Characteristics of NK cell activity in patients with systemic sclerosis

Patricia Hartstein Salim[1], Mariana Jobim[2], Markus Bredemeier[3], José Artur Bogo Chies[4], João Carlos Tavares Brenol[5], Luiz Fernando Jobim[6], Ricardo Machado Xavier[7]

ABSTRACT

Introduction: Previous studies have shown an increased expression of natural killer (NK) cells in the peripheral blood of patients with systemic sclerosis (SSc). NK cells are part of innate immunity, recognizing infected cells through killer immunoglobulin-like receptors (KIR), which show marked polymorphism. A novel model has been proposed predicting the activity of NK cells, evaluating whether there is excessive activation (EA), excessive inhibition (EI) or balance (B) (neutral). **Objective:** To evaluate the activity of NK cells in patients with SSc and compare it with that of a control group. **Method:** This study comprised 110 patients with SSc and 115 healthy controls. A novel model that predicts the activity of NK cells was used. For that, cells with their respective KIR/HLA-C and Bw4 ligands were considered. The activity of NK cells was defined as EA, EI, or B. **Results:** Our results showed that 63.5% of healthy controls had the KIR phenotype characterized by EI, as compared with 39.1% of the patients with SSc ($P = 0.001$). Considering only KIR2DL2-positive individuals, 34.7% of EI was found in healthy controls and 10.9% in patients with SSc ($P < 0.001$). **Conclusion:** In our study, the model that predicts the action of NK cells showed that healthy controls have higher frequency of EI as compared with SSc patients, suggesting a protective effect of the EI profile against the development of SSc. These results suggest a potential role of NK cells in the pathogenesis of SSc, but further studies should be conducted to confirm our data.

Keywords: killer immunoglobulin-like receptor, natural killer cell, systemic scleroderma, autoimmunity.

INTRODUCTION

Systemic sclerosis (SSc) is a rare autoimmune disease characterized by endothelial dysfunction and tissue fibrosis. It is a diffuse connective tissue disease that can affect several organic systems, especially the digestive and respiratory systems. Systemic sclerosis has two presentation forms, limited and diffuse, which are differentiated based on the extension of skin involvement.[1] Its major characteristics are excessive collagen deposition, vascular lesions, and changes in cellular and humoral immunity.[2]

There is evidence that certain genetic characteristics favor the progression of chronic inflammation to fibrosis. Participation of the immune system has been suggested due to the presence of mononuclear cellular infiltrates in lesions, abnormalities in T helper cells and in monocyte function,[3] release of several cytokines, and reduced activity of natural killer (NK) cells.[4]

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Serviço de Reumatologia and Serviço de Imunologia, Hospital de Clínicas de Porto Alegre, Medical School, Universidade Federal do Rio Grande do Sul – HCPA-UFRGS.

1. PhD candidate in Medical Sciences, Universidade Federal do Rio Grande do Sul – UFGRS
2. PhD in Medicine, UFGRS; Immunologist Physician, Hospital de Clínicas de Porto Alegre – HCPA, UFGRS
3. PhD in Medicine, UFGRS; Physician, Rheumatology Service, Hospital Nossa Senhora da Conceição, Grupo Hospitalar Conceição – HNSC-GHC
4. PhD in Life Sciences, Specialist in Immunology, Université de Paris V; Associate Professor, Department of Genetics, UFGRS
5. PhD in Medicine, UFGRS; Associate Professor, Department of Internal Medicine, UFGRS
6. PhD in Medicine, Immunologist Physician, Chief, Service of Immunology, HCPA-UFRGS; Associate Professor, Department of Internal Medicine, UFGRS
7. PhD in Immunology, Shimane Medical University; Associate Professor, UFGRS; Chief, Service of Rheumatology, HCPA-UFRGS

Correspondence to: Ricardo Machado Xavier. Serviço de Reumatologia do Hospital de Clínicas de Porto Alegre. Rua Ramiro Barcelos, 2350, sala 645. CEP: 90035-003. Porto Alegre, RS, Brazil. E-mail: rmaxavier@hcpa.ufrgs.br

The NK cells have receptors, denominated 'killer immunoglobulin-like receptors' (KIR), which belong to the family of the immunoglobulins present on cell surface. According to their functional groups, those receptors are classified as inhibitory (prevent target cell lysis) or activatory (cause target cell lysis).[5] The inhibitory receptor recognizes the specific HLA class I antigen, preventing the attack of NK cells against normal cells; conversely, the activatory receptor is triggered when inhibitory KIR receptors do not recognize the target cell, activating NK cells for destruction.[6] The ability to attack 'self' cells that do not express HLA-I is known as 'missing self-recognition'. That hypothesis has been supported by several independent observations, demonstrating that HLA antigens really protect cells against lysis by NK cells, providing negative signs that inhibit the activity of NK cells.[7]

Theoretically, any inhibitory ligand combination KIR-HLA should be able to neutralize activation. The function of NK cells is regulated by positive and negative signals transmitted by pairs of activatory and inhibitory receptors. In vivo, NK cells are controlled by inhibitory receptors for self HLA-I ligands.[8] Thus, effector functions occur only when activation signals can surpass inhibitory signaling. That is obtained through either the predominance of the activation of receptor-ligand interactions or lack of the inhibitory ligand of the receptor.[9]

A novel model has been recently proposed by Nelson et al.,[10] predicting that, depending on the genotype, individuals could be classified into one of three groups, according to the activity characteristics of their NK cells: 1) mainly controlled by inhibitory receptors (major inhibition); 2) controlled equally by inhibitory and activating receptors (relatively neutral); or 3) mainly controlled by activating receptors (major activation). Similarly, individuals lacking ligands for inhibitory receptors (such as homozygous for HLA-Cw group 1 or 2 ligands) will have fewer NK cells under inhibitory control than individuals with all ligands present.

So far, only two studies have assessed the activity of NK cells according to that novel model – one in diabetes[11] and the other in psoriasis.[12] Because of the scarcity of studies on the subject, this study aimed at assessing the activity of NK cells in a group of patients with SSc as compared to a control group.

PATIENTS AND METHODS

All participants were instructed about this research. They provided written informed consent, and their decision did not affect the physician-patient relationship. This study was approved by the Ethics Committee of the Hospital de Clínicas de Porto Alegre (HCPA).

Patients

This study included 110 patients with SSc originating from the Rheumatology Outpatient Clinic of the HCPA. All patients had been diagnosed according to either the 1987 American College of Rheumatology criteria[13] or the LeRoy and Medsger's criteria for the classification of early SSc.[14] Patients with overlap syndromes with other diffuse connective tissue diseases (except for Sjögren's syndrome) were excluded from the study.

Controls

The control group comprised 115 unrelated individuals, originating from the Immunology Service of the HCPA, who voluntarily registered to donate bone marrow (REDOME). Individuals with chronic and acute diseases were excluded from the sample, as were those with a family history of genetic disorders (X chromosome-linked diseases, autosomal diseases or chromosomal abnormalities).

Immunogenetic study

The DNA samples were extracted according to the salting out[15] method and amplified according to the polymerase chain reaction (PCR) technique. The sequence of primers for PCR has been described by Gómez-Lozano et al.[16]

The following mixture was used for DNA amplification: 1 μL of 10x buffer; 50 nM of $MgCl_2$; 25 mM of dNTPs; 0.5 U taq polymerase; 10 ηg of DNA; 100 nm of internal control; and 500 mM of specific primer. The initial temperature for amplification was 94ºC for 3 minutes. Then, there were 4 cycles of 15 seconds at 94ºC, 15 seconds at 65ºC, and 30 seconds at 72ºC. Then, there were 21 cycles of 15 seconds at 94ºC, 15 seconds at 60ºC, and 30 seconds at 72ºC. To finish, 5 cycles of 15 seconds at 94ºC, 60 seconds at 55ºC, and 120 seconds at 72ºC. The product was maintained for 420 seconds at 72ºC.

The PCR product was analyzed by use of 1% agarose gel electrophoresis (p/v) and tris-acetate-EDTA (TAE) buffer. Electrophoresis ran for 20 minutes at 200 V and room temperature. After electrophoresis was complete, the gel was stained with ethidium bromide, and the bands visualized and photographed under ultraviolet light.

Statistical analysis

The model that predicts the activity of NK cells was applied as follows: a) KIR2DS1 and/or KIR2DS2 with homozygous HLA-Cw for group 1 or 2 (combination of susceptibility – excessive activation); b) KIR2DS1 and/or KIR2DS2 with heterozygous HLA-Cw group; c) lack of KIR2DS1 or KIR2DS2 with homozygous HLA-Cw group (relatively neutral combination – balance);

and d) lack of KIR2DS1 or KIR2DS2 with heterozygous HLA-Cw group (combination of protection – excessive inhibition). The results were assessed by use of Pearson's chi-square test and the SPSS 16.0 software. Statistical significance was adopted as a P value ≤ 0.05.

RESULTS

Table 1 shows the patients' genetic profile. Presence of all KIR genes tends to provide protection against the development of SSc. That genetic profile was evidenced in 1.77% of the patients with SSc as compared to 13.9% of the controls (P < 0.001). The other genetic profiles showed no significant difference.

Activation of NK cells can be predicted by the possible combination of receptor activation or inhibition with the HLA-C molecule. That indicates that, depending on the genotype, an individual can have more over-active or balanced or over-inhibited NK cells. In this study, patients with SSc had excessive activation as compared with controls (Table 2). Of the 110 patients with SSc, 34 (29.6%) had over-active NK cells as compared with 22 of 115 (19.1%) controls. Analyzing excessive inhibition in each group, the control group had that profile more often.

Previous studies have evidenced that the presence of the inhibitory KIR2DL2 gene might be related to protection against the development of SSc. To test that hypothesis, we stratified the patients according to the presence or absence of that gene (Table 3). Considering only 2DL2-positive patients, we observed that the control group had excessive inhibition (34.7%) as compared to patients with the disease (10.9%), with a statistically significant difference (< 0.001). Analyzing balanced or over-active NK cells in the presence of the 2DL2 gene, no statistically significant difference was observed. Patients with SSc had 10.2% of excessive activation and 10% of balance, similarly to controls, who had 16.5% and 11.3%, respectively. Assessing patients who lack the KIR2DL2 gene, different results were found. The frequency of excessive inhibition in patients (29.1%) is similar to that of the control group (26.1%). Thus, the balanced or excessively activated state of NK cells is less frequent in healthy individuals (2.6% and 6.1%, respectively) as compared to patients with SSc (20.9% and 20.9%, respectively).

DISCUSSION

Systemic sclerosis is a complex multifactorial disease. The most accepted hypothesis for its pathogenesis is that immune system activation is triggered by the interaction between environmental factors and genetic predisposition.[17] Some genetic factors can influence the susceptibility to the development of SSc. Family history is the major risk factor identified; however, the absolute risk for each family member is low (< 1%). The relative risk for

Table 1
Frequency of the genetic profile of the KIR system in patients with systemic sclerosis (n = 110) and controls (n = 115)[¥]

KIR profile	2DL1	2DL2	2DL3	2DS1	2DS2	2DS3	2DS4	3DS1	3DL1	SSc (%)	Control group (%)
#1	+	–	+	–	–	–	+	–	+	23.4	24.3
#2	+	–	+	+	–	–	+	+	+	4.34	7.0
#3	+	–	+	-	+	–	+	–	+	6.08	0.0
#4	+	–	+	+	+	–	+	+	+	1.77	0.0
#5	+	+	+	+	+	+	+	+	+	1.77[a]	13.9[a]
#6	+	+	+	–	+	–	+	–	+	9.73	13.9
#7	+	–	+	–	–	–	+	+	+	9.73	0.0
#8	+	+	–	–	–	–	+	–	+	0.0	6.1
#9	+	+	+	+	+	–	+	+	+	0.0	4.3
#10	+	+	+	–	+	+	+	–	+	2.6	4.3
#11	+	+	–	–	+	+	+	–	+	4.34	3.5
#12	+	–	+	+	+	+	+	+	+	5.21	0.0
#13	+	–	–	–	–	–	+	–	+	3.5	0.0
Others*										22.6	21.7

SSc: systemic sclerosis.
[¥]Statistical analysis performed with Fisher exact and Pearson's chi-square tests.
*Genetic profile observed in only one person was combined (others).
[a]P = 0.00085; odds ratio = 0.11; 95% confidence interval (0.012–0.497). The other genetic profiles showed no statistical significance.

Table 2
Analysis of the activity of natural killer cells in patients with systemic sclerosis (n = 110) and control group (n = 115)

	Controls		SSc		P*
	n	%	n	%	
Excessive activation	22	19.1	34	29.6	
Balance	20	17.4	36	31.3	0.001
Excessive inhibition	73	63.5	40	39.1	

SSc: systemic sclerosis.
*Pearson's chi-square test.

Table 3
Prediction of the activity of natural killer cells in patients and controls stratified according to the presence or absence of KIR2DL2

	KIR2DL2 positive			KIR2DL2 negative		
	EA	B	EI	EA	B	EI
Controls	16.5%	11.3%	34.7%	2.6%	6.1%	26.1%
Patients	10.2%	10.0%	10.9%	20.9%	20.9%	29.1%
P*	NS	NS	< 0.001	< 0.001	0.001	NS

EA: excessive activation; B: balance; EI: excessive inhibition; NS: non-significant.
*Pearson's chi-square test.

first-degree relatives ranges from 10 to 16, and, for monozygous twins, from 10 to 27.[18] Several studies have suggested that genetic susceptibility alone is not enough to induce disease.

In our study, 9.73% of the patients with SSc had activating 2DS2 and inhibitory 2DL2, and lack of activating 2DS1, 2DS3 and 3DS1, as compared to 13.9% of the control group, and, thus, protection might also be provided by that profile. The profile 'absence of inhibitory 2DL2 and of activating 2DS1, 2DS2 and 2DS3' was found only in patients with SSc (9.73%). Conversely, the profile 'presence of inhibitory 2DL2 and absence of the activating genes' was only found in the control group (6.1%), and the presence of all genes (including the activating 2DS2 and inhibitory 2DL2) was more often found in the control group than in patients with SSc. Such data show the importance of inhibitory 2DL2 for the development of SSc, and are in accordance with a previous study that showed an increase in the frequency of activating KIR2DS2 in the absence of inhibitory KIR2DL2 in patients with SSc.[19] Recently, using data of the same patients involved in the present study, we have reported a protective effect of the inhibitory 2DL2 gene against the development of SSc.[20] That combination of KIR genes has also been observed in the pathogenesis of other rheumatic diseases. In rheumatoid arthritis, the presence of KIR2DS2 has been related to vasculitis;[21] in psoriatic arthritis, KIR2DS2 in the absence of KIR2DL2 ligands has been associated with a higher risk of developing that disease.[11] In addition, the involvement of the KIR2DS2+/KIR2DL2− combination in the pathogenesis of Sjögren's syndrome has been suggested.[22]

Recent studies have suggested that HLA-I genes might play a role in susceptibility to autoimmune diseases and their expression, such as rheumatoid arthritis, ankylosing spondylitis and systemic lupus erythematosus, through the interaction with KIR receptors. Based on the idea that an activating KIR, such as KIR2DS2, can favor the development of SSc in the absence of the ligand for any KIR2DL1 or KIR2DL2/3 (that is, homozygous for a group of HLA-Cw ligands), the novel model proposed by Nelson et al.[10] was used. That is in accordance with our understanding of KIR expression and function, and has a more robust statistical support for the role played by KIR in susceptibility to SSc than that of the previous model.

Previous studies associating the KIR genes in SSc have evidenced important results regarding susceptibility to that disease. However, no previous study has assessed the activity profile of NK cells in patients and controls – genes were assessed in isolation only. Our study showed that healthy individuals have excessive inhibition as compared to patients with SSc ($P < 0.001$). That is in accordance with a study performed with patients with diabetes,[23] in which excessive inhibition was found in controls (25.71%) as compared to patients (1.02%). However, another study assessing that model in patients with psoriasis has reported no statistical difference between patients and controls ($P = 0.822$).[12]

Stratifying patients according to the gene associated with SSc (KIR2DL2), we found excessive inhibition in KIR2DL2-positive controls ($P < 0.001$). When that gene was absent, there was prevalence of excessive activation ($P < 0.001$) and balance ($P = 0.001$) in patients with SSc, suggesting an important role of that gene in the development of SSc. Such observations further corroborate the hypothesis of a dominant protection provided by some inhibitory KIR genes.

CONCLUSION

Imbalance between the number of activating/inhibitory KIR genes seems to play an important role in susceptibility to and protection against SSc. When certain models are used in data analysis, the interaction between KIR/HLA-C genes might indicate the role of NK cells in the pathology of SSc. Additional levels of variations, such as allelic polymorphisms, require further investigation, and new studies on the association of KIR genes with other autoimmune disorders are necessary. The results suggest that experiences with the function of NK cells can provide more information.

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