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EXPRESSION OF FCεII/CD23 ON HUMAN NEUTROPHILS ISOLATED FROM RHEUMATOID ARTHRITIS PATIENTS

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Abstract—CD23, the low affinity receptor for IgE, is a 45 kilodalton molecule belonging to the C-type lectin family, some members of which have been identified as adhesion molecules. Since it has been described upregulated in different cells in chronic inflammatory diseases and in rheumatoid arthritis in particular, where neutrophils are directly involved in tissue damage, our interest, in this work, has been focused on the expression and regulation of this antigen on neutrophil membrane. We studied 22 patients suffering from rheumatoid arthritis and 22 healthy control subjects. CD23 expression on neutrophil membrane was analyzed by immunofluorescence. Neutrophils of 9 out of 22 patients expressed CD23 molecules, neutrophils of 11 out of 22 patients expressed CD23 only after 24 h of incubation in RPMI; only 2 out of 22 patients did not express the CD23 antigen on neutrophil membrane either after isolation or after a 24 h incubation. On the contrary neutrophils isolated from healthy subjects did not express CD23 molecules upon isolation. Only in 7/22 control subjects neutrophils resulted positive after 24 h of incubation in RPMI. Moreover, we found that in our experimental conditions the presence of IFN-g or GM-CSF alone or in combination with IL-4 inhibited CD23 expression during the 24 h incubation. Our results show that there is a strong association between neutrophil ability to express CD23 and rheumatoid arthritis, and that such expression may be regulated by GM-CSF, IFN-γ and IL-4.

INTRODUCTION

The etiology and pathogenesis of rheumatoid arthritis (RA) are not yet fully understood, but it has become increasingly clear that a series of locally pro-

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duced cytokines play a central role in disease progression. Indeed, cytokines are responsible both for the mobilization and continuous activation of the inflammatory cell infiltrate and for inducing production of the enzymes that destroy bone and cartilage (1). Recently it was discussed that CD23 (45 kD), a low affinity receptor for IgE (FCεII), might play an important role during the immune-inflammatory response (2). The CD23 molecule is a type II transmembrane protein that belongs to the C-type lectin family, some members of which have been identified as adhesion molecules, expressed on many haematopoietic cell types (3–5). CD23 has pleiotropic roles. By interacting with CD21, CD23 appears to mediate cell-cell adhesion, B-cell survival in germinal centers, histamine release from basophils and regulation of IgE synthesis (6). Recently it was found that CD23 also interacts with the alpha chain of the B2 integrin family (CD11b/c) with subsequent release of inflammatory mediators (cytokines and radicals) (7). As an integral membrane protein on B cells, CD23 serves as an IgE receptor that traps antigens on B cells for internalization, processing and presentation to T cells (8).

CD23 was shown to be involved in IgE-dependent cytotoxic effects against parasites (9, 10) phagocytosis of IgE-coated particles (11) and the release of IL-1, TNF-α (12) and nitric oxide (13). CD23 also exists in a 25–30 kilodalton soluble form (sCD23), which may be secreted or formed by cleavage at the membrane level (14); soluble CD23 was found to be involved in B-cell growth, pro-thymocyte maturation and myeloid precursor proliferation (15, 16).

Increased levels of CD23, either membrane or soluble, have been reported in various chronic diseases including multiple sclerosis, systemic lupus erythematosus, Sjogren's syndrome, bullous pemphigoid, inflammatory bowel diseases, glomerulonephritis, autoimmune chronic active hepatitis and RA (2).

These findings suggest that cells expressing FCεRII/CD23 reflect selective activation capacities which might be abnormally triggered in chronic inflammatory diseases through IgE-dependent or independent mechanisms. At the moment it is unclear if neutrophils are able to express CD23: in this regard, Truong et al. studied patients with eosinophilia and healthy donors (17); neutrophils isolated from peripheral blood and immediately analyzed did not express FCε/CD23. On the other hand, Yamaoka et al (18) reported that GM-CSF was able to induce positive signals for the generation of CD23 expression on human neutrophils from healthy donors after 24 h of incubation, whereas it was undetectable in unstimulated neutrophils.

In this work our interest has been focused on the expression of CD23 on human neutrophils recovered from peripheral blood of healthy donors and of RA patients. In this disease the CD23 antigen was previously found to be upregulated in B cells, monocytes and synovial T cells (19), but little is known about neutrophils. Here we report that neutrophils express CD23 only in a sub-popu-

lation of donors and that the ability to express this antigen is strongly associated with RA disease.

MATERIALS AND METHODS

Patients. For this study we enrolled 22 patients (6 males, 16 females, mean age 38.6 ± 8.2) consecutively observed in our Clinic who met the following criteria: 1) diagnosis of RA according to American Rheumatism Association 1987 criteria (20), 2) age between 18 and 65 years, 3) recent onset of RA (<1 year), 4) disease in active phase, 5) absence of any treatment. The disease was considered active in the presence of 6 or more swollen joints and at least 2 of the following data: 9 or more tender joints, duration of morning stiffness = or >45 minutes, ESR value = or >30 mm/hr. Twenty-two sex and age matched healthy subjects were enrolled as controls.

Cell Preparation. Human blood (20 ml) was collected into EDTA-containing tubes. In our experimental protocol, neutrophils were isolated, under sterile and apyrogenic conditions, by centrifugation over Percoll discontinuous gradients and hypotonic lysis to remove remaining erythrocytes, according to previously described techniques (21). This procedure yields a neutrophil population containing 98–99% of viable cells immediately after isolation and still viable 24 h later, as confirmed by trypan blue exclusion and by an in vitro adhesion and superoxide production assay, as an index of neutrophil activation after in vitro stimulation (21).

The purity of neutrophils obtained by discontinuous Percoll density gradient centrifugation was 98–99%, as determined by Sismex-9000 Hematological Analyzer and then checked by double color immunostaining with anti-CD16, an antibody that recognizes the FcgRIII receptor (present on freshly isolated neutrophils and not on eosinophils) (22) and anti-CDw65-FITC, an antigen present only on granulocytes and weakly expressed on monocytes; moreover, the antibody does not react with T and B cells (23).

Neutrophils were resuspended in a complete medium (RPMI 1640 with 10% FCS, 2 mM L-glutamine and antibiotics) to provide 1×10^6 cells/ml, and cultured at 37°C with 5% CO_2 for 24 h with and without IL-4 (5, 10, 20, 30, 40 ng/ml), IFN-γ (100, 500, 1000 U/ml), GM-CSF (10, 100, 500, 1000, 2000 U/ml). Moreover, in other experiments, cells without cytokines were cultured with cycloheximide (10^{-5} mol/L); after 24 h of incubation, cell suspensions were washed in PBS and placed for immunofluorescent staining. All cells both from patients and healthy donors were treated in the same way and with the same stock of medium, cytokines and antibodies in order to eliminate differences attributed to reagents or different experimental conditions.

Monoclonal Antibodies (MAb) and Chemicals. The PE-IgG1 for isotypic control, anti-human-CD23-PE were obtained from Becton Dickinson (San José, California), anti-human-CD69-PE, FITC-IgG1, PE-IgG1, PE-CY5-IgG1 for isotypic control, anti-human-CDw65FITC, anti-human-CD16-PE-CY5 were from Immunotech (Miami, Florida); Percoll was from Pharmacia (Upsala, Sweden), Trypan blue was from Sigma (St. Louis, Missouri); RPMI 1640 and FCS were from Seromed Biochrom (Berlin KG); r-human-IL-4 was from Pepro Tech EC LTD (London UK); r-human-GM-CSF, r-human-IFN-γ were from Genzyme Diagnostics Cambridge, Massachusetts).

Cell Surface Staining and Flow Cytometric Analysis. Purified neutrophils (2×10^6 cells) were placed in prewetted polypropylene tubes (Becton Dickinson). The cells were washed in PBS and stained directly with preconjugated MAbs at 4°C for 30 min, then washed in PBS 0.01% sodium azide 0.02% BSA and resuspended in 0.5 ml of total volume.

Expression of CD23 molecules was analyzed by Epics XL-MCL cytometer (Coulter-Instrumentation Laboratory); values for surface molecule expression were obtained as the percentage of positive cells. Percentage of positive cells were obtained after subtraction of the expression level of this isotype control Abs.

RESULTS

Neutrophils isolated from 9/22 RA patients expressed CD23 on their membrane and such expression increased after incubation: neutrophils from most patients expressed CD23 after 24 h of incubation; in our experimental conditions, we were not able to detect CD23 antigen only in 2 patients, either after isolation or after 24 h of incubation. Neutrophils of all 22 healthy subjects always resulted negative for CD23 expression after isolation and only 7 of these became positive after 24 h of incubation (Figure 1).

Both GM-CSF and IFN-γ, at different concentrations, were not able to induce CD23 expression on neutrophils isolated from CD23 negative subjects, whereas both cytokines were able to modulate CD23 expression on cells isolated from CD23 positive subjects, in a dose dependent manner (Figure 2A, B).

IL-4, alone, at different concentrations were not able to induce or regulate CD23 expression on neutrophils (Figure 2C), whereas it showed a synergic

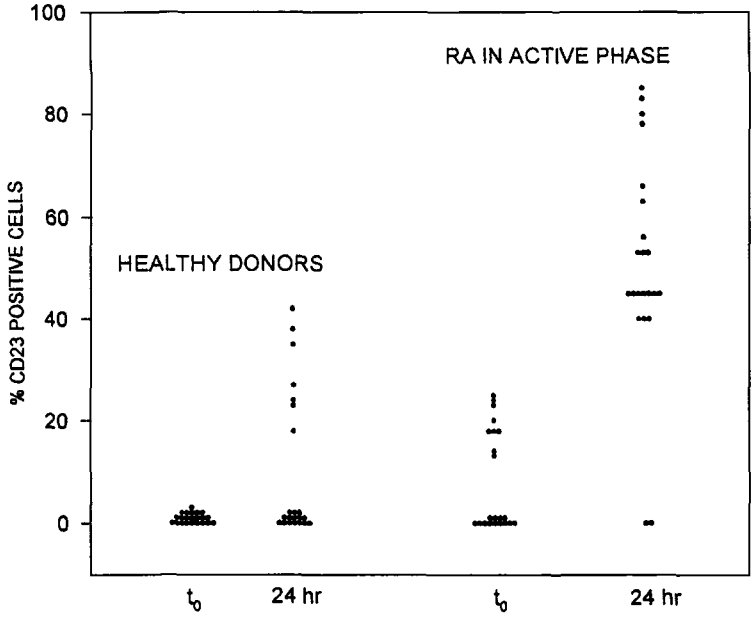

Fig. 1. Representation of CD23 positive neutrophils expression of patients (n = 22) and healthy donors (n = 22) early after isolation and 24 hour later as detected by direct immunofluorescence with a PE-conjugated MAbs anti-CD23 and analyzed on cytofluorimeter Epics XL-MCL (Coulter-Instrumentation Laboratory). Results are expressed as percentages of positive cells after subtraction of non-specific background fluorescence with respective MAb isotype control.

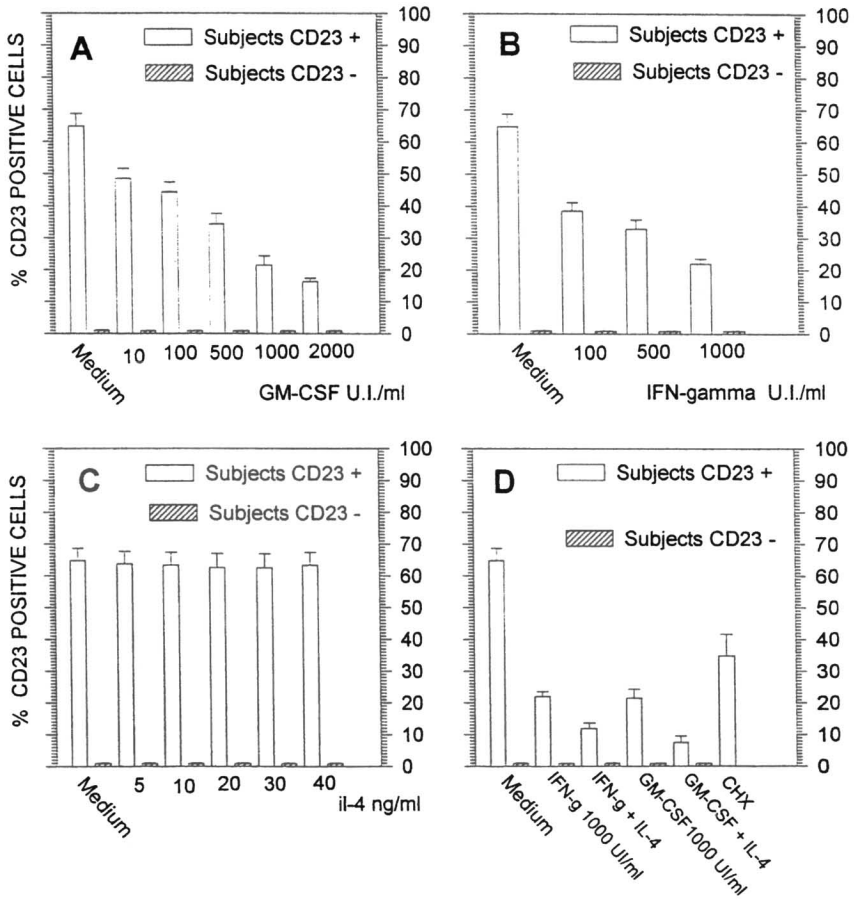

Fig. 2. Analysis of CD23 expression on GM-CSF (10, 100, 500, 1000, 2000 U/ml) (A), IFN-γ (100, 500, 1000 U/ml) (B), IL-4 (5, 10, 20, 30, 40 ng/ml) (C) and GM-CSF1000 U.I./ml+ IL-4 (10 ng/ml), IFN-γ (1000 U.I./ml) + IL-4 (10 ng/ml), cicloeximide (D) treated neutrophils for 24 h as detected by direct fluorescence with a PE-conjugated MAbs anti-CD23 and analyzed on cytofluorimeter Epics XL-MCL (Coulter-Instrumentation Laboratory). Results are expressed as percentages of positive cells after subtraction of non-specific background fluorescence with respective MAb isotype control. All donors were classified as CD23 negative subjects and CD23 positive subjects. Data are reported as mean ± *SD* (n = 22).

effect, in combination with IFN-γ or GM-CSF, on CD23 modulation by GM-CSF and IFN-γ (Figure 2D). Cycloheximide added to CD23 positive cells (10^{-5} M) reduced the expression in a significant manner after a 24-h incubation (Figure 2D).

DISCUSSION

CD23 expression was identified in synovial biopsies from patients with chronic synovitis, and sCD23 can be measured at concentrations exceeding the normal range in the serum and synovial fluid of RA patients. In addition, levels of serum sCD23 in RA patients are related to disease activity (24).

In this work we report that human neutrophils isolated from peripheral blood of 20/22 RA patients and 7/22 healthy donors were able to express CD23 in vitro. Recently Yamaoka et al. reported (18) that human neutrophils, after stimulation with GM-CSF, expressed CD23 and that unstimulated cells remained negative for such antigen. Our experiments gave different results showing two groups of donors: one named "CD23 positive subjects" whose neutrophils were able to express CD23 after 24 h of incubation without stimuli, a second named "CD23 negative subjects" whose neutrophils were not able to express CD23 antigen after 24 h of incubation both in the absence of any stimulations and in the presence of GM-CSF at different concentrations. The reason for this discrepancy is not clear but it may be related to differences in the experimental conditions (neutrophils isolation and/or culture), or to the existence of different sub-populations of donors, with the ability or not to express CD23 on neutrophil membrane. As other blood-derived cell populations can be induced to CD23 expression, the purification of neutrophils has to be carried out with particular care in order to avoid a possible contamination with lymphocytes, monocytes or eosinophils. In addition, to avoid a possible contamination with LPS and subsequent cellular activation, we always used the same stocked reagents in all experiments.

Interleukin-4 and IFN-γ, that have been described as potent CD23 inducers on monocytes and other cells (25), failed to induce this antigen on neutrophils isolated from CD23 negative subjects.

Both cytokines, contrary to all experiments, downregulated CD23 expression. IL-4, if added with IFN-γ or GM-CSF, had a synergic effect in modulating CD23 expression.

At the moment we do not know the positive or negative signals that make cells, in vitro and probably in vivo, resistant or susceptible to CD23 expression; also the mechanisms of its regulation are not completely understood, but our findings lead us to speculate that an important negative control by IFN-γ and/or GM-CSF alone or in combination with IL-4 could exist in vivo, as observed in vitro.

It is worth noting that animals treated with anti-CD23 antibody showed an improvement in clinical severity, confirmed by histological examination of the arthritic feet and by decrease in cellular infiltration of the synovia (26).

Our work showed that RA appears strongly associated to the ability of CD23 expressing neutrophils. In our opinion, CD23 expression on neutrophils

may allow, like on macrophages, homotypic or heterotypic interactions through CD11b/c molecules or unknown ligands in the RA inflamed synovium. In addition, the diffusion of sCD23 molecules through the synovium and their binding to the integrin ligands or other molecules, with positive feedback, might contribute to the inflammatory reactions that lead to neutrophil migration to synovial tissue. These considerations might explain some of the pathogenic aspects of disease exacerbation and chronicity in RA disease and support the hypothesis that, once localized to the joints, neutrophils can maintain and exacerbate inflammation via a pathway involving CD23 molecules, $\beta 2$ integrins, proinflammatory cytokines and radicals. If this hypothesis is correct, therapies aimed to inhibiting the interactions between CD23 and its ligands might break the inflammatory cycle and represent an important advance in controlling inflammation.

We need further investigation to define which isoform of CD23 is present on neutrophils, as it was reported that the two isoforms are associated to the transduction of different signals: isoform B was associated to Nitric Oxide release and cytotoxicity (27), whereas isoform A to the antigen capture by IgE and presentation to specific T cells after internalization, degradation and recyling of fragments associated to HLA to cell surface (28, 29). Recently it was reported that human neutrophils are able to release nitric oxide (30, 31) and that they are able to express HLA-DR molecules involved in antigen presentation to T cells (32, 33). Our findings lead us to speculate that CD23 expressed on human neutrophils could be involved either in antigen presentation to specific memory or naive T-cells or in proinflammatory neutrophil activation by specific IgE-mediated dependent or independent reactions or by reactions mediated by sCD23.

At the moment we do not known if CD23 expression is correlated to an activated or differentiated cellular phase, because 7/22 healthy subjects expressed the CD23 antigen and 2/22 patient did not express it; one explanation might be that CD23, if expressed on neutrophils, could be associated with a type of immune-response that renders subjects, in some unknown circumstances, susceptible to chronic diseases and RA in particular. These considerations are supported by the involvement of CD23 in the murine model of RA (26) and provide a role for this molecule in inflammatory diseases. In conclusion, our data show that human neutrophils are able to express CD23 in vitro only in a sub-population of donors, and that the ability to express CD23 is strongly associated with RA disease, even if further investigations are required to understand the real involvement of this antigen in the disease. The implication of CD23 in inflammation, and our data regarding its expression and regulation in neutrophils, lead us to speculate that this molecule could be implicated in the development of RA, and may represent a marker of its progression.

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