Upregulation of Myxovirus-resistance Protein A: A Possible Marker of Type I Interferon Induction in Systemic Sclerosis

PAOLO AIRÒ, CLAUDIA GHIDINI, CINZIA ZANOTTI, MIRKO SCARSI, ROBERTO CATTANEO, LUIGI CAIMI, and LUISA IMBERTI

ABSTRACT. ***Objective.*** To examine whether myxovirus-resistance protein A (MxA) mRNA expression, commonly considered a reliable marker of Type I interferon (IFN) bioactivity, is modified in patients with systemic sclerosis (SSc); if it is associated to specific clinical features; and if its modulation is accompanied by modulation of mRNA for the Type I IFN receptor (IFNAR).

Methods. Quantification of mRNA for MxA and the subunit IFNAR1 and isoforms of IFNAR2 was performed by real-time polymerase chain reaction in 50 patients with SSc. Results were compared with those obtained from healthy controls and patients with another autoimmune disease such as multiple sclerosis.

Results. Levels of MxA mRNA above the 99th percentile of values found in healthy controls were observed in 9 out of 50 patients with SSc ($p < 0.001$). Induced MxA expression was significantly associated with some features of more severe disease, such as lower forced vital capacity and the presence of ischemic digital ulcers. No differences in the levels of IFNAR were found within MxA-induced and MxA-non-induced patients, but there was a direct correlation between levels of MxA and the soluble isoform of IFNAR2.

Conclusion. Our results show induction of MxA expression in some patients with SSc, which correlates with the presence of ischemic ulcers and other signs of worse disease, suggesting a potential role of Type I IFN in the pathogenesis of this disease and/or its complications. (First Release Oct 1 2008; J Rheumatol 2008;35:2192–200; doi:10.3899/jrheum.080418)

Key Indexing Terms:
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TYPE I INTERFERON
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Human interferons (IFN) are a group of naturally occurring cytokines with important immunomodulatory, antiviral, antiangiogenic, antiproliferative, and antitumor activities. They are classified in 3 major subfamilies based on their biological and physical properties. Type I IFN family includes IFN-α, -ß, -ε, -κ, -ω, -δ, and -τ. IFN-α and IFN-ß are the main types of interest from an immunologic viewpoint, since IFN-ε and IFN-κ are expressed in the placenta and in keratinocytes, whereas IFN-δ and IFN-τ are not found in humans[1]. There are more than 20 different IFN-α genes, of which 13 encode functional polypeptides, whereas there is only 1 type of IFN-ß. The Type II IFN family includes only IFN-γ, while the new family, now called Type III IFN, has 3 subtypes of IFN-λ (also termed IL-28A, -28B, and -29), which are coproduced with IFN-ß[2].

Studies using genetically manipulated mouse strains, microarray gene expression profiling, and other approaches have provided uncontested evidence that IFN-α/ß-producing cell populations, potential IFN-α/ß-induced factors, and signaling events are relevant to the development of systemic and organ-specific autoimmune diseases[1]. This led to new paradigms for disease induction that position IFN-α/ß among the major influences in several autoimmune syndromes, including systemic lupus erythematosus (SLE), diabetes, multiple sclerosis (MS), and, to a lesser extent, rheumatoid arthritis, Sjögren's syndrome, myasthenia gravis, autoimmune hemolytic anemia, thyroiditis, uveitis and Behçet's disease[1].

Some recent data have also shown a correlation between Type I IFN and the development and pathogenesis of systemic sclerosis (SSc)[3]. Clues for an important detrimental role for Type I IFN in SSc first arose from clinical studies. In the 1990s a double-blind, randomized controlled trial was designed in the UK to evaluate a possible benefit of IFN-α in patients with diffuse SSc[3]. This was expected on the basis

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of some *in vitro* studies that demonstrated inhibition of collagen synthesis and fibroblast proliferation by IFN-α. However, the study was ended after an interim analysis that showed no evidence of benefit in the IFN-α-treated patients. To the contrary, in this group there was a significant worsening, as compared to the placebo-treated group, of important measures such as forced vital capacity (FVC) and diffusing capacity for carbon monoxide (DLCO). Moreover, in the IFN-treated patients there were a higher number of deaths, more withdrawal for disease progression, and worse changes of cutaneous scores, although these differences were not statistically significant[4]. Another clue to suggest an association of Type I IFN and SSc[3] was the description of *de novo* onset of severe Raynaud's phenomenon and related complications, including full-blown SSc, in several patients treated either with IFN-α for hematological disorders or chronic viral hepatitis[5-8] or with IFN-ß for MS[9,10], in most cases with a strict temporal relationship. Resolution of these complications was observed after withdrawal of IFN in some but not all of these patients[7,10].

In recent years, the link between IFN and SSc was investigated in experimental studies. Increased expression of Type I IFN-inducible genes (an "interferon signature") was found in unfractionated peripheral blood cells from patients with SSc[11], and similar results were obtained evaluating purified monocytes and CD4+ T cells[12]. In particular, increased protein expression of the IFN-stimulated gene, *Siglec-1*, was detected in circulating monocytes[13] and enhanced expression of IFI16, another IFN-inducible protein, was found in the epidermis and in the dermal inflammatory infiltrate from SSc lesions[14]. Direct evidence of enhanced IFN-α transcription in the skin of patients with SSc in vascular and perivascular cells was also provided[12].

Although the direct measurement of serum IFN-α and/or IFN-ß has been used for markers of HIV disease progression[15], SLE severity[16,17], or efficacy of therapy in patients with MS[18], there are serious limitations for such an approach in a clinical setting, considering the biochemical and biological properties of the cytokines, including their short half-life and their limited distribution to blood circulation[19-21]. After binding with their receptors, called IFNAR, Type I IFN trigger a signaling cascade that results in a tight regulation of several hundred genes[22]. Therefore, measurement of mRNA or protein expression of IFN-stimulated genes may constitute a more sensitive and reliable assay for measuring the extent of Type I IFN biological activity. Among these genes, myxovirus-resistance protein A (MxA) is considered by many to be the most appropriate, because it is induced in a dose-dependent manner by Type I IFN[23]. In our study, we took advantage of MxA mRNA quantification by real-time polymerase chain reaction (PCR) to evaluate the presence of Type I IFN activation in patients with SSc. Further, since IFNAR appears to be modulated after injection of exogenous or increased production of endogenous Type IFN[24,25], we analyzed the expression of mRNA for the IFNAR1 subunit and for the functional (IFNAR2.2), nonfunctional (IFNAR2.1), and soluble (IFNAR2.3) isoforms of the IFNAR2 subunit. Results were compared with those obtained in healthy controls and in patients with another autoimmune disease, such as MS.

MATERIALS AND METHODS

Patients and controls. Our study was approved by the local ethical committee. Fifty patients with a diagnosis of SSc, classified as limited or diffuse disease subset (lSSc or dSSc), according to the criteria of LeRoy, *et al*[26], gave their informed consent to blood withdrawal. Disease duration was defined according to Medsger and Steen[27]: early disease, as less than 3 years from the onset of the first symptom other than Raynaud's phenomenon for dSSc, and less than 5 years for lSSc; the other patients were defined as having late disease. Most patients received low-dose corticosteroids, calcium-channel blockers or other vasodilators, and low-dose aspirin. No patient showed fever of unknown reason or other signs suggestive of acute viral infections at the time of the analysis; with respect to chronic viral infections, patients with hepatitis B virus, HCV, or HIV infection were excluded.

Fifty-five patients with relapsing/remitting MS naive for therapy, and 47 healthy blood donor controls were included in our study. None of them showed fever or symptoms of infectious illness within 2 weeks from blood sampling.

Real-time PCR for MxA and IFNAR quantification. RNA was isolated from peripheral blood from patients and controls and drawn into PAXgene tubes (PreAnalytiX GmbH, Hombrechtikon, Switzerland), containing an additive that reduced the *in vitro* RNA degradation. RNA was prepared using PAXgene Blood RNA kit (PreAnalytiX) and, to remove contaminating DNA, samples were treated with RNase-Free DNase Set (Qiagen Inc., Valencia, CA, USA). Five hundred nanograms of total RNA were converted to cDNA using TaqMan reverse transcription reagents (Applied Biosystems, Foster City, CA, USA) that was subjected to real-time PCR in an Applied Biosystems 7500 FAST PCR system. Primer and probe sequences for MxA, IFNAR, and for the housekeeping gene GAPDH are shown in Table 1. Primer selectivity testing was done by comparing primer sequences against genomic and transcriptomic databases to assess their potential for multiple amplicons, while their specificity was assessed by amplifying the cDNA obtained from randomly chosen subjects by conventional PCR. All these primers gave rise to bands of the expected size when run by electrophoresis on 2% ethidium bromide stained agarose gel. The bands of each PCR product were purified and direct-sequenced using its forward primer and the Big Dye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) according to the manufacturer's instructions. The sequences, obtained by capillary electrophoresis on an Abi Prism 310 automated sequencer (Applied Biosystems), were compared to reference sequences by Blast search, which confirmed the specificity of amplification products.

Real-time PCR reactions were set up in 96-well optical reaction plates (Applied Biosystems) in a final volume of 25 µl with 12.5 µl of double-concentrated TaqMan Universal PCR Master Mix (Applied Biosystems), 3 µl of cDNA, 900 nM of forward and reverse primers, and 200 nM of probes for IFNAR quantification, and 200 nM of forward and reverse primers and 400 nM of probe for GAPDH amplification. Real-time PCR started with a first step at 50°C for 2 min performed with AmpErase uracil-N-glycosylase (included in the TaqMan Universal PCR Master Mix) that eliminates possible contaminations carried over from previously generated PCR products, followed by an initial heating at 95°C for 10 min to activate AmpliTaq Gold polymerase. Samples were then subjected to 45 cycles of denaturation at 95°C for 15 s and a combined annealing and elongation at 60°C for 1 min. mRNA for MxA and for IFNAR components was measured by real-time PCR only after assay standardization in terms of precision, accuracy, and

Table 1. Primers and probes for real-time PCR of genes for MxA, IFNAR1 subunit, IFNAR2.1, IFNAR2.2, and IFNAR2.3 isoforms.

Gene		Primer Sequence 5'---------→3'
MxA	Forward	AAG CTG ATC CGC CTC CAC TT
	Reverse	GCA ATG CAC CCC TGT ATA CC
	Probe	FAM-CCA GAT GGA ACA GAT TGT CTA CTG CCA G-TAMRA
IFNAR1	Forward	GAA ACC ACT GAC TGT ATA TTG TGT GAA A
	Reverse	CAG CGT CAC TAA AAA CAC TGC TTT
	Probe	FAM-CCA GAG CAC ACA CCA TGG ATG AAA AGC-TAMRA
IFNAR2.1	Forward	CTA TTC ACA GGT GCA GTC ATA ATG C
	Reverse	GCA CGC TTG TAA TCC CAG CTA
	Probe	FAM-CAG TCG TCC TGC CTA AGC TTC CCC A-TAMRA
IFNAR2.2	Forward	TGA CAA GCA CCA TAG TGA CAC TGA
	Reverse	TAG GAA ATG GCC AGG CTA AAA A
	Probe	GAM-TGG ATT GGT TAT ATA TGC TTA AGA AAT AGC CTC CCC A-TAMRA
IFNAR2.3	Forward	GGC CAG GAA TCA GAA TTT TCA T
	Reverse	CCC ACA CTT TCT TCT TTC TGT TGA
	Probe	FAM-CTA ACC TGC CAC CGT TGG AAG CCA T-TAMRA
GAPDH	Forward	GAA GGT GAA GGT CGG AGT C
	Reverse	GAA GAT GGT GAT GGG ATT TC
	Probe	FAM-CAA GCT TCC CGT TCT CAG CC-TAMRA

reproducibility[25,28], and results were expressed as normalization ratio (NR) calculated with the comparative Ct (cycle threshold) method. With this method, the level of target gene expression was obtained by the formula $2^{-\Delta\Delta Ct}$, where ΔΔCt value was calculated as the difference between the ΔCt of the sample under evaluation and the ΔCt of a calibrator, which is a sample taken from the same subject and is evaluated in every experimental session. According to the formula, the NR of the calibrator in each run is always 1. After verifying that the amplification efficiency of the target and housekeeping gene was approximately equal, the values of IFNAR1, IFNAR2.1, IFNAR2.2, and IFNAR2.3 mRNA were expressed both as NR and as ΔCt, which is defined as Ct of the target gene minus the Ct of the housekeeping gene evaluated in the same biological sample.

Statistical analysis. If not otherwise indicated, data are expressed as the median (25th-75th percentile). The comparisons between 2 groups for continuous variables were made using the Mann-Whitney test. The comparisons of categorical data were made by the Fisher exact test or, between more than 2 groups, by means of contingency tables, with Holm's correction for multiple tests. Kruskal-Wallis test and Dunn's post-hoc test were used when evaluating real-time PCR results expressed as NR, and analysis of variance for repeated measures followed by Bonferroni post-hoc test, when assessing results expressed as ΔCt. Correlations were evaluated by Spearman's rank test. A level of $p < 0.05$ was considered significant.

RESULTS

Expression of MxA by peripheral blood of patients with SSc, controls, and patients with MS is shown in Figure 1. Considering the 99th percentile of the results observed in controls (NR = 3.68) as the cutoff value, raised levels of MxA were observed in 9 out of 50 patients with SSc (18%), compared with one of 47 controls (2%), and none of 55 patients with MS ($p < 0.001$). Comparing patients with SSc

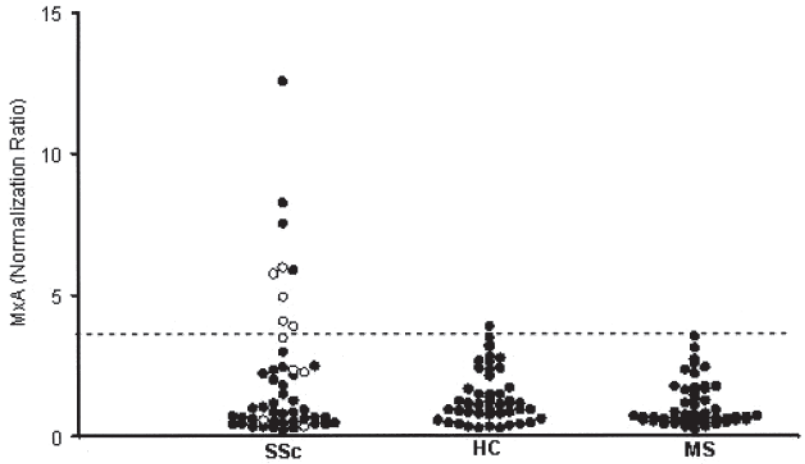

Figure 1. MxA mRNA levels under or above the cutoff value of samples obtained from patients with systemic sclerosis (SSc), healthy controls (HC), and patients with multiple sclerosis (MS). Results are expressed as normalization ratio (NR); broken line indicates 3.68 NR, which represents the cutoff, calculated as 99th percentile of values obtained in controls; white circles indicate patients with SSc and ischemic ulcers. $p < 0.001$ was calculated by contingency tables for categorical data, with Holm correction for multiple tests.

and raised MxA mRNA expression to those with normal levels of MxA, there was no significant difference in sex, age, or disease duration, or in disease subset or SSc-specific autoantibodies (Table 2). Moreover, there was no difference in the number of patients receiving immunosuppressive drugs, iloprost, or bosentan. However, FVC was significantly lower in the group of patients with high MxA expression than in other patients with SSc ($p = 0.02$; Table 2). There were other signs of a worse disease in this group, since DLCO was lower ($p = 0.07$) and the skin score was slightly higher ($p = 0.14$), although not significantly. More interestingly, patients with raised levels of MxA had ischemic digital ulcers in a significantly higher proportion than patients with normal MxA expression ($p = 0.002$; Table 2).

Next, we evaluated whether MxA induction in patients with SSc was associated to any abnormality in the expression of Type I IFN receptor. For this purpose, after the standardization of the assay, real-time PCR for the quantification of the IFNAR1 subunit and of truncated IFNAR2.1, full-length IFNAR2.2, and soluble IFNAR2.3 isoforms were performed on blood samples from the 9 MxA-induced SSc patients, and from 9 individuals randomly chosen within the group of MxA non-induced SSc without ulcers, 18 patients with MS, and 18 controls. Figure 2 shows the levels of RNA of the different IFNAR components in the 4 groups of subjects, expressed as ΔCt and obtained by subtracting the Ct of the reference gene GAPDH from the Ct of the target mRNA, without further normalizing on the calibrator value. We used this approach because we have previously demonstrated the low interassay variability of the assay[25]. By definition, the lower the ΔCt value of any sample, the higher its mRNA expression. Indeed, in controls and patients with SSc, independently of MxA induction or not, mRNA levels for the IFNAR1 subunit and functional IFNAR2.2 isoform had a higher level of expression ($p < 0.001$) compared to inactive IFNAR2.1 and soluble IFNAR2.3 isoforms (Figure 2). In the patients with MS, IFNAR2.2 was the most expressed IFNAR subunit. It is notable that in all groups the expression of the functional IFNAR2.2 isoform was greater than the nonfunctional one, and that the soluble isoform was barely detectable, as shown by the highest ΔCt values.

The results were also analyzed using the ΔΔCt method, in which all experimental data of target mRNA are normalized in respect to values obtained from a "calibrator" sample, represented by the cDNA of a healthy subject whose absolute level of expression of each IFNAR element is not known. Indeed, to give rise to the ΔΔCt value, the ΔCt value of the calibrator must be subtracted from the ΔCt of every target sample run in the same plate and, consequently, its NR is always set to 1. Using this approach we found no dif-

Table 2. Main demographic, clinical, and laboratory data of SSc patient groups.

	MxA Induced, n = 9	MxA Non-induced, n = 41	p
Sex, f/m	8/1	37/4	NS
Age, yrs	51 (43–62)	60 (55–68)	NS
Disease duration, yrs	8 (5–12)	9 (5–15)	NS
Disease subset, diffuse/limited	6/3	19/22	NS
Diffuse SSc, early (%)	1 (11)	2 (5)	NS
Diffuse SSc, late (%)	5 (56)	17 (41)	NS
Limited SSc, early (%)	0 (0)	8 (20)	NS
Limited SSc, late (%)	3 (33)	14 (34)	NS
Antitopoisomerase I antibodies (%)	5 (56)	16 (39)	NS
Anticentromere antibodies (%)	1 (11)	15 (37)	NS
Anti-RNA polymerase III (%)	1 (11)	5 (12)	NS
Modified Rodnan skin score	11 (10–17)	8 (4–12)	NS
FVC, % predicted	82 (61–99)	103 (88–119)	0.02
DLCO, % predicted	59 (32–66)	65 (48–83)	NS
ILD (%)	5 (56)	20 (48)	NS
Isolated PAH (%)	0	4 (10)	NS
Renal crisis (%)	0	1 (2)	NS
Ischemic ulcers (%)	5 (56)	3 (7)	0.002
Joint contractures (%)	5 (56)	12 (30)	NS
Current treatment (%)			
Cyclophosphamide	1 (11)	7 (17)	NS
Methotrexate	3 (33)	4 (10)	NS
Azathioprine	1 (11)	0	NS
Iloprost	6 (67)	17 (41)	NS
Bosentan	3 (33)	4 (10)	NS

FVC: forced vital capacity, DLCO: diffusing capacity of carbon monoxide, ILD: interstitial lung disease, PAH: pulmonary arterial hypertension, NS: not significant.

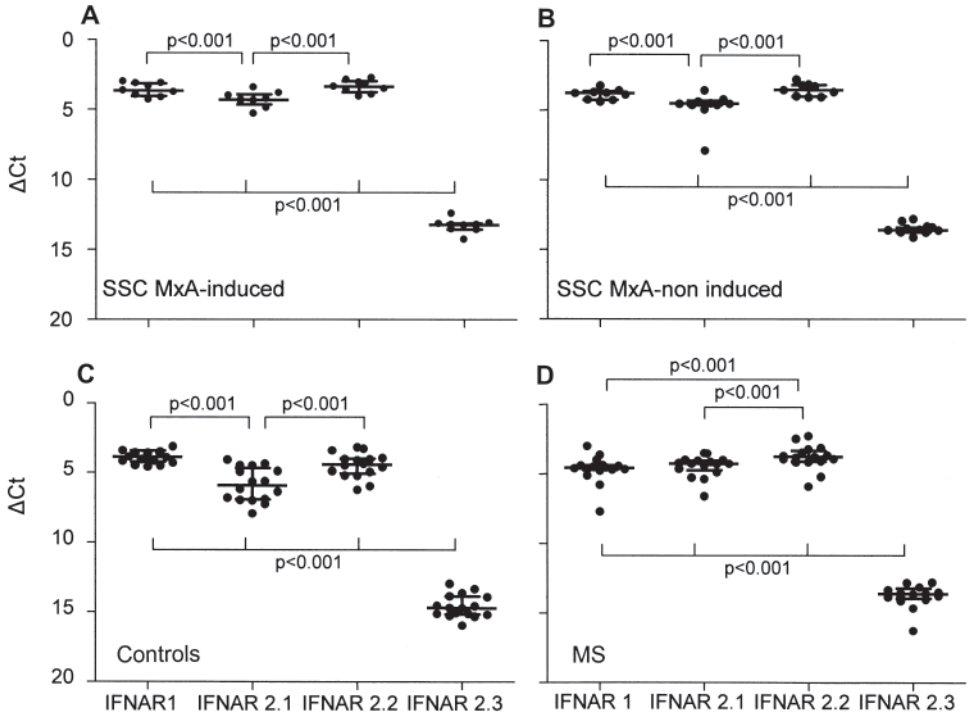

Figure 2. Expression of IFNAR components in SSc patients with MxA above the cutoff (A), SSc patients with MxA below the cutoff without ulcers (B), healthy controls (C), and patients with multiple sclerosis (D). Real-time PCR results are expressed as ΔCt. Statistical analysis by Bonferroni post-hoc test.

ference in the quantification of all IFNAR components in MxA-induced and MxA-non-induced SSc patients or controls, while as reported[29], patients with MS showed a reduced expression of IFNAR1 subunit compared to controls and patients with SSc (Figure 3). These data suggest that variations in MxA induction between SSc patients with or without ischemic digital ulcers and controls were not associated with variation of Type I IFN receptor expression. Finally, we correlated the levels of MxA and of IFNAR components in 18 patients with SSc, and we found a significant ($p < 0.05$) positive correlation between levels of MxA mRNA and IFNAR2.3 subunits (Figure 4).

DISCUSSION

In our study, we evaluated whether enhanced MxA expression is associated with the presence of SSc and specific clinical features of this disease.

Since Type I IFN are difficult to measure in biological samples, several assays have been developed to detect their activity, and among them, mRNA expression of MxA has been successfully used as a marker of Type I IFN activity in a variety of settings, like the assessment of the bioavailability of therapeutically administered IFN-α and IFN-ß[28,30].

Recent data have questioned the strict relationship between MxA and Type I IFN. Indeed, although Type III IFN do not use the IFNAR used by Type I IFN, but rather signal through a heterodimeric receptor composed of a specific R1 chain (also termed IL-28R), and a second chain (IL-10R2) that also serves for the IL-10, IL-22, and IL-26 receptors, it has been shown that they can activate signal transduction pathways similar to those activated by Type I IFN[31-33]. Consequently, it is plausible that the different biological activities ascribed to Type I IFN can be shared by these proteins. Indeed, some data demonstrate that Type III IFN can also induce MxA, albeit at a weaker level than Type I IFN[34,35]. The data presented here, obtained using MxA as a marker of Type I IFN bioactivity, may therefore support the hypothesis that SSc, like other systemic autoimmune rheumatic diseases[1,3], is associated with activation of the Type I IFN system in some patients, but a role of another IFN family is also possible.

SSc is a complex disease, whose pathogenetic mechanisms are still incompletely understood. However, a distinctive triad of autoimmunity, small-vessel vasculopathy and inflammation, and interstitial and vascular fibrosis in the skin, lungs, and other organs is thought to be involved[36]. Type I IFN might be implicated in all these mechanisms through their complex biological activities[3]. Indeed, Type I IFN are considered to play a pivotal role in the pathogenesis of both organ-specific and systemic autoimmune diseases, given their potent modulating effect of the immune system. These cytokines, upregulating the expression of HLA class I and II, adhesion molecules, and chemokines, can enhance antigen presentation, homing to lymphoid organs, activation

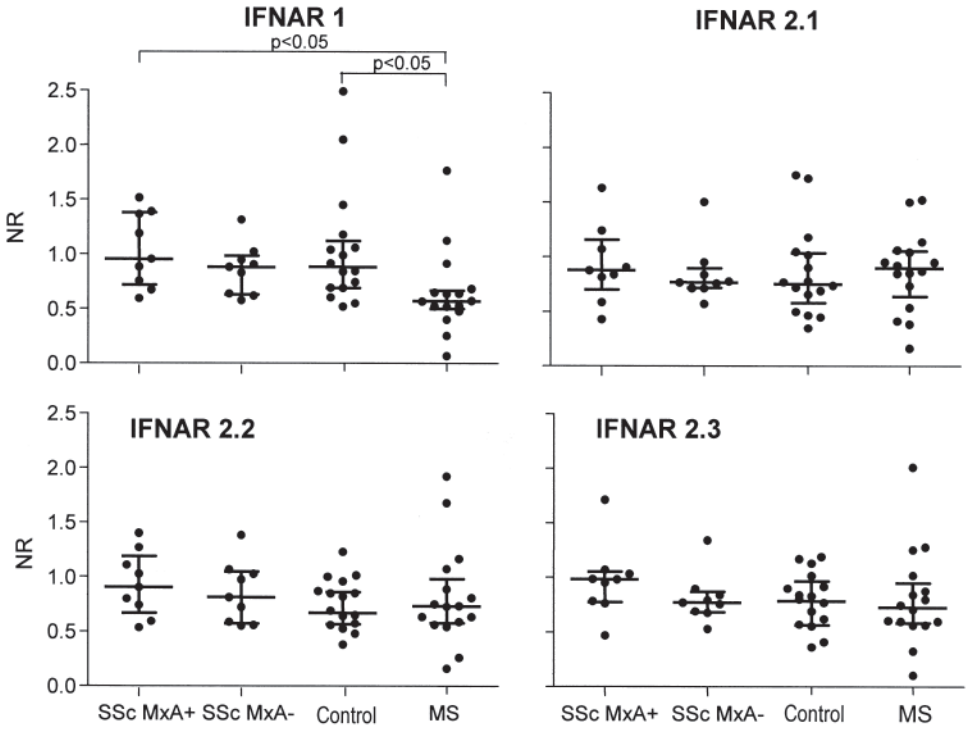

Figure 3. Expression of IFNAR components in SSc patients with MxA above the cutoff, SSc patients with MxA below the cutoff without ulcers, healthy controls, and patients with multiple sclerosis (MS). Real-time PCR results are expressed as normalization ratios (NR). Statistical analysis by Kruskal-Wallis test.

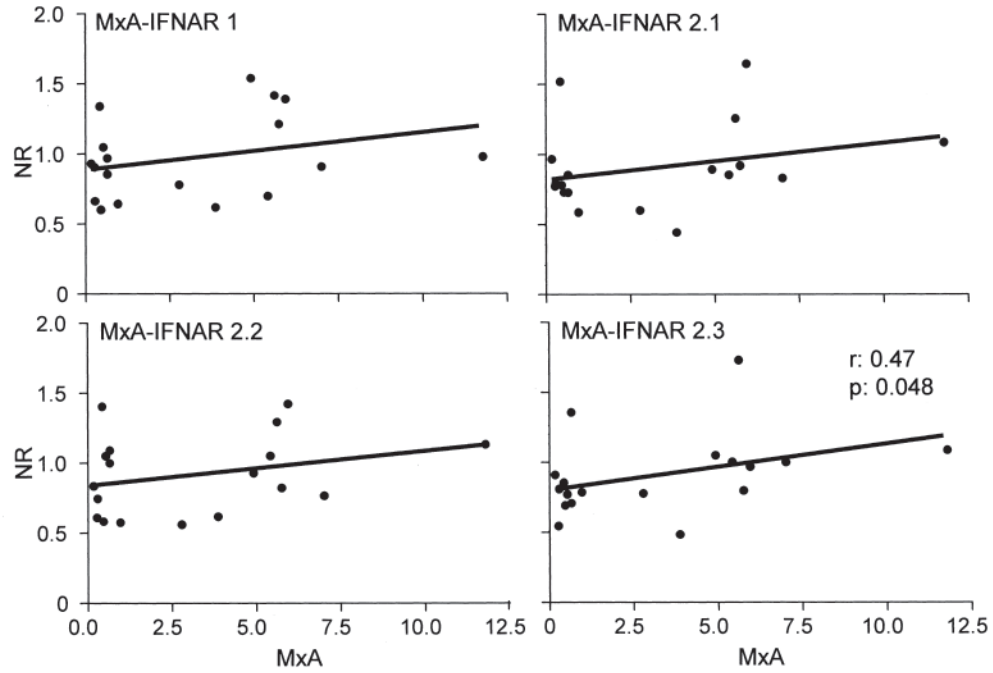

Figure 4. Correlation between MxA normalization ratio (NR) values and levels of IFNAR components in 18 patients with SSc (9 with MxA values above the cutoff, 9 below the cutoff). Data evaluated by Spearman's rank test.

of immune cells, and stimulation of Th1 responses[1]. In addition, they might promote the production of autoantibodies, decreasing the threshold for B cell activation and enhancing their differentiation, antibody production, and immunoglobulin isotype class switching[1]. It has been suggested that the appearance of antibodies in patients receiving treatments with Type I IFN, or the rise of their titer, may be a risk factor for the development of an overt autoimmune disease[37,38]. Interestingly, in one case report, the development of severe Raynaud's phenomenon in a patient with MS treated with IFN-ß was preceded by the appearance of antinucleolar antibodies, a marker for SSc[39].

The interplay between autoimmunity, inflammation, and obliterative vasculopathy in the pathogenesis of SSc is complex and may include a sort of vicious circle[36]. It has been suggested that endothelial damage, induced either by vasospasm-induced ischemia[40] or by antiendothelial autoantibodies[41], might lead to apoptosis, thereby providing a source of autoantigens, and enhancing autoantibody production in SSc. Interestingly, SSc sera containing anti-Topo I or antinucleolar autoantibodies are able to induce high levels of IFN-α production by normal plasmacytoid dendritic cells (pDC), through uptake of immune complexes via FcγRII, and presumably interaction of RNA molecules, associated with autoantigens, with Toll-like receptor 7[42].

IFN induction may cause further vascular injury[42] since these cytokines have important effects on endothelial cell properties, amplifying activation and damage, and triggering the expression of genes encoding intracellular adhesion molecule-1 (ICAM-1) and other adhesion molecules, such as E-selectin, or chemokines, such as interleukin 8 and monocyte chemoattractant protein-1 by endothelial cells[43,44]. Moreover, IFN have been shown to induce replicative senescence of endothelial cells[45] with an antiangiogenic effect, and to retard wound healing[46].

Finally, Type I IFN can act also on fibroblasts, upregulating the expression of adhesion molecules, including ICAM-1. This molecule is already overexpressed on scleroderma fibroblasts and may be important in mediating lymphocyte-fibroblast interactions in the disease[4].

Interestingly, increased MxA mRNA expression in our patients with SSc was correlated with some features of more severe disease, in particular the presence of ischemic digital ulcers and worse lung function test results. The association with ischemic ulcers was not reported in other studies on Type I IFN markers and SSc, but this is the most frequent SSc-related clinical feature described in patients treated with Type I IFN for other diseases[5-10]. The hypothesis that the vasculopathic action of IFN might be implicated in this complication should therefore be explored with further studies. The association with reduced FVC observed in our series is also interesting, since the same observation was made in patients with SSc receiving treatment with IFN-α, as compared with placebo-treated patients[4]. Moreover, Kim, *et al*[42] observed higher IFN-α-inducing activity in the sera of patients with SSc and lung fibrosis, as compared with those without lung fibrosis. This observation led these authors to suggest that IFN-α may contribute to tissue injury.

Increased MxA production can be due to several factors: an augmented number of pDC, the "professional" IFN-producing cells, producing 10–100 times more Type I IFN than other cell types[47]; increased synthetic activity of pDC or of other cells producing Type I IFN; or altered interaction of IFN with IFNAR. IFNAR is a dimer composed of 2 transmembrane chains: the α (IFNAR1) and ß (IFNAR2) subunits[48,49]. IFNAR1 occurs in a short and long variant, which are both capable of signal transduction[50]. In contrast, IFNAR2 can be expressed in 3 different isoforms, generated from the same gene by exon skipping, alternative splicing, and differential usage of polyadenylation sites: the functional full-length isoform, defined IFNAR2.2; the second isoform, IFNAR2.1, with a truncated cytoplasmic tail and therefore incapable of complete signaling[51,52]; and IFNAR2.3, which, lacking both the transmembrane and intracytoplasmic domains, is regarded as a soluble receptor subunit[53]. *In vitro* studies showed that a soluble IFNAR2 can also be generated by cleavage of transmembrane IFNAR2 by intramembrane proteases in response to IFN and other stimuli[54], but there is no definitive *in vivo* evidence for this effect. Indeed, the cell-surface concentration of IFNAR and lateral organization of the 2 chains into microdomains might be important cellular measures that shape responsiveness to individual IFN[55]. For example, the autocrine production of IFN-ß from lipopolysaccharide- and poly I:C-matured DC can induce a marked decline in the level of the 2 IFNAR subunits[55]. Therefore, the activity of this complex receptor machinery may modulate the cellular responsiveness to Type I IFN, probably depending on the concentration of the ligand and on other immunomodulatory events. To date, only a few studies have investigated the role of all IFNAR components in human diseases or under conditions of receptor stimulation. Downmodulation of IFNAR1 was observed in patients with HCV who responded to IFN-α therapy[56], and IFNAR2 decreased over time in patients with chronic myelogenous leukemia displaying a good cytogenetic response to IFN-α[57]. In patients with MS we found that exogenous IFN-ß induced a decrease of IFNAR1 mRNA that might serve as a mechanism for counterbalancing the loss of IFN receptor on the cell surface[29].

In our study, we observed that in patients with SSc, soluble IFNAR2.3 was expressed far less than IFNAR2.1 and IFNAR2.2, and among the latter 2, the functional IFNAR2.2 was significantly more represented. This result is in accord with the functional role of IFNAR2.2 receptor whose expression must be privileged over the truncated nonfunctional IFNAR2.1 and soluble IFNAR2.3 forms. Comparing SSc patients with or without MxA induction, there were no

differences in the level of mRNA for IFNAR components, but we observed a positive direct correlation between MxA and soluble IFNAR2.3 levels. Depending on its relative concentration, on the stability of binding with the ligand, and on the rate of discharge, the soluble receptor was regarded either as a competitive antagonist or as an agonist of the activity of its cognate ligand[58]. Soluble IFNAR2.3 was identified in body fluids[53,59], and it may compete with the transmembrane IFNAR2.2 isoform for binding with the target cytokine[60]. However, in a mouse model, soluble IFNAR2.3 was able to bind Type I IFN, and even in the absence of the other IFNAR2 subunits, it was able to form a complex with IFNAR1 and to transducer signal[61]. Increased levels of soluble IFNAR2.3 have been reported in cancers[57], MS[62], AIDS, and vasculitis associated to SLE[63]. In the case of SSc we have found that patients with the highest MxA mRNA were those with higher soluble IFNAR2.3, but we do not know if this was a strategy made up by the organism to defend itself from the deleterious effect of Type I IFN.

In our study we observed that the MxA expression is activated in some patients with SSc, and is correlated with the presence of ischemic ulcers and other signs of worse disease. This observation might support the hypothesis of a potential role of the Type I IFN system in the pathogenesis of this disease and its complications, as suggested by recent observations[3]. Future studies to evaluate the potential clinical relevance of MxA determination in patients with SSc are warranted.

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