference in endothelial cells inhibits the activation of both caspase 2 and caspase 3 by IFN-β priming and synthetic double-stranded RNA treatment. Expression of a dominant-negative mutant of IKK2 kinase or treatment with AS602868, an inhibitor of IKK2 activity, results in a strong reduction of NF-kB activation along with absence of caspase 2 and caspase 3 activation and apoptosis induction. Collectively, our findings provide new insights into the role of IFI16 in the pathogenesis of autoimmune diseases by demonstrating that in addition to the stimulation of pro-inflammatory molecules, IFI16 also leads to apoptosis in endothelial cells.

1. Introduction

Apoptosis or programmed cell death is a fundamental developmental process in animals and plants essential for the regulation and maintenance of tissue growth and homeostasis [1,2]. A number of investigations, ranging from animal models to human pathology, support the view that apoptosis plays an important role in the development of autoimmunity [3–6]. Under normal circumstances apoptotic cells are rapidly cleared by macrophages, "non-professional" phagocytes in the surrounding tissues, and dendritic cells (DC) [7]. This clearance process is, in general, non-inflammatory and may even result in active tolerance towards autoantigens. Defects in this clearance process and the microenvironment in which it occurs may affect the way in which these autoantigens are presented and ultimately result in autoimmunity [8,9]. Moreover, recent data have demonstrated the presence of autoantigens within apoptotic bodies and show that apoptotic cells are critical for antigen presentation [10–12], activation of innate immunity and regulation of cytokine secretion by macrophages [13]. Apoptotic bodies have even been described as "B cell autoantigens" [14].

Interferons (IFNs) are known to have a multitude of immunological functions in both innate and adaptive immunity. Given their pleiotropic effects upon the immune system, it is conceivable that excess quantities of type I IFN or aberrant regulation of its signaling could contribute towards autoimmunity. Several lines of evidence link IFNs with autoimmune disorders, in particular with Systemic Lupus Erythematosus (SLE), Sjogren Syndrome (SjS), and Systemic Sclerosis (SSc) [15,16]. The expression of a spectrum of genes which constitutes an "IFN signature" is the most significant line of evidence indicating that IFNs may be the dominant pathogenic mediators involved in at least some autoimmune diseases [17,18].

HIN-200 comprises a family of structurally related IFN-inducible genes, of which both human (IFI16, IFIX, MNDA, and AIM2) and

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mouse (Ifi202a, Ifi202b, Ifi203, Ifi204, Ifi205/D3 and Ifi210/AIM2) genes have been sequenced [19–22]. The encoded proteins are primarily nuclear phosphoproteins involved in the transcriptional regulation of genes that are important for cell cycle control, differentiation, immunomodulation, and apoptosis [23–26].

Immunohistochemical analysis in normal human tissues has revealed prominent IFI16 expression in stratified squamous epithelia, particularly intense in basal cells in the proliferating compartments, whereas it gradually decreased in a more differentiated suprabasal compartment. In addition, endothelial cells from vascular and lymph vessels have been found to strongly express IFI16 [27,28]. This physiological expression of IFI16 in endothelial cells and stratified squamous epithelia (both of which are targets for the clinical manifestations of autoimmune diseases) indicates that IFI16 may be involved in the early steps of inflammation. In support of this hypothesis, overexpression of IFI16 in primary human umbilical vein endothelial cells (HUVEC) efficiently suppressed tube morphogenesis *in vitro* and increased the expression of pro-inflammatory molecules [29,30]. In addition, autoantibodies against the IFN-inducible IFI16 protein have been detected in patients suffering from SLE, SjS, and SSc suggesting an abnormal IFI16 presentation to the afferent limb of the immune system [31].

Since the role of HIN-200 genes in programmed cell death is widely accepted, IFI16 overexpression in apoptotic cells as a further trigger for autoimmunity has been suggested [31]. However, the molecular pathways underlying endothelial cell apoptosis have remained elusive. In particular, it is unknown whether IFI16-mediated apoptosis is dependent on caspase activation. If it is, then it will be important to identify the nature of the initiator and executioner caspases and transduction signals that lead to caspase activation.

In this study, a systematic analysis of the signal transduction pathways upregulated by IFI16 as endothelial cells approach apoptosis upon treatment with Interferon type I (IFN-β) and synthetic double-stranded RNA was performed. Consistent with previous studies, the pathway involving NF-kB, caspase 2, and caspase 3 appeared to play a critical role in IFI16-mediated apoptosis.

2. Materials and methods

2.1. Cells lines and reagents

Human umbilical vein endothelial cells (HUVEC) obtained by trypsin treatment of umbilical cord veins were cultured in endothelial growth medium (EGM-2, Lonza, Basel, Switzerland) containing 2% fetal bovine serum (FBS) (Sigma, Milan, Italy), human recombinant vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), human epidermal growth factor (hEGF), insulin growth factor (IGF-1), hydrocortisone, ascorbic acid, heparin, gentamycin, and amphotericin B (1 μg/ml each; all from Sigma), then seeded into 100-mm culture dishes coated with 0.2% gelatin. Experiments were performed with cells between passages 2nd and 6th. Cells were kept in logarithmic growth phase by 1× Citric Saline detachment and replating every 2–4 days. AS602868 is an anilinopyrimidine derivative and adenosine triphosphate (ATP) competitor that has been selected for its inhibitory activity *in vitro* on IKK2, the constitutively active form of IKK2 [32,33]. Stocks of AS602868 were prepared in 100% DMSO. In all experiments in which AS602868 was used, each dish of cells received an equal volume of DMSO. When infections were performed in presence of AS602868, the cells were treated with the compound 10 h after the infection. Caspase 2 inhibitor Z-Val-Asp(O-Me)-Val-Ala-Asp(O-Me) fluoromethyl ketone (Z-VDVAD-FMK) and caspase 3 inhibitor Z-DEVD-FMK were purchased from Sigma and R&D system, respectively. When infections were performed in presence of caspase 2 or caspase 3 inhibitors, the cells were treated with the compound 1 h before the infection. Doxorubicin was purchased from Sigma and was used at 1 μM as indicated.

2.2. Recombinant adenovirus preparations and HUVEC infection

Human embryo kidney 293 cells (HEK-293, Microbix Biosystems Inc., Toronto, Canada) were cultured in MEM (Invitrogen, Milan, Italy) supplemented with 10% FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin sulfate (Sigma). The pAC-CMV IFI16 containing the human IFI16 cDNA linked to a FLAG-tag at the NH2-terminal was co-transfected with pJM17 into human embryonic kidney 293 cells as previously described [34,35]. After several rounds of plaque purification, the adenovirus containing the IFI16 gene (AdVIFI16) was amplified on 293 cell monolayers and purified from cell lysates by banding twice on CsCl gradients. Desalting was performed using G50 columns (GE Healthcare, Milan, Italy), and viruses were frozen in PBS-10% glycerol at −80 °C. The infectious titers (PFU) were determined by a standard plaque assay on 293 cell monolayers. The physical particles of the vector preparations were measured by spectrophotometry, and our viral preparations showed a 1:10–1:20 ratio between PFU and physical particles. Endotoxin contamination was excluded by testing with the E-Toxate kit (Sigma, sensitivity > 1.4 pg/ml). For cell transduction, pre-confluent HUVEC were washed once with phosphate-buffered saline (PBS) and incubated with AdVIFI16 or AdVLacZ (used as a control) at a multiplicity of infection (MOI) of 300 in EGM-2. After 60 min at 37 °C, the virus was washed off and fresh medium was added.

2.3. Immunoblotting

Whole-cell protein extracts were prepared by resuspending pelleted cells in lysis buffer containing 125 mM Tris–Cl (pH 6.8), 1% SDS, 20 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 4 μg/ml leupeptin, 4 μg/ml aprotinin, and 1 μg/ml pepstatin (all from Sigma). After a brief sonication, soluble proteins were collected by centrifugation at 15,000 × g. Supernatants were analyzed for protein concentration using a Bio-Rad D_c protein assay kit (Milan, Italy) and stored at −70 °C in 10% glycerol. Proteins were separated by SDS-PAGE then transferred to Immobilon-P membranes (Millipore, Milan, Italy). Filters were blocked in 5% non-fat dry milk in 10 mM Tris–Cl (pH 7.5), 100 mM NaCl, and 0.1% Tween 20, and immunostained with rabbit anti-IFI16 Ab (diluted 1:2000) or mouse anti-actin mAb (Chemicon, Milan, Italy) (diluted 1:4000).

2.4. In vitro analysis of caspase activities

Caspase 1, 2, 3, 8, and 9 protease activity was measured in a fluorometric assay, by measuring the extent of cleavage of a fluorometric peptide substrate using SensoLyte AFC Caspase Sampler Kit Fluorimetric (Anaspec, CA, USA). Experiments were performed according to the manufacturer's instructions. After a 1 h of incubation at 25 °C, fluorescence was measured at an excitation wavelength of 405 nm and an emission wavelength of 500 nm using the VICTOR^3 1420 multilabel counter (Perkin–Elmer, Milan, Italy). Protease activity was expressed as RFU (relative units of fluorescence).

2.5. Nuclear extract isolation and Electrophoretic Mobility Shift Assay (EMSA)

HUVEC were plated at a density of 3×10^5 cells/100-mm diameter dish and 24 h later infected with either AdVIFI16 or AdVLacZ, or mock infected. At the indicated times post-infection (hpi), cells were washed in cold PBS and nuclear proteins were