Concise Report

CD95-Mediated control of anti-citrullinated protein/peptides antibodies (ACPA)-producing plasma cells occurring in rheumatoid arthritis inflamed joints

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Objective. Serum anti-citrullinated protein/peptides antibodies (ACPA) are a valuable diagnostic parameter that might be involved in rheumatoid arthritis (RA) pathogenesis. CD95-dependent apoptosis is defective in RA synovium. The present study explores the occurrence of ACPA IgG, and the CD95-mediated control of ACPA IgG-secreting plasma cells (PC) in RA patients.

Methods. Mononuclear cells (MC) were purified from synovial fluid (SF) and peripheral blood (PB) of 15 RA patients. PC capable of secreting ACPA IgG were detected in MC cultures. ACPA IgG present in serum and SF, and PB and SF MC culture supernatant was measured by ELISA. CD95, CD27 and CD138 expression was examined on RA PC identified as CD19low CD38high cells by flow cytometry. CD95-ligation was obtained by treatment of cultured MC with the anti-CD95 Ab CH11. Apoptotic PC were identified as Annexin-V þ.

Results. ACPA IgG level was found higher in patients’ SF than in their serum. PC were detectable in SF and PB, and exhibited high CD95 and CD27 expression. In contrast, SF, but not PB, PC expressed elevated levels of CD138. SF, but not PB, PC actively secreted ACPA IgG in cultures, in a linear fashion for at least 14 days, and CD95-ligation markedly reduced this activity and provoked PC apoptosis.

Conclusions. The results suggest that RA synovium is a prominent site for ACPA IgG formation and for the accumulation of ACPA IgG-secreting PC exhibiting prolonged survival, probably due to RA defective CD95-mediated control.

KEY WORDS: Human, RA, SF, Anti-citrullinated protein/peptides antibodies (ACPA) IgG, plasma cell, CD95-mediated apoptosis.

Introduction

Rheumatoid arthritis (RA) is a chronic inflammatory disorder mainly affecting multiple joints, and leading to cartilage and bone lesions. RA synovial tissue becomes a prominent site of immune activation, and local maturation of B lymphocytes into plasma cells (PC), including PC capable of secreting RA-associated auto-antibodies (Ab), has been demonstrated in affected joints [1]. The occurrence of serum anti-citrullinated protein/peptides antibodies (ACPA) is increasingly used as a specific parameter for the prediction and diagnosis in many cases of RA [2]. In addition, PC-secreting ACPA have been demonstrated in the synovium of RA patients [3, 4], and a pathogenic role has recently been proposed for this auto-Ab [5].

Apoptosis through engagement of the death receptor Fas (CD95) plays pivotal roles in maintaining peripheral immune self-tolerance and in controlling a variety of cellular components of the inflammatory response [6]. Defects in apoptosis, namely in the CD95-dependent death receptor pathway, have been demonstrated to participate in RA pathogenesis [7]. However, a possible role of CD95-mediated apoptosis on RA-associated auto-Ab-producing PC has not yet been explored. The aim of this study is to analyse the susceptibility of ACPA-secreting PC from RA patients to CD95-mediated apoptosis.

Patients and methods

Patients

Fifteen patients who fulfilled the American College of Rheumatology (ACR) criteria for RA were included in the study. Twelve were female and three male, with mean age 60.5 ± 2.9 yrs (range 38–79). Ten patients were taking methotrexate (range 10–17.5 mg/week) associated or not to oral prednisolone (range 5–10 mg/day). Three patients received leflunomide (20 mg/day) and prednisolone (10 mg/day). Two patients were taking only prednisolone (10 mg/day). None of them were receiving biological agents. All patients underwent therapeutic arthrocentesis, and synovial fluid (SF) and peripheral blood (PB) samples were obtained at the time. The patients were informed of
the objective of the study and gave their consent. The study was approved by the Institutional Review Board (Comisión Ética).

Reagents and antibodies

RPMI 1640 medium, L-glutamine and fetal calf serum (FCS) were obtained from Gibco BRL (Bethesda, MD, USA). Lymphocyte isolation medium was purchased from ICN (Costa Mesa, CA, USA). Mouse mAbs conjugated to fluorescein isothiocyanate (FITC)-anti-CD19, phycoerythrin (PE)-anti-CD3, -CD27, -CD38, -CD95, peridinin chlorophyll protein (PerCP)-anti-CD3, -CD38, allophycocyanin (APC)-anti-CD19 and FITC-PE-PerCP- and APC-conjugated mAb of appropriate isotype used as negative controls were provided by Becton Dickinson (San Jose, CA, USA). PE-anti-CD138 and purified anti-CD95 mAb (clone CH11, IgM) were obtained from Beckman Coulter (Fullerton, CA, USA). FITC-Annexin-V was purchased from Roche (Penzberg, Germany). Purified mouse IgM was obtained from Zymed Laboratories (San Francisco, CA, USA).

Cellular purification and identification of PC

Mononuclear cells (MC) from PB and SF were obtained by centrifugation on a Ficoll-Hypaque density gradient. Three and four-colour labelling experiments and flow cytometry analysis were performed as previously reported, and PC were identified as CD19 low CD38 high MC [8].

Cell culture

PB and SF MC cells (2 × 10⁶/ml) were cultured in a medium consisting of RPMI 1640 supplemented with 10% FCS, L-glutamine (2 mM) and gentamycin (0.05 mg/ml) for indicated times. For induction of CD95-mediated apoptosis, the anti-CD95 stimulating mAb CH11 (400 ng/ml) or mouse purified IgM (400 ng/ml) used as an isotypic control were added to the indicated cultures. Apoptotic T lymphocytes (CD3+ cells), B lymphocytes (CD19+ cells) and PC were detected by determining the percentage of FITC-Annexin-V+ cells after 24 h of culture [9].

ACPA IgG detection

ACPA IgG were determined in serum, SF and the supernatants (SN) of SF and PB MC cultures by using an ACPA IgG-specific ELISA (INOVA Diagnostic Inc., San Diego, CA, USA). Results were expressed as units/ml (serum and SF) and absorbance units (SN).

Statistical analysis

Differences were established by using the Mann–Whitney U-test and Wilcoxon rank test.

Results

Occurrence of ACPA IgG in RA patients

ACPA IgG was measured in serum and SF from RA patients. Fourteen patients showed detectable quantities in serum of these auto-Ab. In these cases, ACPA IgG levels were higher in SF than in serum samples (227.1 ± 17.4 vs 181.2 ± 15.5 U/ml, respectively; \( P < 0.05 \)). A positive correlation was found between the level of ACPA in serum and SF (Pearson’s \( R = 0.778, P < 0.001 \)).

The death receptor CD95 is expressed by PC of RA patients

PB and SF MC obtained from RA patients contained similarly low but detectable proportions of PC (0.08 ± 0.02% and 0.09 ± 0.0% for PB and SF, respectively; mean ± S.E.M.; \( n = 12 \)) that were determined as CD19 low CD38 high MC (Fig. 1). These percentages were similar to those detected for PB PC obtained from normal volunteers (0.08 ± 0.02%; mean ± S.E.M.; \( n = 14 \)). Interestingly, the majority of RA PC from both locations

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**Fig. 1.** Identification of PB and SF PC from RA patients, and expression of CD95 and other PC markers. RA patients PB (upper part) and SF (lower part) PC were identified in the CD19 vs CD38 dot plot of the corresponding MC population as CD19 low CD38 high cells (indicated circle). The compared expression of additional PC markers including CD95, CD27 and CD138 are depicted in the right part of the figure. A representative experiment is shown.
expressed similarly high levels of the CD95 death receptor (Fig. 1; 94.7 ± 0.5% and 96.0 ± 2.3% for RA PB and SF PC, respectively; mean ± s.e.m.; n = 8). A well defined PC marker such as CD27 high expression was also exhibited by RA PC (Fig. 1; 91.3 ± 3.2% and 86.0 ± 4.1% for RA PB and SF PC, respectively; n = 5). In contrast, CD138, a PC specific molecule whose expression enhances during the PC maturation process [10], was exhibited in higher proportion by RA SF PC (Fig. 1; 78.7 ± 8.2% and 42.3 ± 6.7% for RA SF and PB PC respectively; n = 5; P < 0.04).

RA PC from SF, but not from PB, produce ACPA IgG with prolonged kinetics

The presence of ACPA IgG-secreting PC was also explored in ACPA-seropositive RA patients. Figure 2A shows that the SF MC population contained PC capable of spontaneous production of ACPA IgG in culture. This production was dependent on active protein synthesis, as cycloheximide addition to the cultures markedly reduced ACPA IgG production (up to 29 ± 8% of the control production; mean ± s.e.m., n = 6; P < 0.01). However, PB samples obtained from the same RA patients contained very few, if any, spontaneous ACPA IgG-secreting cells (Fig. 2A). ACPA IgG production by SF PC linearly accumulated during at least 14 days (Fig. 2B). ACPA IgG production was very low or absent in either 7- or 14-days RA PB cell cultures (Fig. 2B). ACPA IgG secretion was not detected in SF MC cultures from the only ACPA-seronegative RA patient (data not shown).

**SF PC ACPA IgG production can be inhibited by CD95-ligation through the induction of PC apoptosis**

The effect of CD95 ligation on ACPA IgG secretion by cultured SF PC was also investigated. As can be seen in Fig. 2C, the inclusion of the agonistic anti-CD95 mAb CH11 markedly inhibited spontaneous ACPA IgG secretion (up to 38.3 ± 10.4% of control production; mean ± s.e.m.; n = 6; P < 0.002). A similar quantity of purified mouse IgM was used as an isotypic control for CH11 Ab, and did not inhibit ACPA IgG secretion (Fig. 2C, IgM control, grey colour). Total spontaneous IgG secretion was also reduced by CH11 treatment in these RA SF cell cultures (from 655.3 ± 71.3 ng/ml in control cultures to 417 ± 50.2 ng/ml in treated cultures; mean ± s.e.m.; n = 4). To determine whether this inhibitory phenomenon was caused by CD95-mediated apoptosis of PC, the proportion of annexin-V+ PC present in CH11-treated and in untreated cultures...
was compared. Figures 2D–F show that SF PC exhibited low levels of Annexin-V+ PC in control cultures after 24 h. CD95-ligation enhanced by more than six times the percentage of apoptotic PC (Fig. 2E; 4.0 ± 1.0 and 25.0 ± 9.4, for control and anti-CD95 Ab treated cultures, respectively; mean ± S.E.M., n = 5; P < 0.01). The limited number of SF PC did not allow us to test PC apoptosis by alternative methods. Nevertheless, it has been recently reported that annexin-V staining correlates very well with other techniques for detecting human apoptotic PC [11, 12]. CH11 treatment did not induce apoptosis (Annexin V+ cells) in other SF MC cells during this culture period (2 ± 1% and 2 ± 0.5% of apoptotic CD3+ T lymphocytes, and 3 ± 1% and 3 ± 2% of apoptotic CD19+ B lymphocyte, in the absence and in the presence of 24 h CH11-treatment, respectively; mean ± S.E.M., n = 5). Direct demonstration of CD95-induced apoptosis by ACPA IgG-secreting PC could not be explored because soluble labelled citrullinated proteins/peptides were not available.

**Discussion**

An increasing body of evidence reveals the complex biology of the human PC compartment, and this can be relevant for focusing our understanding of autoimmune pathogenic humoral responses. In this regard, upon antigen challenge, early PC are first generated in local lymphoid tissues, and most of these are short-lived (2-3 days) cells that die by apoptosis. Later, high-affinity specific Ab-secreting locally generated PC travel through the circulation toward appropriate deposit organs, such as the bone marrow (BM) for systemic humoral responses; here, they are recruited into particular niches providing signals that allow the PC to survive for much longer, and achieve maximum maturity and Ab production [10]. In this context, the occurrence in a humoral autoimmune response of either short-lived, or long-lived, or both types of auto-Ab secreting PC could have clinical and therapeutic consequences.

The present study demonstrates that ACPA IgG-secreting PC were detectable in the SF, but not in the blood, of all ACPA-seropositive RA patients. In addition, patients’ ACPA IgG levels were higher in SF than in serum, and a good and positive correlation was observed between the quantities of this auto-Ab detected in both locations. These findings support the view that RA inflamed synovium is a prominent site for the local generation, and accumulation of ACPA-secreting PC and for the production of these auto-Ab.

The transition from early short-lived to long-lived PC is associated with phenotypical changes that include increasing CD138 expression on more mature stages [8, 10]. The mechanisms that determine the rapid apoptosis of early PC are starting to be elucidated. In humans, this process is mediated, at least in part, through the CD95 pathway, since early PC present in tonsil and blood have been shown to express this death receptor, and its agonistic activation induces rapid PC apoptosis; in contrast, human long-lived PC accumulated in the BM exhibit low levels of this receptor, and its triggering does not lead to PC apoptosis [9]. A role for the interaction of CD95 with its natural ligand FAS-L (CD178) in the control of the PC compartment is also based on the observation that its failure leads to the development of hyper-gammaglobulinemia, autoimmunity and PC tumors in humans and murine systems [6, 13, 14]. Present data demonstrate that PC recruited in the RA SF exhibited peculiar properties. Specifically, they showed high CD138 expression, a feature of mature PC from deposit organs such as the BM and the mucosal lamina propria, but not in cultures of tonsil and blood early PC [15]. Therefore, SF ACPA IgG-secreting PC seem to exhibit features of terminally-differentiated PC (high CD138 expression and prolonged Ig-secreting capacity), together with high expression of CD95. This latter characteristic led us to test the possibility that RA SF PC were susceptible to being regulated through CD95 ligation, as happens in normal human early PC [9]. Present results demonstrated that CD95 cross-linking of SF PC markedly reduced their capacity of ACPA IgG secretion, and this phenomenon was associated to a rapid induction of PC apoptosis, as demonstrated by increased annexin-V+ PC in these cultures. The apparent discrepancy between the percentage of apoptotic PC (~20%; Fig. 2E–F) and the ACPA-IgG production after CH11 treatment (~40%; Fig. 2C) is probably due to the different time of performance for every technique: annexin-V binding of apoptotic PC was determined after 24 h of CH11-treatment and ACPA-IgG secretion was measured at the end of the whole culture period (14 days).

Taken together, these results strongly suggest that ACPA IgG-secreting PC present in RA synovial tissue are different from long-lived normal BM PC, since these auto-Ab secreting PC are still susceptible to elimination via CD95-dependent apoptosis.

It is well-established that RA inflamed synovium exhibits defective CD95-dependent control of immune and inflammatory cells, a phenomenon that is due to the lack of expression of FAS-L/CD178 [16], apparently caused by the inflammatory-induced FAS-L gene promoter repressor Foxo-3a, as has been recently demonstrated [17]. Interestingly, the generation of ACPA-secreting cells in mice requires defects in the B-cell apoptotic control, including the CD95 system [18]. In this context, from the present results, we can hypothesize that ACPA IgG-secreting PC accumulating in the RA synovial tissue, despite being susceptible to CD95-dependent apoptotic control, lack this regulatory mechanism, and thus could acquire abnormally prolonged survival capacity. In consequence, RA synovium might behave as a ‘deposit’ for ACPA IgG-secreting PC, a phenomenon that could explain the enhanced ACPA Ab response observed in RA SF.

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### References