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CD69 expression on neutrophils from patients with rheumatoid arthritis

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ABSTRACT

Objective. *Since the early activation antigen CD69 has been implicated in the pathogenesis of some inflammatory diseases, we evaluated the expression of the molecule on peripheral blood (PB) and synovial fluid (SF) neutrophils obtained from RA patients and its possible correlation with PB and SF cytokine concentration.*

Methods. *CD69 membrane expression (and CD11b as control marker) was assessed by indirect immunofluorescence and flow cytometry analysis on purified PB and SF neutrophils. Cytokine levels (GM-CSF, IFN-γ, TNF-α) in plasma and SF supernatants were measured by ELISA.*

Results. *CD69 was absent on control neutrophils, while it was expressed on PB neutrophils from RA patients although no detectable GM-CSF, IFN-γ or TNF-α was observed in their plasma. CD69 expression was still more evident on SF neutrophils from RA patients; 59% had detectable levels of INF-γ in their SF while GM-CSF and TNF-α were detectable in SF from 95% and 33% of RA patients, respectively. However, no correlation was observed between cytokine concentrations and CD69 expression on SF neutrophils. SF but not PB neutrophils from RA patients expressed increased amounts of CD11b when compared to control PB neutrophils without any correlation with CD69 membrane expression.*

Conclusion. *The activation antigen CD69 is significantly expressed on PB and SF neutrophils from RA patients. However, the mechanism(s) of induction and its possible role in the pathogenesis of RA remain to be defined.*

Introduction

CD69 is a disulfide-linked homodimer that was initially described as an antigen induced very early during lymphoid activation (1). It is a type II integral membrane glycoprotein included in the natural killer cell gene complex (NKC) family of cell surface receptors (2) and in humans it is the product of a single gene located on chromosome 12 (3). CD69 expression is not restricted to activated lymphocytes, being constitutively expressed on platelets, epidermal Langerhans cells, monocytes, and bone marrow mast cells (reviewed in 4) while in eosinophils and neutrophils CD69 may be induced by several cytokines, above all GM-CSF and IFN-γ (5-7).

Although a specific ligand for this molecule has not been identified, its broad cellular distribution, its ability to generate intracellular signals upon cross-linking and its enhanced expression in certain inflammatory diseases have suggested a possible pathogenetic role for CD69 (reviewed in 4). Arole in the pathogenesis of rheumatoid arthritis (RA) was previously hypothesized by Laffon *et al.* (8) who found that CD69 positive T cells were detectable at high levels in synovial fluid (SF) and synovial membrane from RA patients and that molecule expression correlated with disease activity.

Even if RA is considered to be a T-cell driven disease, the role of neutrophils in RA pathogenesis is well known (9). Neutrophils heavily infiltrate synovial fluid and the pannus-cartilage junction where they can release cytotoxic compounds such as reactive oxygen intermediates, granule enzymes and proinflammatory cytokines. Recently, a possible critical role for CD69 on neutrophils in the pathogenesis of collagen-induced arthritis in mice has been suggested (10). Therefore, in the present work we examined the expression of CD69 on neutrophils obtained from peripheral blood (PB) and SF of RApatients and attempted to correlate it with SF or plasma cytokine concentration. We found that both SF and PB neutrophils from RApatients exhibited increased membrane expression of CD69, even if a direct correlation with cytokine concentration was not evident.

Materials and methods

Patients

PB and SF samples were collected from 42 RApatients who satisfied the American College of Rheumatology 1987 criteria (11); the mean age was 51.3 (range 13-81), 30 were rheumatoid factor (RF) positive and 12 were RF negative. All patients were taking disease-modifying antirheumatic drugs (DMARDs) (hydroxychloroquine in 8 patients, methotrexate in 20 patients, hydroxychloro-

quine + methotrexate in 14 patients), nonsteroidal anti-inflammatory drugs (NSAIDs) and no more than 10 mg/day of prednisone; none had received intra-articular corticosteroids within 3 months of the sampling. Controls were 25 healthy laboratory workers, matched with the patients for age and sex.

Expression of CD69 and CD11b on PB and SF neutrophils

PB and SF neutrophils were obtained by density gradient centrifugation (Lymphoprep, Nyegaard, Oslo, Norway) (12). Neutrophil membrane expression of CD69 and CD11b was evaluated as previously reported (13). Briefly, purified neutrophils were stained with predetermined saturating concentrations of the anti-CD69 or anti-CD11b monoclonal antibodies (mAbs) (60 min at 4°C), washed and then resuspended in buffer containing FITC-conjugated goat anti-mouse IgG in a saturating concentration (30 min at 4°C). The cells were then washed twice and resuspended in 0.5 ml of ice-cold 2% paraformaldehyde in PBS (pH 7.2). The percent of neutrophils positive for CD69 or CD11b was quantified on a FACSscan flow cytometer (Becton Dickinson). A relative measure of the antigen expression was obtained using the mean fluorescence intensity (MFI), converted from log to linear scale, after subtraction of the fluorescence of cells incubated with irrelevant isotype control mAbs.

In some experiments control neutrophils were cultured for 18 h in complete medium supplemented with RA serum or SF supernatants (1:1) before being assayed for CD69 or CD11b membrane expression.

Measurement of cytokines in plasma and SF

GM-CSF, IFN-γ and TNF-α levels were measured in plasma from all subjects and in the SF supernatants from RA patients by a quantitative ELISA (Biotrak, Amersham, U.K.). The assay was performed as specified by the manufacturer.

Statistical analysis

CD69 and CD11b values are presented as mean ± s.e.m. and P values for comparison of the means were calculated using Student's t-test for unpaired values. As the cytokine values included a proportion of zero values, non-parametric tests were used for the statistical analysis and medians, and the absolute ranges were reported. Correlations between the quantitative measurements (cytokine concentrations and CD69 expression) were tested using Spearman's rank correlation coefficient. P values < 0.05 were accepted as significant.

Results

PB control neutrophils stained with anti-CD69 mAb gave very low fluorescence (% CD69-positive cells: 1.2 ± 0.5; MFI: 1.1 ± 0.4), just above that of unstained cells. On the other hand, CD-69 was significantly expressed on PB neutrophils from RA patients (% CD69-positive cells: 18.22± 2.93; MFI: 5.27 ± 3.73; $p < 0.001$ vs controls) (Fig. 1). The membrane expression of the molecule was even more evident when SF neutrophils from RA patients were considered (% CD69-positive cells: 28.15 ± 4.01; MFI: 6.13 ± 1.03; $p < 0.001$ vs controls) (Fig. 1). In 33/42 RA patients (78%) CD69 expression on PB neutrophils was higher than the upper 95% confidence limit of the 25 healthy

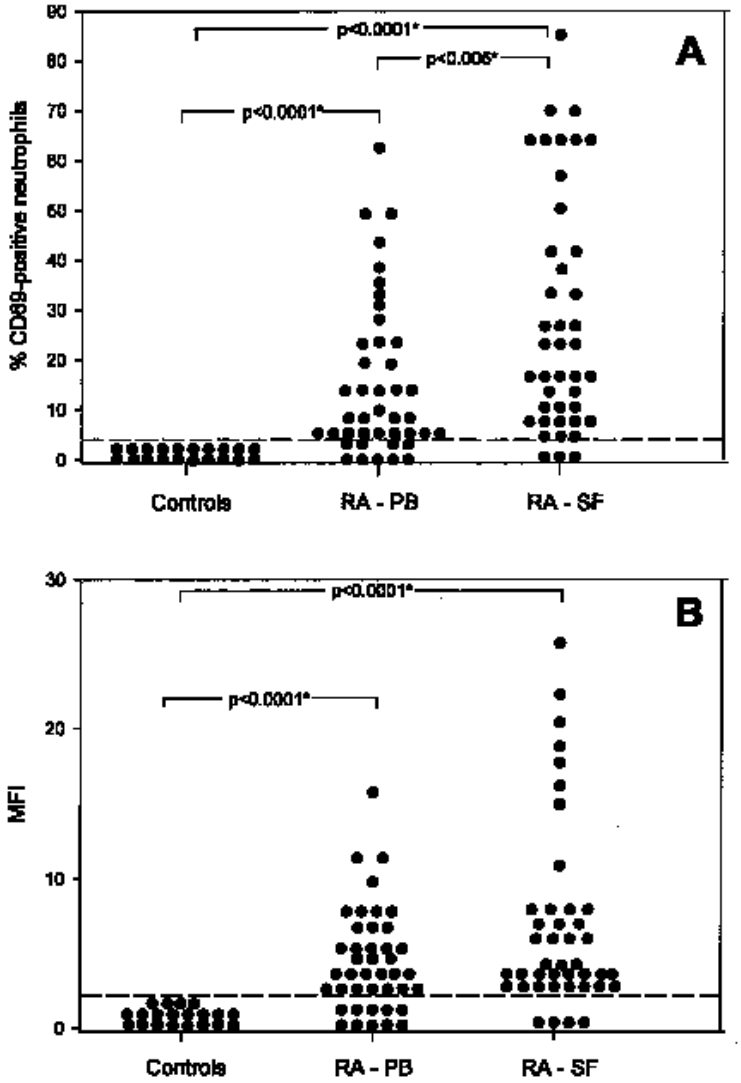

Fig. 1. CD69 membrane expression on neutrophils. Neutrophils were purified from the peripheral blood (PB) of 25 controls and from the PB and synovial fluid (SF) of 42 RApatients. Cells were stained with anti-CD69 mAb and FITC-labelled goat anti-mouse IgG. Values represent the % CD69-positive cells (**A**) or the mean linear fluorescence intensity (MFI) (**B**) corrected for non-specific staining. The dotted lines indicate the 95% confidence limits for the controls. * Student's t test.

controls; when SF neutrophils were considered 39 of the 42 RA patients (92%) showed CD69 expression higher than the upper 95% confidence limit of the 25 healthy controls.

The well known activation marker CD11b was expressed on more than 90% of both control and RA PB neutrophils, with MFI values being similar between the two groups (45.1 ± 4.2 for controls and 43.4 ± 5.4 for RA PB) (Fig. 2). On the other hand, neutrophils from the SF of RA patients expressed significantly increased amount of CD11b (MFI: 209.7 ± 24.7) when compared to PB neutrophils from patients or controls ($p < 0.001$; Fig. 2). No correlation between CD69 and CD11b membrane expression was observed on SF neutrophils from RA patients (not shown), suggesting the existence of different mechanisms of induction for the two molecules.

The increased expression of CD69 on both PB and SF neutrophils form RA patients did not correlate with age, RF or therapy (not shown) .

Since GM-CSF and IFN-γ have been recently reported to induce CD69 on normal neutrophils (6, 7) we wondered whether a correlation existed between the levels of these cytokines and CD69 membrane expression. None of the patients had detectable GM-CSF or IFN-γ in their plasma. On the other hand, GM-CSF was detectable in the SF of all but 2 of the RA patients (median, 5.55 pg/ml; range, 0-18.8) and IFN-γ in the SF of 25 patients (median, 1.7 pg/ml; range 0-84.6), even if the cytokine concentrations were about 100 times below those required *in vitro* to induce CD69 expression on neutrophils (7). No significant correlations were shown between cytokines concentrations and CD69 expression on SF neutrophils (not shown).

To verify whether other CD69 inducers were present in the SF from RA patients, in some experiments control neutrophils were incubated for 18 h in medium supplemented with RA serum or SF supernatants, before being assayed for CD69 or CD11b expression. Under these experimental conditions no CD69 membrane expression was observed, while a low but significant expression of CD11b was induced by SF supernatants (Table I).

Since we have recently reported that *in vitro* CD69 may act as a co-stimulus during neutrophil activation and TNF-α production (7), we attempted to correlate CD69 positive neutrophils with the TNF-α concentration in the SF of our patients. TNF-α was detectable in SF of 22 patients (median, 3.2 pg/ml; range, 0-131) but in this case as well no significant correlation with CD69-positive neutrophils was observed.

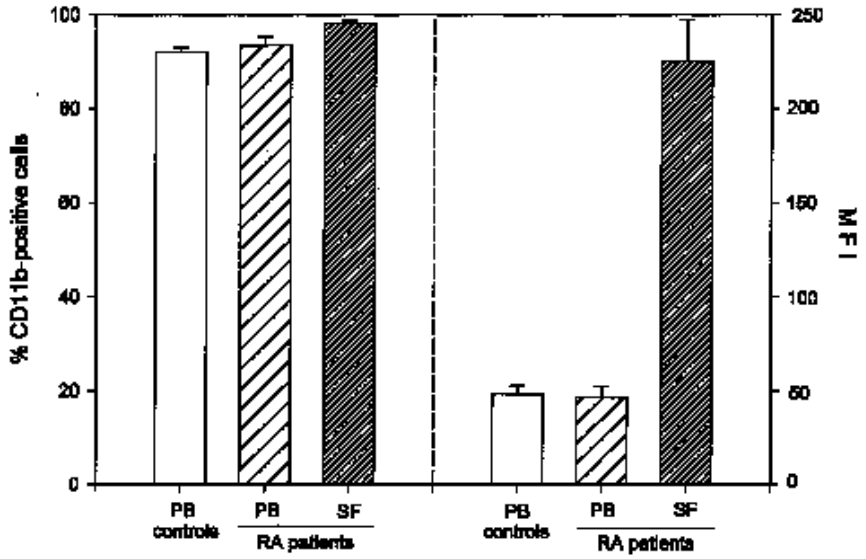

Fig. 2. CD11b membrane expression on neutrophils from controls and RApatients. Neutrophils from the peripheral blood (PB) of 25 controls and from the PB and synovial fluid (SF) of 42 RA patients were stained with anti-CD11b mAb and FITC-conjugated goat anti-mouse IgG. Results are expressed as the mean ± SE of % positive cells or as the mean linear fluorescence intensity (MFI) corrected for non-specific staining.

Table I. Effect of RAsera or synovial fluid (SF) supernatants on CD69 and CD11b expression on neutrophils.

Incubation	CD69+ cells		CD11b+ cells	
	%	MFI	%	MFI
Medium	10.9 ± 1.9	2.1 ± 0.4	93.4 ± 2.9	52.3 ± 3.9
RAserum	11.7 ± 1.3	1.9 ± 0.3	92.9 ± 3.0	55.2 ± 4.1
RASF supernatant	13.8 ± 1.7	2.4 ± 0.5	96.9 ± 2.1	68.1 ± 4.9*

Control neutrophils were incubated for 18 h in medium alone or medium supplemented with RAsera (1:1) or RASF supernatants (1:1) obtained from 10 patients, washed and then used in the assay. Values are shown as the mean ± s.e.m. of the percentage of positive cells or the mean linear fluorescence intensity (MFI). * $p < 0.05$ compared to medium.

Discussion

In this work we showed that PB and SF neutrophils from RApatients expressed significant levels of membrane CD69. This observation suggested a role for GM-CSF and/or IFN-γ as inducers in these patients, since both cytokines have been detected in SF from RA patients (14) and both have been recently reported as active inducers of CD69 on neutrophils *in vitro* (6, 7). Fifty-nine percent of the patients had detectable levels of INF-γ and 95% had detectable GM-CSF in their SF, even if at concentrations below those required *in vitro* to induce CD69 expression on neutrophils (7). No correlation was observed between cytokine concentrations and CD69 expression on SF neutrophils. Moreover, PB neutrophils from RA patients significantly expressed CD69 on their membrane although no detectable GM-CSF or IFN-γ was observed in their plasma. These observations suggest

that other, not yet defined CD69 inducers could be active *in vivo* during an inflammatory process. However, preincubation of control neutrophils with RA sera or SF did not induce CD69 membrane expression, whereas a low but significant increase in CD11b expression was observed, suggesting – in agreement with previous studies (15, 16) – that CD11b is induced within the joint while different mechanism(s) are implicated in the CD69 induction in RAneutrophils.

Other hypotheses may be proposed to explain the poor correlation between CD69 expression and the cytokine concentration. First, CD69 expression could be a more sensitive or lasting marker of the biological activity of the cytokines *in vivo* than their direct measurement by specific ELISA. Otherwise, it is possible that CD69 membrane expression may be induced on neutrophils in the bone marrow by sustained levels of GM-CSF. In agreement with this hypothesis is the recent observation of increased bone marrow granulopoiesis in RApatients (17).

Until a specific ligand for CD69 is identified, the biological significance of the molecule will remain uncertain. We recently showed that *in vitro* CD69 cross-linking on GM-CSF-primed neutrophils synergized with LPS and increased TNF-α production (7). Furthermore, Yu *et al*. (18) demonstrated that autoAb to CD69 exist in the sera of patients with RA and they were associated with severe disease. These autoAb might realize CD69 crosslinking *in vivo* and neutrophil activation at inflammatory sites, where leukocytes may be induced to express CD69 by GM-CSF or others inducers. Our attempt to correlate CD69 positive neutrophils with the TNF-α concentration in the SF of our patients was unsuccesful, but clearly TNF-α does not represent the sole component in the inflammatory mechanism of neutrophils. The combination therapy in our patients might be responsible for the low cytokine concentrations in RA synovial fluid and the lack of correlation with CD69 expression.

Of great interest is the recent observation that CD69-null mice are protected from collagen-induced arthritis, and that in these animals arthritis could be restored by transfer of neutrophils from wild-type mice (10). Even if the precise role of CD69 in the induction of this model of experimental arthritis was not defined, the data suggested a crucial role for CD69 in the pathogenesis of arthritis in mice and indicate the molecule as a possible therapeutic target for human arthritis. By the light of these observations, the significant expression of CD69 on neutrophils from RA patients suggest a possible role of the molecule in the pathogenesis of joint disease in RA. To further define this possibility it will be of interest in future studies to evaluate neutrophils CD69 expression and cytokine concentrations in patients with early RA before and during pharmacological treatment.

Our present observations indicate that CD69 is expressed by circulating and synovial fluid neutrophils of RA patients as a marker of a cellular activation state *in vivo*. However, further studies are required to define the mechanisms of induction of the molecule as well as its possible role in the pathogenesis of this inflammatory disease.

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