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Original article

Correlation between cellular expression of complement regulatory proteins with depletion and repopulation of B-lymphocytes in peripheral blood of patients with rheumatoid arthritis treated with rituximab

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ARTICLE INFO

Article history:
Received 22 December 2015
Accepted 31 July 2016
Available online 18 October 2016

Keywords:
Rheumatoid arthritis
Complement regulatory proteins
Rituximab
Biomarkers

ABSTRACT

Objectives: To correlate the basal expression of complement regulatory proteins (CRPs) CD55, CD59, CD35, and CD46 in B-lymphocytes from the peripheral blood of a cohort of 10 patients with rheumatoid arthritis (RA) initiating treatment with rituximab (RTX) with depletion and time repopulation of such cells.

Methods: Ten patients with RA received two infusions of 1 g of RTX with an interval of 14 days. Immunophenotypic analysis for the detection of CD55, CD59, CD35, and CD46 on B-lymphocytes was carried out immediately before the first infusion. The population of B-lymphocytes was analyzed by means of basal CD19 expression and after 1, 2, and 6 months after the infusion of RTX, and then quarterly until clinical relapse. Depletion of B-lymphocytes in peripheral blood was defined as a CD19 expression $<0.005 \times 109$/L.

Results: Ten women with a median of 49 years and a baseline DAS28 = 5.6 were evaluated; 9 were seropositive for rheumatoid factor. Five patients showed a repopulation of B-lymphocytes after 2 months, and the other five after 6 months. There was a correlation between the basal expression of CD46 and the time of repopulation (correlation coefficient = −0.733, $p = 0.0016$). A similar trend was observed with CD35, but without statistical significance (correction coefficient = −0.522, $p = 0.12$).

Conclusion: The increased CD46 expression was predictive of a faster repopulation of B-lymphocytes in patients treated with RTX. Studies involving a larger number of patients will be needed to confirm the utility of basal expression of CRPs as a predictor of clinical response.

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http://dx.doi.org/10.1016/j.rbre.2016.09.007

Correlação entre expressão celular de proteínas reguladoras do complemento com a depleção e repopulação de linfócitos B no sangue periférico de pacientes com artrite reumatoide tratada com rituximabe

Palavras-chave:
Artrite reumatoide
Proteínas reguladoras do complemento
Rituximabe
Biomarcadores

RESUMO

Objetivos: Correlacionar a expressão basal das proteínas reguladoras do complemento (PRC) CD55, CD59, CD35 e CD46 nos linfócitos B do sangue periférico de uma coorte de 10 pacientes com artrite reumatoide (AR) iniciando tratamento com rituximabe (RTX) com a depleção e tempo de repopulação dessas células.

Métodos: Dez pacientes com AR receberam duas infusões de 1g de RTX com intervalo de 14 dias. Análises imunofenotípicas para detecção de CD55, CD59, CD35 e CD46 nos linfócitos B foram feitas imediatamente antes da primeira infusão. A população de linfócitos B foi analisada por meio da expressão de CD19 basal e após um, dois e seis meses após a infusão de RTX e então trimestralmente até a recaída clínica. Depleção de linfócitos B no sangue periférico foi definida como expressão de CD19 < $0{,}005 \times 10^9$/l.

Resultados: Dez mulheres com mediana de 49 anos e DAS 28 basal de 5,6 foram avaliadas; nove eram soropositivas para o fator reumatoide. Cinco pacientes apresentaram repopulação de linfócitos B após dois meses e as outras cinco aos seis meses. Houve correlação entre a expressão basal de CD46 e o tempo de repopulação (coeficiente de correlação -0,733, p = 0,0016). Tendência semelhante foi observada com CD35, porém sem significância estatística (coeficiente de correção 0,522, p = 0,12).

Conclusão: Expressão aumentada de CD46 foi preditora de repopulação mais rápida de linfócitos B em pacientes tratados com RTX. Estudos com um número maior de pacientes serão necessários para confirmar a utilidade da expressão basal das PRC como preditora de resposta clínica.

Introduction

Rheumatoid arthritis (RA) is an autoimmune disease that affects about 1% of the adult population, with a prevalence three times higher in women, that causes a chronic and persistent polyarthritis, mainly of peripheral joints.[1] The inflammatory infiltrate composed of macrophages, CD4+ T-cells, B-cells, dendritic cells, granulocytes and mast cells leads to synovial proliferation, resulting in swollen and tender joints, with high rates of functional limitation.[2] High levels of complement activation products such as membrane attack complex (MAC), release of anaphylatoxins C3a and C5a, and increased C3 and C4 consumption can be detected in the synovial fluid of patients with RA, suggesting an overactivation of the complement system (CS) in these patients.[3] Normal cells are resistant to the complement-mediated lysis because they have regulatory mechanisms consisting of soluble proteins in biological fluids, such as properdin and factor H, and anchored to the membrane, such as CD55 (decay-accelerating factor [DAF]), CD59 (membrane inhibitor of reactive lysis [MIRL]), CD46 (membrane cofactor protein [MCP]) and CD35 (complement receptor type 1 [CR1]).[4]

Rituximab (RTX) is a chimeric monoclonal antibody directed to CD20 which is expressed on the surface of pre-B-cells to mature B-lymphocytes, leading to a transient, almost complete, depletion of B-cells in blood and to a partial depletion in bone marrow and in the synovial tissue.[5–10] Its mechanism of action is based on the signaling and subsequent B-cell death by induction of apoptosis, complement-mediated lysis, or cell death by macrophages.[11,12] The clinical response seems to correlate to the level of B-cell depletion in peripheral blood and synovium.[6] The treatment of RA with RTX showed significant efficacy in controlling signs and symptoms, improving physical function, in addition to benefits in the prevention of radiological damage.[13–19]

Although a large number of patients present a satisfactory response to treatment with RTX, about 40–50% are refractory and the mechanism of this failure is not fully understood.[5,18,19] In this context, the search for biomarkers that can predict which subgroups of patients have the greatest potential to be benefited from this therapy has been one of the goals of the most recent studies related to the drug. This strategy, in addition to reducing costs, could minimize periods of disease activity and of exposure to possible side effects of an ineffective treatment.[20]

Several studies have investigated the association between increased expression of complement regulatory proteins (CRPs) and the mechanism of treatment failure with RTX in patients with lymphoproliferative disorders. Golay et al. correlated the increased expression of CD55 and CD59 on the surface of lymphoma tumor cells with increased resistance to complement-mediated lysis.[21] This same group, in a study involving neoplastic cells isolated from patients with chronic lymphocytic leukemia (CLL) and lymphoma, detected a two-to-three-fold increase in cell lysis, when anti-CD55 and anti-CD59 monoclonal antibodies were used.[22] Other authors have found an increased resistance to complement-mediated

lysis triggered by RTX in the face of the increased expression of CD55[23] and CD59.[24]

Although the literature includes consistent findings suggesting that CRPs expression, particularly CD55 and CD59, can be used as a biomarker of response to treatment with RTX on lymphoproliferative diseases, no study in the literature has made this correlation in patients with RA.

The aim of this study is to correlate the basal expression of CRPs CD55, CD59, CD35, and CD46 on B lymphocytes from peripheral blood from a cohort of RA patients initiating treatment with RTX with the depletion level of these cells in peripheral blood and their time of repopulation. In this study, the correlation between the expression of these proteins with a clinical response will be evaluated, according to the American College of Rheumatology (ACR).

Materials and methods

Study population

We included in this study 10 consecutive RA patients with clinical indication for initiation of treatment with RTX, according to the guidelines of the Brazilian Society of Rheumatology (SBR): failure or intolerance to at least two schemes of traditional disease-modifying drugs (DMARDs) and an anti-TNF agent.[25] The other inclusion criteria were: age under 18 years; RA diagnosis for at least 6 months according to ACR criteria[1]; DAS28 score (Disease Activity Score-28) greater $\geq$3.2; use of an adequate contraception method to fertile patients; and desire to participate voluntarily and ability to understand the protocol, documented by the signing the Consent Form. Patients with overlapping of autoimmune rheumatic disease, lymphoproliferative disorders, and neoplastic diseases; presence of active infection; use of cytotoxic drugs; seropositivity for HIV, HBV, or HCV; active tuberculosis; allergy or hypersensitivity to RTX; pregnant or breastfeeding women; subjects participating in another interventional clinical study; IV functional class defined based on Steinbrocker functionality criteria for RA[26] and prior therapy with RTX. The patients received two infusions of RTX 1 g separated by an interval of 14 days. All patients were pretreated with methylprednisolone 100 mg, paracetamol 1 g, and dexchlorpheniramine 2 mg. Patients were followed for a maximum period of 24 months.

Clinical assessment

All patients underwent a baseline visit immediately before the infusion of RTX, with a monthly visit 6 months after the infusions, and then quarterly. At each visit, routine laboratory tests (blood count, transaminases, creatinine, routine urinalysis, erythrocyte sedimentation rate (ESR), and C-reactive protein) were carried out. On the first visit, rheumatoid factor (RF), antinuclear (ANA) factor, complement, anti-HCV, anti-HIV, anti-HBc, and HBsAg serology, chest X-rays of hands and feet, and a Mantoux test were also carried out. The following clinical parameters were evaluated at each visit: counting of 28 swollen and/or painful joints (always performed by the same examiner); overall score of disease activity (0–100 mm on a visual analog scale [VAS] assigned by both the examiner and the patient); a pain score (0–100 mm), the Health Assessment Questionnaire (HAQ) and DAS28 score using ESR.

Clinical response assessment

The clinical response to treatment was assessed 6 months after infusion of RTX, being considered as positive when the patient reached a response >20%, according to the criteria established by ACR.[27] A response failure was considered when an only an improvement <20% was perceived, compared to baseline parameters according to the same criteria. Clinical relapse was considered when there was a loss of ACR20 response in patients considered responders.

Immunophenotypic analysis

The analyses of peripheral blood flow cytometry were performed immediately before the first infusion of RTX, and 1, 2 and 6 months after the 2nd infusion of RTX and after quarterly intervals, according to a standardized technique in leukocytes,[28] during a period of fewer than 24 hours after the collection. Briefly, 100 μL of whole blood were placed into polystyrene tubes and stained with 8 μL of each fluorochrome-conjugated monoclonal antibody to CD19-PerCP, CD55PE, CD59FITC, CD35PE, and CD46FITC (BD Biosciences, San Diego, CA, USA). After incubation, 1.0 mL of FACSlyse (BD Biosciences, San Diego, CA, USA) was added, and lysis was allowed during 10 min at room temperature. Samples were washed and resuspended in 0.5 mL of phosphate buffered saline (PBS) and analyzed in CellQuestTM software of FACSCalibur flow cytometer – BD (Becton Dickinson). The intensity of the membrane fluorescence was calculated from the mean fluorescence intensity (MFI). The definition of positive or negative cells was defined when the staining of control isotype was performed in order to define the gates and to distinguish a positive staining versus autofluorescence or of nonspecific antibody binding. In each of these collections, CD19-marked cells were quantified. The expression of CD55, CD59, CD35, and CD46 was investigated before the infusion of RTX, only in CD19+ subpopulations.

100,000 events in the region of lymphocytes by flow cytometry were acquired. Data were analyzed with Infinicyt software in the region of lymphocytes (in terms of relative values and of fluorescence intensity); to do so, we used data from routine blood counts to calculate the absolute values.

B-cell depletion in peripheral blood was defined by a value of CD19+ $<0.005 \times 10^9$/L of total leukocytes. B-cell repopulation was defined when the concentration of B-cells was $>0.005 \times 10^9$/L of total leukocytes.

Statistical analysis

Due to the lack of previous studies evaluating CRPs expression and B-lymphocyte depletion and repopulation after RTX in patients with RA, we chose a pilot cohort of 10 patients who would provide the necessary information for a more accurate calculation of sample size in subsequent studies to confirm the observations of this study, in the case of some association trend.

Data were analyzed using the SPSS 16.0 program for Windows. In order to compare the means of the parametric values, the Student's *t*-test for paired samples the Mann–Whitney test for nonparametric values were performed. Correlations were analyzed using the Pearson test for parametric data and the Spearman correlation for nonparametric data. The correlation was considered as statistically significant with a *p*-value <0.05, and clinically significant when a correlation coefficient >0.4 was obtained.

Ethical aspects

This study was approved by the HCPA Research Ethics Committee under number 09–585 and was funded through the Fundo de Apoio à Pesquisa e Eventos (FIPE) of HCPA. All participating patients signed an Informed Consent Term. The Roche Laboratory contributed with a donation of RTX for use in 10 patients through a research initiative protocol, without interference in the design, analysis and preparation of this study.

Results

In total, 10 female patients with a median of 49 years were included in this study. Their baseline characteristics are shown in Table 1. The median for disease duration was 8 years. All patients were being medicated with methotrexate (median 25 mg/week) and prednisone (median 10 mg/day); in addition, these patients had made use of at least one anti-TNF agent. Nine patients (90%) were RF-positive and 8 of them (80%) had joint erosions.

Table 1 – Baseline characteristics of patients taking rituximab.

	RA patients (*n* = 10)
Age (years), median (range)	49 (37–56)
Female gender, n (%)	10 (100)
White, n (%)	8 (80)
Positive rheumatoid factor, n (%)	9 (90)
Disease duration (years), median (range)	8 (2–18)
Erosions, n (%)	8 (80)
ESR (mm/h), median (range)	27.5 (8–120)
CRP (mg/L), median (range)	10.8 (4–42.2)
HAQ (0–3), median (range)	1 (0.750–2.125)
DAS28-ESR, median (range)	5.6 (4.4–6.82)
Current use of MTX, n (%)	10 (100)
MTX dose (mg/week), median (range)	25 (20–25)
Current use of corticosteroids, n (%)	10 (100)
Prednisone dose (mg/day), median (range)	10 (5–15)
Anti-TNF, previous, n (%)	
1	9 (90)
≥1	1 (10)
Anti-TNF used, n (%)	
Adalimumab	5 (50)
Etanercept	2 (20)
Golimumab	1 (10)
Infliximab	3(30)

RA, rheumatoid arthritis; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein; HAQ, Health Assesment Questionnaire; DAS28-ESR, Disease Activity Score in 28 joints using the erythrocyte sedimentation rate; MTX, methotrexate.
Range = minimum and maximum values found.

In evaluating the clinical response, 8 patients (80%) reached ACR20 four months after infusion of RTX; but only 3 (30%) maintained this response after 6 months. When considering the DAS28 activity index, four months after infusion of RTX 3 patients (30%) had clinical remission, 2 patients (20%) showed low clinical activity, and 5 patients (50%) showed moderate disease activity. In the evaluation performed 6 months after infusion of RTX, only 1 patient (10%) exhibited low clinical activity.

The repopulation of B-cells in peripheral blood was identified in the analysis carried out 2 months after infusion of RTX in 5 patients (50%), and in the other patients at the time of the 6-month analysis.

Table 2 shows the median of CD55, CD59, CD35, and CD46 expressions, as measured by MFI in B-cells of the whole group of patients and of subsets with repopulation identified in 2 and 6 months. Patients with repopulation of B lymphocytes in the peripheral blood at 2 months showed an increased expression of CD46 (median of MFI = 72) compared to those with repopulation at 6 months (median of MFI = 47), confirming a correlation between an increased expression of CD46 with an earlier repopulation of B lymphocytes in the peripheral blood (correlation coefficient = −0733, $p = 0.016$). A similar trend was found with CD35, but with no statistical significance (median of MFI = 444 for patients with earlier repopulation versus MFI = 289 for those with a later repopulation, with a correlation coefficient = −0522, $p = 0{:}12$). No correlations were found between the expressions of CD55 and CD59 on B lymphocytes and the time of repopulation of B lymphocytes in this sample of patients (correlation coefficients = −0383 and −0174, and $p = 0.275$ and 0.631, respectively).

Although a variation in the expression of CD55, CD59, CD35, and CD46 on B lymphocytes of the patients studied has been observed, we found no correlation between this expression and the clinical response measured by ACR20 at 6 months. The expressions of CRPs measured by the median of MFI among responders (CD59 = 39; CD55 = 456; CD35 = 444; and CD46 = 79) were not statistically different compared to non-responders (CD59 = 40; CD55 = 322; CD35 = 346; and CD46 = 49). These data are presented in Table 3.

Discussion

In this study, we detected a correlation between an increased expression of CD46 and earlier repopulation of B-lymphocytes in peripheral blood following the treatment with RTX. A similar trend with CD35 was observed, but with no statistical significance. This association supports the hypothesis that an increased expression of CD46 could reduce complement-mediated lysis, one of the mechanisms of action of RTX, thus reducing the effectiveness of the drug. However, there was no correlation between the expression of CRPs in B lymphocytes and clinical response. Contrary to the expected biological reasoning that the patients with an increased expression of CRPs in peripheral B-lymphocytes treated with RTX would have an

Table 2 – Correlation between baseline expression of CD55, CD59, CD35, and CD46 in B-lymphocytes before treatment with rituximab and repopulation time of these cells.

Antigen	Total, $n = 10$	B-cell repopulation		Correlation coefficient	p-Value
	MFI median (range)[a]	2 months, $n = 5$ MFI median (range)[a]	6 months, $n = 5$ MFI median (range)[a]		
CD59	39.5 (27–66)	40 (29–66)	39 (27–44)	−0.174	0.631
CD55	386.5 (148–619)	430 (148–619)	322 (276–456)	−0.383	0.275
CD35	353.5 (148–557)	444 (240–557)	289 (148–429)	−0.522	0.122
CD46	52.5 (36–137)	72 (49–137)	47 (36–53)	−0.733	0.016[b]

MFI, mean of fluorescence intensity.
[a] Range between minimum and maximum values found.
[b] $p < 0.05$ = statistically significant.

Table 3 – Correlation between baseline expression of CD55, CD59, CD35, and CD46 in B-cells before treatment with rituximab and clinical response at 6 months.

Antigen	Clinical response at 6 months (ACR20)		p-Value
	Responders, $n = 3$ MFI median (range)[a]	Nonresponders, $n = 7$ MFI median (range)[a]	
CD59	39 (29–66)	40 (27–60)	1.0
CD55	456 (430–619)	322 (148–551)	0.067
CD35	444 (289–456)	346 (148–557)	0.383
CD46	79 (53–137)	49 (36–72)	0.067

MFI, mean of fluorescence intensity.
[a] Range between minimum and maximum values found.

earlier lymphoid repopulation and a lower clinical response to therapy, in our study, we found a tendency for higher expression of CD46 and CD55 on peripheral lymphocytes B of patients that reached ACR20 at 6 months, compared to nonresponders, but without statistical significance ($p = 0.067$). We believe that this seemingly contradictory finding is due to the small sample size leading to a lack of power of this study, in order to properly evaluate this outcome. This limitation also probably affected the results that showed no correlation between CD55 and CD59 expression on peripheral B lymphocytes with the earlier repopulation of these cells in peripheral blood after treatment with RTX.

Another important issue in this study was the low sustained response rate at 6 months (30%). This response was lower than previous studies evaluating patients with RA whose prior anti-TNF treatment failed,[18,19] where ACR20 responses were obtained at a mean of about 50% at 6 months. Our sample, when compared to the populations studied in these studies, had lower rates of HAQ (1.0 × 1.8–1.9, respectively), DAS28 (5.6 × 6.8), and disease duration (8 years × 10–12 years), but used higher doses of methotrexate (25 mg × 15 mg) and glucocorticoids. All our patients were taking glucocorticoids, while only 65% used this drug class in the study of Cohen et al.[19] We do not know, however, whether these differences have influenced somehow our results.

Despite the limitations related to sample size and the low rate of therapeutic response at 6 months, this is the first study to correlate the expression of CRPs of CD55, CD59, CD35, and CD46 with the repopulation time of B lymphocytes in peripheral blood and clinical response in a cohort of RA patients treated with RTX. Previous studies of lymphoproliferative diseases had already hypothesized that an increased expression of CRPs could reduce the complement-mediated cytotoxicity and influence in the response to treatment with RTX.[21,23,24,29] These studies have involved in vitro models, animals, and humans. Dalle et al. investigated the expression of CD55, CD59, and CD46 in rats that received cells derived from human follicular lymphoma and detected an increased expression of CD59 in those animals with RTX-resistant lymphoma.[24] Terui et al. studied the expression of CD55 in non-Hodgkin's Lymphoma (NHLs) cells from 30 patients, showing that this protein contributed to the resistance to complement-mediated cytotoxicity and to the resistance to RTX.[23] Although there is evidence that CRPs may have a response-biomarker role to RTX in lymphoproliferative diseases, this association has never been studied in patients with RA.

Our findings reinforce the importance of further studies to evaluate the role of variation in CRPs in the depletion of B lymphocytes and their impact on the therapeutic efficacy of RTX, ideally involving more numerous cohorts of RA patients.

The potential for the clinical significance of this correlation can only extrapolate the prediction of therapeutic response since, the combination of RTX with CRP inhibitors, such as recombinant protein Ad35KK++ and anti-CD55 and anti-CD59 monoclonal antibodies could be taken into account. This combination has been studied in lymphoproliferative diseases using in vitro models and animals, with favorable responses. The use of anti-CD55 and anti-CD59 increased the complement-mediated cytotoxicity, increasing the sensitivity of tumor cells to the action of RTX.[22,30–32] In a recent study, Beyer et al. studied the use of a pretreatment with Ad35K++ (a

recombinant protein which induces CD46 internalization and degradation) in monkeys treated with subclinical RTX, observing a complete cell depletion of peripheral B lymphocytes.[33]

We can conclude that the increase of CD46 expression in B lymphocytes from RA patients can predict an earlier repopulation of such cells in the peripheral blood of patients treated with RTX. Further studies are needed to confirm this result and to evaluate the correlation of other CRPs with repopulation and clinical response to treatment, thus enabling the use of these proteins as biomarkers of response to RTX.

Funding

This study was supported by the Fundo de Apoio à Pesquisa e Eventos (FIPE), from the Hospital de Clínicas of the city of Porto Alegre, Brazil, linked to the Group for Research Postgraduate Studies. The laboratory Produtos Roche Químicos e Farmacêuticos S.A., Brazil, contributed with the donation of rituximab for use in 10 patients through a protocol at the initiative of the investigator, without interference in the design, analysis, and preparation of the study.

Conflicts of interest

The authors declare no conflicts of interest.

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