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ROLE OF PROTEIN KINASE C ISOENZYMES IN SYSTEMIC LUPUS ERYTHEMATOSUS AND IN THE REGULATION OF CELLULAR PROCESSES OF MONOMAC-6 CELLS

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INTRODUCTION

Systemic lupus erythematosus

Systemic lupus erythematosus is a polisystemic autoimmune disease characterized by dysfunction of monocytes/macrophages, T-lymphocytes and antigen presenting cells (APC). The altered cellular and humoral immune responses lead to pathological autoantibody production, developing inflammation of organs, like joints, skin, kidney, central nervous system, etc. The disease occurs dominantly in females, and is characterized by alternating serious exacerbations and remissions.

Numerous laboratory abnormalities were justified in SLE; to the most frequent aberrations belong hypersedimentation, leucopenia, lymphopenia, thrombocytopenia, and anemia. The most recurrent immunserological alterations are positive antinuclear antibody, increased anti-dsDNA, anti-cardiolipin levels, lupus anticoagulant positivity, low complement protein concentrations, and total complement activity. The etiology and pathogenesis of the disease is mostly unknown. It is proposed that a variety of environmental, hormonal and other factors lead to the expression of the disease in a genetically predisposed host. Recently it was proved that the formation of autorective T-cells possess (one of) the most important initiating and preserver role in the pathomechanism of the disease, which leads to B-cells producing pathological autoantibodies.

T cell activation triggered via contact of the T-cell receptor with Ag/MHC is mediated by a series of biochemical events that transduce signals from the surface TCR to the nucleus, leading to activation of gene transcription for T cell proliferation and differentiation. TCR ligation by Ag/MHC results in the phosphorylation of TCR-associated CD3 subunits and activates the T cell-specific ζ-associated protein-70 (ZAP-70). ZAP-70 acts as a tyrosine kinase, which then phosphorylates the critical linker/adapter molecules linker for activation of T cells (LAT), which serve to form molecular complexes for

coupling proximal phosphorylation to distal events. These distal events include activation of mitogen-activated protein kinases (MAPKs), and calcium flux, which together culminate in nuclear gene transcription

Protein kinase C izoenzymes

Protein kinase C (PKC) comprises a family of serine threonine kinases that play key roles in the regulation of various cellular functions. Up to date, at least 11 different PKC isoenzymes have been identified, which can be classified into the groups of the calcium- and phorbol ester-dependent "conventional" cPKCs (PKCα, βI, βII, and γ), the calcium-independent "novel" nPKCs (PKCδ, ε, η, and θ), the calcium- and phorbol ester-independent "atypical" aPKCs (PKCζ and λ/ι) isoforms, and the unique PKCμ. These isoforms, possessing characteristic tissue and cellular distribution, isoform-specifically regulate various cellular functions such as proliferation, apoptosis, differentiation, cytokine production, mediator release, phagocytosis and receptor-mediated signal transduction. Recently more and more evidences argue for that PKC isoforms own not only structural and distribution heterogeneity, but their regulation and biological roles show remarkably difference too. It is also confirmed that the different isoforms could variously participate in the development of a cellular function (i.e. proliferation, differentiation) and, in addition, their effect can occur often antagonistic.

Role of PKC isoforms in lymphocytes of patients with SLE

Numerous studies have verified that the PKC isoforms possess central role in the regulation of lymphocytes cellular functions. Previously it was proved that in the T-cells of SLE patients, a decreased PKC activity exists. It was also shown, that T-cells from SLE patients possess reduced TCRζ chain expression, which results in a more potent FcRγ chain ligation to the receptor,

altering the distal signal transduction cascade and leading to augmented calcium response, abnormal cAMP regulation and increased intracellular phosphorilation. The suppressed TCRζ chain expression is possibly due to decreased transcription of its gene, which is regulated by transcription factor Elf-1 (E-74-like factor), a member of Ets (E-26-specific) transcription family. It was proved, that in SLE T-cells there exist a molecular defect of the active Elf-1, which is a consequence of abnormal post-translational modification of the molecule. Several hypotheses argue for that the defect of Elf-1 (and hereby the decreased TCRζ chain expression) is the result of reduced phosphorilation of the molecule, caused by the suppressed PKC activity of SLE T-cells.

The aforementioned signal transduction abnormalities of SLE T-cells lead to altered cytokine production, where the decreased interleukin-2 (IL-2) release is of great importance. It was also detected, that cPKCα and nPKCθ facilitate the IL-2 receptor expression of T-cells from healthy individuals, whereas cPKCβ and nPKCδ and ε increase IL-2 synthesis of the cells. Antigen-induced translocation of nPKCθ to membrane rafts is required for T cell activation, which reveals the central role of nPKCθ. In addition, the fact, that transfection of Jurkat T cells with nPKCθ significantly increased IL-2 production, strongly support this hypothesis.

Role of PKC isoenzymes in healthy and SLE monocytes

In the monocytes, the activation of the cells results in induction of numerous signal transduction pathways. However, there are only few reports in the literature, regarding the role of PKC isoenzyme family. It was proved, that PKC isoforms play central, but antagonistic role in the regulation of Fcγ receptor mediated intracellular killing mechanisms of human monocytes (cPKCβ stimulated, whereas cPKCα and nPKCε inhibited). It was also shown that the function of cPKCα and β, and nPKCδ and ε are essential for the proinflammatory cytokine production (TNFα, IL-1 β, IL-6) of monocytes.

Controversially, the mediator release of monocytes mediated by lipopolisacharides (LPS), namely, TNFα, IL-1 β, IL-6 production, goes through activation of aPKCζ.

It is important to observe, that phagocytosis and inflammatory mediator release of the cells depend to a great extent on phospholipase A_2 isoenzyme family, thus the arachidonic acid (AA) release. It is well known, that PLA_2 enzymes, participating in the AA synthesis –such as cytosolic phospholipase A_2 ($cPLA_2$), calcium independent phospholipase A_2 ($iPLA_2$) and diacylglycerol (DAG) lipase – may be potential downstream targets of different PKC isoforms. It was also verified, that in a human monocytoid cell line, in MonoMac-6 cells, PKC has fundamental role in IgG mediated phagocytosis, activating the monocytic signal transduction pathway: PKC→PLA_2→AA→phagocytosis. It was supported by the paper, where cPKCα was shown, using isoform-specific antisense oligonucleotids, to regulate the phosphorilation and enzymatic activity of $cPLA_2$ in human peripheral monocytes. Our laboratory previously demonstrated that AA production is markedly decreased in the monocytes of SLE patients compared to the healthy control. Moreover we confirmed that corticosteroid treatment elevate (normalize) the AA release of the cells.

MonoMac-6 cells

Cell lines developed from various transformed tissues provide valuable models for studying normal cellular processes in cell types that are difficult to obtain and/or isolate in large quantities. However, biochemical elucidation of the critical signaling molecules involved in this process has been limited by the difficulties associated with isolating large numbers of relatively pure monocytes. A number of human cell lines of the monocyte lineage have been described, the best characterized of which include U937 and THP-1 cells. These cells represent early stages in monocytic development and thus require differentiation with phorbol esters or interferon-γ to express such functional properties of mature

monocytes as IgG-mediated phagocytosis and antibody-dependent cellular cytotoxicity. Zeigler-Heitbrock established the MonoMac-6 cell line, which express many of the functional and phenotypic characteristics of mature human monocytes. Unlike U937 and THP-1, MonoMac-6 cells constitutively express the CD14 molecule, phagocytose IgG-opsonized particles, produce reactive oxygen metabolites, and express nonspecific esterase activity. Based on these criteria, MonoMac-6 cells are the most differentiated of the monocytic cell lines and, as such, may provide researchers with a human cell line in which to study monocytic cell functions.

There are only few data in the literature however, about the roles of PKC isoenzymes in the MonoMac-6 cells. In contrast, numerous cell lines in the early stage of myelo-monocytoid differentiation have been widely investigated. As we previously mentioned, PKC isoenzyme family has fundamental function in regulation of phagocytosis and AA production of the MonoMac-6 cells. Controversially, data are missing about isoform-specific characterization of PKC; moreover, there is no information about the role of the PKC isoenzymes in the regulation of proliferation of MonoMac-6 cell line.

AIMS

We wanted to carry out the following objectives during our experiments:

1. We wanted to determine the PKC expression profile of healthy human lymphocytes and monocytes.
2. We aimed to compare these results with PKC profiles of cell populations from patients with different autoimmune diseases.
3. Thereafter we investigated the effect of steroid therapy (frequently administered in treatment of patients with immune diseases), then we determined the role of steroid therapy in the AA release of cells from healthy individuals and SLE patients.

4. In the second part of our experiments we studied the expression profile of PKC in MonoMac-6 cells both at mRNA and protein level.
5. We analyzed the effects of different PKC activators and selective inhibitors, as well as of molecular biological techniques (transient overexpression and RNA interference) on the proliferation and AA release of MonoMac-6 cells.
6. Finally, we aimed to study the connection of PKC isoforms and enzymes, participating in the synthesis of AA in MonoMac-6 cells.

PATIENTS AND METHODS

Patients

The SLE study population consisted of 22 patients, 20 women and 2 men, average of age 36 years, range 21–58 years. They all fulfilled at least four of the American College of Rheumatology classification criteria. Eleven patients were free of any glucocorticosteroid, whereas 11 patients received 2–40 mg/day corticosteroid treatment. One woman with freshly diagnosed active SLE was tested before the treatment with pulse dose of glucocorticosteroid (4 × 500 mg methylprednisolone/day, 1 × 250 mg i.v., then 64 mg/day for a month) and also 8 days later. All the nine patients with primary Sjögren's syndrome (SS) were women; they all were treated according to the scheme of 8 mg methylprednisolone/48 h. All the six patients with mixed connective tissue disease (MCTD) were women; two of them were without any steroid and cytostatic treatments, two took 4 mg/day of steroid and two were treated by 8 mg/48 h of methylprednisolone + 100 mg of Cytoxan/day. As controls, the peripheral blood samples of 21 healthy Caucasian subjects (12 women, 9 men) were studied. Approval was given through the Institutional Review Board, and informed consent was obtained from all patients.

Preparation of purified T-cell and monocyte populations

A Dynal magnetic-bead cell separation technique was used to obtain purified cell suspensions. The average monocyte purity was >85% whereas the average of T-cell purity was >95%, as was assessed by flow cytometry analysis of specific surface markers.

Cell culturing

Peripheral monocytes and MonoMac-6 cells were cultured under lipopolysaccharide-free conditions in RPMI-1640 media containing 10% fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM nonessential amino acids, 200 U/ml penicillin and 200 µg/ml streptomycin.

Transient transfection of PKC isoforms

Cells were transfected using the Amaxa nucleofection technology. Cells were resuspended in solution from nucleofector kit V, also available as part of the Amaxa cell optimization kit, following the Amaxa guidelines for cell line transfection. Briefly, 100 µl of $3.5x10^6$ cell suspension mixed with 4 µg eGFP conjugated cDNA vectors (encoding the sequence of various PKC isoforms as well as the empty vector) was transferred to the provided cuvette and nucleofected with an Amaxa Nucleofector apparatus. Cells were transfected using the U-01 pulse protocol and were immediately transferred into 12-well plates containing 37 °C pre-warmed culture medium. After transfection, cells were cultured from 2 to 48 hrs and then the efficacy of overexpression was analyzed by fluorescent microscopy, flow cytometry, and Western blotting.

Flow cytometry

To assess the separation and transfection efficiency of eGFP-conjugated PKC constructs and also of possible changes of cell viability, MonoMac-6 cells (4, 24 and 48 hrs after transfection) and the purified cell populations were subjected to flow cytometry analysis (Coulter Epics XL). The separation efficacy was determined based on fluorescent intensity of the cells after appropriate staining (monoclonal antiCD3 and antiCD14 antibody) and fixation of the cells (1% paraformaldehide). The transfection efficacy of MonoMac-6 cells was measured by determining fluorescence intensity values, whereas living and dead cells were identified by forward and side scatter parameters.

RNA-interference (siRNA)

Cells were seeded in 6-well culture plates in RPMI media containing serum, but lacking antibiotics. At 40-50 % confluence, cells were transfected with siRNA probes against PKCs or with fluorescein-labeled control siRNA previously mixed (and incubated at room temperature for 25 min) with a transfection medium also containing the transfection reagent following the manufacturer's protocol. The efficacy of siRNA-driven "knock-down" of the PKCs was daily evaluated by Western blotting for 4 days.

Western blot analysis

Cells were washed with ice-cold phosphate-buffered saline (PBS), harvested in homogenization buffer and disrupted by sonication on ice. Total cell lysates were mixed with SDS PAGE sample buffer and boiled for 10 min at 100°C. The samples were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (8% gels were loaded with 20 30 mg protein per lane) and transferred to nitrocellulose membranes. Membranes were then blocked with 5% dry milk in PBS and probed with the appropriate primary antibodies against the given PKC isoform. Peroxidase-conjugated goat anti-

rabbit IgG antibodies were used as secondary antibodies, and the immunoreactive bands were visualized by an ECL Western blotting detection kit. Immunoblots were subjected to densitometric analysis using an Intelligent Dark Box and appropriate software

Quantitative „real-time" PCR

Q-PCR was carried out on an ABI PRISM 7000 Sequence Detection System by using the 5' nuclease assay. Total RNA was isolated using TRIzol. One μg of total RNA were then reverse transcribed into cDNA by using AMV reverse transcriptase and random primers. PCR amplification was carried out by using the TaqMan primers and probes using the TaqMan Universal PCR Master Mix Protocol. As internal controls, transcripts of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were measured; thereafter to determine the gene expression, results were presented using the ΔCT, or ΔΔCT method.

Determination of cellular proliferation

The proliferation of the cells was determined by measuring the conversion of the tetrazolium salt MTT to formazan. Cells were plated in 96-well multititer plates in quadruplicates and were treated with different concentrations of the reagents for the time indicated. Cells were then incubated with 0.5 mg/ml MTT for 3 h, and the concentration of formazan crystal (as the indicator of number of viable cells) was determined colorimetrically according to the manufacturer's protocol. Data are expressed as mean ± SEM.

Arachidonic acid release

Cells at 10^5 cells/ml densities were preincubated with [3H]AA in CO2 incubator at 37 °C for 20 hr. After extensive washing, the cells were further incubated with either culturing medium (basal production of AA) or with various agents investigated for 4 hr and the released [3H]AA was determined by

scintillation counting. Each value was calculated as the average of triplicates of cultured cells and at least four individual experiments were performed.

RESULTS

Alterations in the expressions of PKC isoforms in T-cells of SLE patients without and with steroid treatment

The possible alterations in the PKC isoform pattern in the different T-cell populations using Western blot analysis were determined. We could detect seven PKC isoforms (cPKCα, β, nPKCδ, ε, η and θ and aPKCζ) in T-cell suspensions of both healthy controls and SLE patients (cPKCγ, aPKCλ/ι and PKCμ was not detected in the T-cells). Then we tried to select various groups among the SLE patients to be able to define whether the status of the disease (active, inactive) or the applied therapy (e.g. corticosteroid) affected the PKC isoform levels.

The PKC isoform expression pattern in the T-cells of SLE patients with no steroid therapy differed remarkably from that of the healthy controls. Whereas the levels of cPKCα showed no measurable changes, there were marked and significant (but variable) decreases in the expressions of PKCβ, δ, ε and θ, and a moderate, yet significant, suppression in the level of PKCζ. Comparison of these data to those obtained with T-cells of SLE patients receiving steroid treatment revealed that the expressions of cPKCβ, nPKCδ, ε and η but not of nPKCθ and aPKCζ were partially or almost completely normalized in this group. These PKC isoenzyme alterations seemed to be characteristic of SLE because we could not detect any significant changes in the expressions of the PKC isoforms in T-cells of SS and MCTD patients.

Alterations in the expressions of PKC isoforms in monocytes of SLE patients without and with steroid treatment

We measured the PKC isoform pattern in monocyte populations of patients with SLE using also Western blotting. As a striking difference compared to the T-cell data, we found that the expressions of several existing isoforms (cPKCα, β and nPKCη) were similar in the monocytes of steroid-free SLE patients to the healthy controls. In contrast, the levels of nPKCε and δ markedly whereas the expression of aPKCζ moderately, yet significantly, decreased in the diseased monocytes (nPKCθ and cPKCγ were not present in these cells). Similarly to the T-cell results, in the monocytes of SLE patients who received corticosteroid therapy, the expressions of some isoforms returned differentially to the levels of controls. Namely, whereas the expressions of PKCδ and ε markedly and significantly increased (compared to the steroid-free group), the level of PKCζ remained lower than seen in monocytes of healthy volunteers. These alterations also were characteristic of SLE because we could not measure any significant change in the PKC pattern of monocytes of patients with SS.

Effect of in vitro steroid treatment on PKC isoform levels

To support the clinical observations regarding steroid treatment and the PKC levels, isolated monocytes from healthy volunteers were *in vitro* treated for 2 days with 10 μM hydrocortisone, total RNA was isolated, and the levels of mRNA transcripts of PKC isoforms were determined by quantitative real-time Q-PCR. We found, that steroid application selectively and significantly up-regulated the level of nPKCε (when compared to the non-treated cells) whereas the expressions of the other isoforms were not affected. In addition, we were able to perform a similar *in vitro* study on peripheral isolated monocytes of a freshly diagnosed SLE patient (free of any treatment). We could detect, that *in vitro* corticosteroid application, identically to the above Western blot data

markedly increased the transcript levels of nPKCδ and ε (but not of the other isoforms) compared to the expressions seen in the non-treated monocytes. These data indicate that, similarly to the *in vivo* effects, *in vitro* corticosteroid application also increases the expressions of certain PKC isoforms.

Our laboratory previously showed that AA production is decreased in peripheral monocytes of SLE patients compared to healthy control. In contrast, this phenomenon was not detected in patients receiving corticosteroid therapy. Therefore we measured the AA release of monocytes from healthy individuals after the *in vitro* corticosteroid treatment. Surprisingly, we found, that hydrocortisone treatment do not significantly alter (despite overexpression of nPKCε) AA production of the cells.

PKC system of MonoMac-6 cell line

SLE associates very often with serious mono- and lymphopenia, in addition, the treatment of the disease may alter the expression of the examined PKC system. Therefore in the second part of our study we used the MonoMac-6 cell line, as a monocyte model to determine the exact roles of PKC isoforms in regulation of cellular proliferation and AA release. Using Western blot technique, we showed that MonoMac-6 cells possess functionally active PKC system. Namely, they express the cPKCα and β, nPKCδ and ε and aPKCζ; interestingly, we were unable to detect any expression of nPKCη – an isoform which does exist in human peripheral monocytes – and other PKC isoforms (cPKCγ, nPKCθ, aPKCλ/τ, or PKCμ). These results were confirmed by Q-PCR, where we were also able to show that the relative expression levels of various PKC isoforms in MonoMac-6 cells are different. Namely, certain PKC isoforms possessed relatively high (nPKCδ>>>>cPKCβ>aPKCζ) whereas others low (cPKCα>nPKCε) expression levels normalized to the endogenous control GAPDH.

Role of PMA in regulation of proliferation and AA production of MonoMac-6 cells

We then intended to determine the potential roles of the PKC system in the regulation of cellular functions of MonoMac-6 cells. We found, that application of the general PKC activator PMA (for two days) resulted in a significant and dose-dependent decrease in the proliferation of cells. In parallel, as was assessed by Western blotting, expressions of cPKCβ and nPKCδ were suppressed (possibly due to down-regulation of the isoforms) by 100 nM PMA whereas the cellular levels of cPKCα and aPKCζ were not changed. Interestingly, the expression of nPKCε increased upon the phorbol ester treatment. These finding argued for that the affected (i.e. down- and up-regulated PKCs) might mediate the growth-inhibitory effect of PMA.

Moreover, we also found that the administration of PMA (in the presence of calcium) induced a remarkable increase in the AA release, which also suggested the involvement of the PKC pathway. To further determine the participation of the existing PKCs in the effect of PMA, certain PKC inhibitors were investigated. We could observe that the cPKC inhibitor (in our case, the inhibitor of the cPKCα and β) Gö6976, as well as Rottlerin (a selective inhibitor of the nPKCδ) significantly prevented the effect of the phorbol-ester to induce AA-release. To further dissect the mechanism, we then modified the endogenous activity of the existing isoenzymes and measured the effect of the inhibitors on the "basal" proliferation and AA release of the cells. Incubation of MonoMac-6 cells with a cPKC inhibitor Gö6976 remarkably impeded cellular division in a dose-dependent fashion. Similarly, the inhibition of the nPKCδ by Rottlerin also dose-dependently suppressed cellular proliferation. It was important to observe, however, that neither Rottlerin nor Gö6976 was able to affect the basal AA release when applied alone.

Overexpression of cPKCβ and nPKCδ increased basal whereas transfection of cPKCα inhibited PMA induced AA release

In the next section of our experiments we investigated the roles of the isoenzymes using various molecular biological techniques. Firstly, we transiently overexpressed the isoforms in a GFP-tagged fusion protein format and then we measured the effect of PKC isoform overexpression on the AA release of the cells. First, we analyzed the effect of the transfection itself on potential cell death and AA leakage of the cells. We found that transient transfection of the empty eGFP vector resulted in similar basal and PMA-induced AA release to those of the control (i.e. non-transfected) cells suggesting that the transfection procedure did not alter the responsiveness of the cells.

We could detect also that the overexpression of cPKCα, nPKCε or aPKCζ did not affect the basal AA release. In contrast to these findings, the overexpression of cPKCβ or nPKCδ significantly elevated the basal AA release (Fig. 4c and d). However, it was intriguing to observe that the overexpression of cPKCα (unlike any of the other four isoforms) significantly affected the PMA-induced AA release; namely, it inhibited the effect of the phorbol-ester.

cPKCβ and nPKCδ exert it's action on AA release via activating calcium independent PLA_2 and DAG lipase pathway

To further evaluate the putative molecular mechanism coupled to the cPKCβ and nPKCδ mediated signaling, we aimed to determine the effects of the inhibitors of the PLA_2 enzymes on the basal AA release of control (eGFP transfected) MonoMac-6 cells, and on the elevated basal AA production of the cPKCβ and nPKCδ transfectants. In control cells, application of the $cPLA_2$ inhibitor AACOCF3 did not modify the basal AA release. In contrast, incubation the cells with the $iPLA_2$ inhibitor PACOCF3 or the DAG lipase inhibitor RHC-80267 significantly decreased the basal AA production of the cells. Importantly, similar phenomena were observed in the cases of the two

overexpressers. Namely, the $iPLA_2$ and the DAG lipase inhibitors significantly inhibited the elevated basal AA release of the cPKCβ and nPKCδ transfectants whereas the $cPLA_2$ inhibitor was ineffective.

cPKCβ and nPKCδ positively whereas cPKCα negatively regulate proliferation of MonoMac-6 cells

Unfortunately, the transient overexpression method did not permit the parallel investigation of the alteration in the proliferation rate of the cells. Therefore, we employed another molecular biological approach (namely the RNA-interference technique) to reveal the specific, endogenous roles of the above three isoforms (i.e., cPKCα and β, and nPKCδ) in the regulation of the proliferation of the cells. Western blot analysis revealed that the expressions of all three PKC isoforms investigated were significantly "knocked-down" by distinct siRNA probes at day 2. Importantly, in analogy to data obtained with the various PKC inhibitors, the "knock-down" of nPKCδ and cPKCβ partially (yet significantly at day 2) suppressed the proliferation rate of the cells. It was also intriguing to observe that, at day 2, the sum of growth inhibition of nPKCδ and cPKCβ "knock-downs" exactly matched the effect of 10-1000 nM PMA (added alone for 2 days) to inhibit proliferation. Conversely, the siRNA-induced down-regulation of cPKCα slightly elevated the growth rate of the cells. Taken together, these results strongly argue for that cPKCβ and nPKCδ promote whereas cPKCα rather inhibits proliferation of MonoMac-6 cells.

Corticosteroid treatment results in up-regulation of nPKCε in MonoMac-6 cells

Then we investigated the effect of *in vitro* steroid application on PKC expression profile and AA release of MonoMac-6 cells. We could observe that administration of 10 μM hydrocortisone for two days (similarly to those found in monocytes of healthy volunteers) selectively up-regulated the level of nPKCε

both at mRNA and protein level. In addition, it is important to emphasize that AA release of the cells (following hydrocortisone treatment) did not changed at any aspect, which strongly suggest the hypothesis that nPKCε do not play any role in the regulation of AA production of monocytoid cells.

DISCUSSION

Expressions of numerous PKC isoenzymes were decreased in T-cells from SLE patients compared to healthy control

In the first part of our experiments we made a comparison between the PKC expression profile of T-lymphocytes from SLE patients with the PKC pattern of T-cells from patients with other immunological disorder or from healthy controls. We established that in the background of suppressed PKC activity, cell- and isoform-specific PKC alterations exist, which are influenced by numerous factors, including corticosteroid treatment. We found that in lymphocytes of SLE patients receiving no steroid therapy, most of the PKC isoform expressions were decreased. Controversially in the patients receiving daily corticosteroid application, the levels of most – but notably not all – of PKC isoenzymes were improved. It is important to emphasize that these alterations were only seen in T-cells from patients with SLE.

To conclude our findings these data from the lymphocytes propose the following considerations: (1) knowing the central role of PKC system in the signal transduction processes, our current finding may (at least partly) explain the previously documented complex abnormalities of T-cells in SLE; (2) since these alterations were only seen in SLE, determination of PKC isoform pattern of autoimmune diseases (beside numerous immunological and biochemical parameters) could provide further diagnostic approach and could bring to light the pathomechanism of the diseases; (3) finally the effect of steroid treatment

reveals that altered expression of some of the PKC isoenzymes in SLE cells is a transient state, and it can be reversed and repaired by corticosteroids.

The decreased expression of TCRζ-chain possess fundamental role in signal transduction abnormalities seen in SLE T-cells, since TCR activation (therefore the signal transduction cascade) leads to development of autoreactive T-cells and to production of pathological autoantibodies. It is important to call attention to that the defect of Elf-1 transcription factor (i.e. alteration in the phosphorilation status of the molecule) may be potential cause of the suppressed expression of TCRζ-chain. It is also well-known that nPKCθ facilitates the posphorilation of Elf-1 and thereby the translocation of the molecule to the nucleus and the connection to TCRζ-chain promoter. Our result, whereas expression of nPKCθ is markedly decreased in SLE T-cells, which can not be improved by corticosteroid treatment, argue for central role of nPKCθ in the decreased level of TCRζ-chain and, furthermore, in altered signal transduction and pathological autoantibody production. Simultaneously, these data point out to limited efficacy of most frequently used corticosteroid therapy of the disease.

Expressions of nPKCδ and ε were suppressed in monocytes of SLE patients, which can be normalized by steroid treatment

Similarly to T-cells, we also investigated the expression profile of PKC isoforms in monocytes of SLE patients. We firstly demonstrated that in SLE isoform specific PKC defects, depending on corticosteroid treatment, exist, which may also contribute to the impaired (and chiefly PKC-dependent) monocyte functions seen in SLE. In our current study we showed using Western blot technique that in monocytes of SLE patients treated *in vivo* with corticosteroids – in contrast to untreated cases – the expressions of nPKCδ and ε were normalized. Similar data were obtained in the experiments with mRNA, where we found that after *in vitro* steroid treatment the levels of nPKCδ and ε were exclusively “up-regulated”, which isoforms were previously dramatically

suppressed, whereas the expressions of other isoenzymes remained unchanged. In addition, it was interesting to observe that in monocytes of healthy individuals *in vitro* steroid treatment produced different effect, namely, the administration of hydrocortisone elevated solely the mRNA level of nPKCε. These data argue for different steroid effects in monocytes of healthy controls and SLE patients.

nPKCδ and cPKCβ promote AA production and proliferation of MonoMac-6 cells

In the second part of our study we investigated the PKC system of a human monocytoid cell line, MonoMac-6, in regulation of numerous cellular processes. Using combined pharmacological and molecular biological techniques we found that these cells possess functionally active PKC system, namely, they express cPKCα and β, nPKCδ and ε and aPKCζ. This is the first demonstration that the nPKCδ (which is, by far, the dominant PKC isoform in MonoMac-6 cells) acts as a positive regulator of both the proliferation and the AA release in human monocytoid cells. This argument is supported by numerous lines of evidence: (1) the isoform was remarkably down-regulated (hence possessed lower expression levels) upon PMA treatment in parallel with the growth inhibitory action of the phorbol ester; (2) pharmacological inhibition of endogenous activity of nPKCδ suppressed proliferation; (3) the selective inhibitor of the isozyme inhibited PMA-induced AA release; (4) siRNA-driven "knock-down" of the isoform significantly inhibited the proliferation of the cells. It should be noted, however, that the nPKCδ inhibitor did not modify the basal AA production suggesting that, in contrast to its role in the regulation of proliferation, the endogenous basal (yet fairly high) expression and activity of the isoform most probably does not participate in the processes of basal AA production. On the contrary, it appears that when nPKCδ was recombinantly overexpressed (hence possessed an even more increased activity), it significantly

elevated the basal AA production of the cells suggesting that, upon exogenous activation, the isoform may mediate the action of agents to promote AA production.

Strikingly similar results were obtained when investigating the function of another highly expressed isozyme, cPKCβ (a previously more extensively studied isoform) in MonoMac-6 cell functions. Namely, the isoenzyme was also down-regulated during PMA-induced growth-inhibition whereas inhibition of its endogenous activity or suppression of its expression level by siRNA significantly inhibited proliferation of the cells. These data are in good accord with numerous previous studies in the literature. With respect to AA production, the overexpression of cPKCβ also resulted in a significant elevation of the basal AA release of the cells whereas inhibition of cPKCβ by Gö6976 significantly prevented the action of PMA to stimulate AA production. In addition, also similarly to the function of nPKCδ, suppression of the endogenous cPKCβ activity did not modify the basal AA production.

Finally, it was also important to observe that increased AA release in cells overexpressing cPKCβ and nPKCδ (similarly to control cells) was fully abrogated by the inhibitor of the $iPLA_2$ enzyme which findings identify this "downstream" molecule as a potential target of these PKC isozymes. In summary, these results strongly argue for the positive role of cPKCβ and nPKCδ in the regulation of proliferation and AA release of the monocytoid MonoMac-6 cells.

cPKCα slightly inhibits whereas nPKCε and aPKCζ do not modify AA release and proliferation of MonoMac-6 cells

Experimental data with cPKCα, however, suggested that this isoform (which possessed a very low expression level in the cells) plays a minor yet (most probably) opposite role in the regulation the MonoMac-6 cell processes

when compared to the actions of cPKCβ and nPKCδ. The only significant finding with this isoform was that its overexpression efficiently prevented the effect of PMA to stimulate the AA production of the cells. Interestingly, even when overexpressed, it did not affect at all the basal AA release suggesting the lack of involvement of this isoforms in the regulation of the process. Moreover, we observed only a slightly increased growth rate of those cells in which cPKCα was "knock-down" using siRNA which may suggest the negative role of this isoform in regulating proliferation of the cells. Finally, the very low endogenous expression level and hence the relatively minor (negative) role of cPKCα in the regulation of proliferation of MonoMac-6 cells may also explain the "unexpected" growth-inhibitory effect of cPKC inhibitor Gö6976 which most probably exerted its inhibitory action on the growth-promoting cPKCβ possessing >7-fold higher expression level than cPKCα.

In our hands, the nPKCε and aPKCζ did not seem to participate in the above cellular processes of the cells. Firstly, transient overexpression of these isozymes did not affect the basal or PMA induced AA release of the cells. Moreover, as expected, PMA treatment did not influence the protein expression of aPKCζ. Interestingly, the level of nPKCε seemed to double after PMA administration; however, since the endogenous level of this isoform was by far the lowest (e.g., less than 1/20 of cPKCβ as revealed by Q-PCR, we think that this alteration in the protein expression has negligible role, especially when compared to the effect of PMA to down-regulate cPKCβ or nPKCδ.

Clinical implications and potential utilizations of our results

Finally, our results may even have clinical implications. As we have previously shown, the diseased monocytes from freshly-diagnosed SLE patients possessed impaired AA production as well as low nPKCδ and ε levels. Of great importance, we have moreover documented that the clinically effective corticosteroid treatment of the patients *in vivo* or the isolated monocytes *in vitro*

"normalized" both the AA release and the expressions of the two isoforms (at both protein and mRNA levels) suggesting that the pathological levels of nPKCδ and/or ε might be responsible for the decreased AA production in SLE monocytes. In addition, our previous results also suggested that the "normalization" of the AA production in the monocytes by corticosteroid treatment of SLE patients was due to the "re-activation" of the $iPLA_2$-dependent pathway. Therefore, our current presentation that, (1) in the monocytoid MonoMac-6 cell line and in healthy human controls *in vitro* steroid treatment up-regulated selectively the level of nPKCε, while the AA production of the cells remained unchanged; (2) in MonoMac-6 cells the overexpression of nPKCδ markedly increased AA production whereas that of nPKCε did not modify the process; and (3) the effect of nPKCδ overexpression to elevate AA release could be completely reversed by the inhibition of the $iPLA_2$ enzyme; invite an attractive hypothesis that the selective activation of nPKCδ may be a fine tool in the therapeutic management of SLE-related alterations of monocyte functions.

It is seems more difficult to determine the exact role of the isoforms (and accordingly, to correct the defects) in the lymphocytes. Our current results argue for that the decreased PKC activity found in lymphocytes is the cause of suppressions of levels of (almost) all isoforms; therefore direct activation of every individual isoenzyme seems fairly complicated. Nevertheless, our data suggest that, following detailed investigation of specific roles of nPKCθ (which's expression is independent on steroid treatment), selective activation of this isoform may have good opportunity to correct the lymphocyte abnormalities seen in SLE.

It is important to emphasize that these alterations have, for the moment, only theoretical aspects. Since PKC isoenzymes possess characteristic tissue and cellular specific distribution and function, it has essential importance that activation or suppression of the distinct isoform occurs exclusively in the target

cell. Activation of an isoform (possessing for example proliferation promoting role in other cell type) would produce unwanted effects and could lead to development of malignant tumors. Furthermore it could give rise to difficulties that – following the example of nPKCδ - one isoform may have different functions in the same cell. Thus activation of the isoenzyme results not only in the elevation of AA release, but in cellular proliferation too. However, the determination of the beneficial (counterbalancing monocytopenia) or detrimental (promoting leukemia) effects of these modifications requires further intensive investigation.

CONCLUSIONS

We have studied the expressions of various protein kinase C (PKC) isoenzymes in T-cells and monocytes from patients with systemic lupus erythematosus (SLE) and in MonoMac-6 cells. We found that the levels of cPKCβ, nPKCδ, η, ε, θ and aPKCζ in T-cells, whereas the expressions of nPKCδ, ε and aPKCζ (but not the expressions of other PKC isoforms) in monocytes of SLE patients were significantly decreased. *In vivo* corticosteroid application, as well as *in vitro* steroid treatment of monocytes, elevated the expressions of most isoforms close to normal values; however, the decreased levels of nPKCθ and aPKCζ were not affected by steroid application. These alterations were characteristic to SLE because we could not detect any changes in the PKC levels in mononuclear cells of primary Sjögren's syndrome and mixed connective tissue disease patients. Experiments with MonoMac-6 cells revealed that the two dominantly expressed isoenzymes, i.e. cPKCβ and nPKCδ promote AA production and cellular proliferation. In addition, we were able to show that the calcium-independent $iPLA_2$ as well as diacylglycerol lipase (but not the cytosolic $cPLA_2$) function as “down-stream” targets of cPKCβ and nPKCδ. We have also found that, among the other existing PKC isoforms, cPKCα plays a minor inhibitory role whereas nPKCε and aPKCζ apparently do not regulate these cellular processes. In conclusion in this thesis we provide the first evidence that (corticosteroid dependent) impaired PKC isoenzyme pattern exist in the T-cells and monocytes of SLE patients and furthermore, PKC isoforms play pivotal, specific, and (at least partly) antagonistic roles in the regulation of AA production and cellular proliferation of human monocytoid MonoMac-6 cells.

PUBLICATIONS

List of publications the thesis based on:

1. ***Griger Z.****, Páyer E., Kovács I., Tóth I.B., Kovács L., Sipka S., Bíró T.* (2007): Protein kinase C-β and δ isoenzymes promote arachidonic acid production and proliferation of MonoMac-6 cells. J Mol Med. 85(9):1031-42. **IF: 5.157**
2. *Biro T,* ***Griger Z****, Kiss E, Papp H, Aleksza M, Kovacs I, Zeher M, Bodolay E, Csepany T, Szucs K, Gergely P, Kovacs L, Szegedi G, Sipka S.* (2004): Abnormal cell-specific expressions of certain protein kinase C isoenzymes in peripheral mononuclear cells of patients with systemic lupus erythematosus: effect of corticosteroid application. Scand J Immunol. 60(4):421-8. **IF: 1.912**

Abstracts:

1. ***Griger Z****, Biro T, Papp H, Aleksza M, Kiss E, Zeher M, Bodolay E, Szegedi G, Sipka S* (2004): Effect of corticosteroid treatment on the protein kinase C system in monocytes of patients with systemic lupus erythematosus and in modell cell lines Tissue Antigens 64 (4): 408-409.
2. *Bodo E, Biro T, Telek A, Czifra G,* ***Griger Z****, Toth IB, Lazar J, Meschalchin A, Ito T, Bettermann A, Pertile P, Kovacs L, Paus R* (2004): A 'hot' new twist to hair biology - involvement of vanilloid receptor-1 signaling in human hair growth control. Exp Dermatol 13 (9): 581-581

Other publications:

1. *Bodo E, Biro T, Telek A, Czifra G,* ***Griger Z****, Toth BI, Mescalchin A, Ito T, Bettermann A, Kovacs L, Paus R.*(2005): A hot new twist to hair biology: involvement of vanilloid receptor-1 (VR1/TRPV1) signaling in human hair growth control. Am J Pathol. 166(4):985-98. **IF: 5.796**
2. *Szántó A, Weisz R, Krenács L, Csiki Z,* ***Griger Z****, Zeher M.* (2006): Korai fázisban felismert Takayasu-arteritis. LAM 16(8-9):762-7.

Posters, lectures:

1. ***Z. Griger****, T. Biró, E. Kiss, S. Baráth, M. Zeher, G. Szegedi, S. Sipka* (2006): Corticosteroid dependent PKC abnormalities bot hat mRNA and€ protein leveli n the mononuclear cells of patients with SLE. International€ Congress of Immunogenomics and Immunomics 2006. oct. 8-12, Budapest, (P 2-24, abstract book p. 360.) poster.
2. ***Griger Z****, Bíró T, Kiss E, Baráth S, Zeher M, Szegedi G, Sipka S.* (2006): Corticosteroid dependent PKC isoform abnormalities both at mRNA and

protein level in the mononuclear cells of patient with SLE. 1st Joint Meeting of European National Societies of Immunology, 16th European Congress of Immunology, 2006. sept. 6-9, Paris, (PC-1712, abstract book p. 162.) poster.

3. *Sipka S,* ***Griger Z****, Bíró T, Aleksza M, Kiss E, Kovács I, Baráth S, Bodolay E, Zeher M, Szegedi G.* (2006): The central role of PKC delta in the impaired production of arachidonic acid in the monocytes of SLE patients. 1st Joint Meeting of European National Societies of Immunology, 16th European Congress of Immunology, 2006. sept. 6-9, Paris, (PC-1713, abstract book, p. 162.) poster.
4. ***Zoltán Griger****, Sándor Sipka, Tamás Biró, Magdolna Aleksza, Emese Kiss, Edit Bodolay, Margit Zeher and Gyula Szegedi* (2005): The role of PKC delta in the impaired production of arachidonic acid in the monocytes of SLE patients. MIT, 2005. okt. 19-22, Sopron, lecture.
5. *Sándor Sipka,* ***Zoltán Griger****, Tamás Biró, Helga Papp, Magdolna Aleksza, Emese Kiss, Margit Zeher, Edit Bodolay, and Gyula Szegedi* (2004): The examination of the protein kinase C system in the mononuclear cells of patients with systemic lupus erythematosus and in a model cell line. International Congress of Immunogenomics and Immunomics 2004 oct. 3-7., Budapest, P 12-14, poster.
6. ***Griger Z****, Biro T, Papp H, Aleksza M, Kiss E, Zeher M, Bodolay E, Szegedi G, Sipka S* (2004): Effect of corticosteroid treatment on the protein kinase C system in monocytes of patients with systemic lupus erythematosus and in modell cell lines. 12th International Congeress of Immunology and 4th Annual Conference of FOCIS, Montreal, Canada, 2004. jul. 18-23. (lecture and poster).