hyporesponsiveness upon engagement of the TCR/CD3 complex despite phenotypic evidence of former activation. We have previously shown that the central and crucial adaptor protein LAT (linker for activation of T cells), which plays a central and crucial role in the T cell receptor (TCR)-mediated signalling pathways, exhibits deficient phosphorylation due to displacement of the integral membrane protein from the plasma membrane in SF T lymphocytes. SF T lymphocytes exhibit several features of chronic oxidative stress, e.g. severely decreased intracellular levels of glutathione (GSH), and our previous studies have indicated that the subcellular localization of LAT is sensitive to changes in the intracellular GSH levels. The cysteine-to-serine substitutions of several cysteine residues (C26/29 or C117) within LAT creates LAT mutants that are resistant to reduced intracellular GSH levels and remain membrane-anchored in GSH-depleted cells.

Objectives

Methods

Results In this study, we have used the redox-insensitive LAT mutants to study the effect of redox balance alterations, like in SF T lymphocytes, on TCR signalling pathways downstream from LAT and on CD28 signalling pathways. In co-transfection experiments, we show that the presence of the redox-insensitive LAT mutants allows for the partial restoration of the TCR-mediated signalling pathways, but not the signalling pathways induced through the CD28 receptor. The data are indicative that the Raf1-ERK and the calcium-calcineurin pathways leading to transcriptional activation of AP-1 and NFAT, respectively, are very sensitive to reduced intracellular GSH levels, while the activation of the p38/Mpk2 pathway leading to AP-1-mediated transcription is mostly unaffected by chronic oxidative stress. A very proximal event in the CD28-mediated signalling pathways seems to be extremely sensitive to GSH depletion since costimulation did not affect the transcriptional activity of either AP-1 or NF- κ B.

Conclusion We conclude that the signalling pathways in SF T lymphocytes from RA patients are affected at several levels by chronic oxidative stress, all contributing to the observed hypo-responsiveness of these cells.

OP0002 DEOXYSPERGUALIN AND SPERMINE INDUCE APOPTOSIS IN QUIESCENT AND ACTIVATED HUMAN LYMPHOCYTES

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10.1136/annrheumdis-2001.516

Background Deoxyspergualin (DSG) is a novel immunosuppressive drug currently undergoing clinical trials for treatment of transplant rejection or autoimmune diseases. The polyamine analogue DSG is a synthetic derivative of spergualin containing a spermidine moiety. Polyamines are known to induce apoptosis in several mesenchymal cell lines in presence of fetal calf serum (FCS).

Objectives The aims of the present study were

- to test whether DSG alone or in combination with other immunomodulating drugs induces apoptosis or necrosis in immunocompetent cells (fresh isolated cells, PHA generated lymphoblasts) and whether this effect depends on the presence of FCS;
- to analyse which cellular mechanisms are involved in the execution of the cell death program induced by DSG.

Methods Freshly isolated quiescent or activated human PBMCs, PHA generated lymphoblasts and several tumour cell lines (Jurkat, SKW-3, U937, RS4.11., MV4.11.) were incubated with different concentrations of DSG in presence of FCS or human AB serum (hABS). The rate of apoptotic cells was analysed after Propidium-Iodide/AnnexinV (PI/AxV) and PI/Triton staining by flow cytometric analysis.

Mitochondrial-membrane-potential ($\Delta\psi$ M) was measured by flow cytometric analysis after staining with Dihexyloxocarbocyanine (DiOC6).

Results By means of PI/Triton and PI/AxV staining we found an induction of apoptosis by DSG in presence of FCS in quiescent PBMCs, PHA generated lymphoblasts and the tumour cell lines Jurkat, SKW-3, U937. In presence of hABS no induction of apoptosis was found. Necrosis was excluded in any of the experiments.

DSG was also capable to induce apoptosis in the tumour cell lines RS4.11., MV4.11. which are refractory to conventional chemotherapeutic agents operating through the induction of apoptosis. In lymphoblasts coactivation with IL-4, IL-7 or IL-15 did not prevent DSG-induced apoptosis, whereas IL-2 had various effects.

A decrease of $\Delta\psi$ M was seen after treatment of cells with DSG in presence of FCS. DNA degradation measured by PI/Triton staining was abolished by adding the caspase inhibitor zVAD FMK.

Combination of DSG with Cycloheximid (CHX), a protein synthesis inhibitor which is described to interact with the regulation of cellular polyamine synthesis, lead to a remarkable increase in the induction of apoptosis by DSG, while CHX alone showed no effects.

Similar effects as described above for DSG were achieved if spermine, a physiological polyamine, was added to the culture.

Conclusion These results show, that DSG in presence of FCS is able to induce apoptosis in several cell systems. Interestingly induction of apoptosis was seen in resting cells and two cell lines, that are resistant to conventional chemotherapeutic agents. Our findings point out that induction of apoptosis by DSG in presence of FCS requires activation of caspases and is associated with a decrease in $\Delta\psi$ M. The structural conjunction of DSG with polyamines and the additive effects in combination with CHX suggest, that alterations of the cellular polyamine contents might be involved in the induction of apoptosis by DSG.

OP0003 STAT1 AND STAT3 ACTIVATION DURING THE PRIMARY AND CHRONIC PHASES OF EXPERIMENTAL ARTHRITIS IN WILDTYPE AND IL-6 DEFICIENT MICE

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10.1136/annrheumdis-2001.517

Background Experimental arthritis in Interleukin-6 deficient (IL-6^{-/-}) mice has shown the importance of this cytokine for chronicity of the synovitis. We^{1,2} and others have found previously that in IL-6 deficient mice the primary inflammation does not develop into a chronic synovitis. The JAK/STAT pathway is important for signaltransduction by IL-6 in the cell. Upon activation of the IL-6 receptor complex, the Signal Transducer and Activator of Transcription molecules STAT1 and STAT3 become phosphorylated and migrate to the nucleus to activate gene transcription.

Objectives In the present study we followed activation of STAT1 and 3 during zymosan-induced arthritis (ZIA) in wildtype (wt) and IL-6-/- mice. We also studied expression of the STAT inhibitors Suppressor Of Cytokine Signalling (SOCS) 1 and 3 during the arthritis.

Methods Zymosan induced Arthritis was elicited by intra-articular injection of zymosan as described in (2). Protein lysates of inflamed and control synovia were subjected to Western blotting for both total and phosphorylated STAT1 and STAT3. RT-PCR was performed for STAT3, SOCS1 and SOCS3.

Results The primary inflammation lasted for a week in both strains. In wt mice this inflammation became chronic until the end of the experiments at week 4, while it decreased rapidly in IL-6-/- mice. In wt mice STAT3 became activated from day 1 until the end of the experiments. Also the level of STAT3 mRNA and protein increased. STAT1 in contrast only became activated during the chronic phase after day7. During the arthritis there was an increase of SOCS1 and SOCS3 mRNA expression. In IL-6-/- mice STAT3 only became activated at day 1. No activation of STAT1 occurred in IL-6-/- mice.

Conclusion STAT3 activation occurred during both the primary and chronic inflammation in wildtype mice. During the first week IL-6-/- mice also developed a primary inflammation. IL-6 is not the only cytokine that activates STAT3, explaining STAT3 activation at day 1 in IL-6-/- mice. Lack of further STAT3 activation during the primary inflammation suggests a major role for IL-6 in STAT3 activation. STAT1 only became activated in the chronic phase. Arthritis increased expression of SOCS1 and 3, but this seemed not enough to inhibit STAT activation. Future research will have to address the importance of activated STAT1 and 3 for chronic synovitis and the possibility of inhibiting these signalling molecules in the inflamed synovium.

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OP0004 PIVOTAL ROLE FOR THE SMALL GTPASE RAP1 IN THE REGULATION OF INTRACELLULAR GENERATED ROS AFTER T CELL STIMULATION

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10.1136/annrheumdis-2001.518

Background Oxidative stress plays a critical role in various diseases including cancer, neuronal degeneration, atherosclerosis and rheumatoid arthritis. Identifying the target proteins for redox modification is crucial in understanding how oxidants mediate the pathological processes. As previously demonstrated activation of T cells with anti CD3 antibody or with phorbol ester results in a rapid production of intracellular reactive oxygen species (ROS). In this paper we further dissect the signalling pathways leading to ROS generation and we propose a possible critical role for the small GTPase Rap1 in antagonising the intracellular generated ROS.

Objectives

Methods In our experiments we made use of the free radical dye 6-carboxy-2',7'-dichlorofluoresceine DCF to measure radical generation at the single cell using flow cytometry. Transfected

Jurkat cells were incubated with the dye 20 min 37° before addition of stimuli. Readings were taken every 5-min over a 20 min period. The positive transfected Jurkat cell population was identified by detection of a co-transfected CD20 receptor.

Results T cells as well as Jurkat cells rapidly produce intracellular ROS in a time dependent manner, after stimulation with either CD3 or PMA+ionomycin. The intracellular ROS generated after T cell stimulation seem to be induced through a signalling pathway downstream of the small GTPase Ras, since Jurkat cells transfected with a constitutively active mutant of p21Ras, RasV12, produced large amounts of intracellular ROS, that could not be augmented by an additional stimulus. The observed ROS production after addition of a stimulus was suppressed by the expression of dominant negative mutant of Ras, RasN17. Jurkat cells transfected with the constitutive active mutant of another small GTPase, Rap1, failed to generate ROS after any stimulus tested. Moreover co-transfection of RapV12 and RasV12 showed that RapV12 abolishes the intracellular ROS generated by RasV12. The inhibition of ROS production appears to involve on a PI3K-dependent signalling pathway as is shown by inhibition of ROS production after Jurkat transfection with RapV12C40 (a Rap1 mutation that, analogue with RasV12C40 could affect specific binding to PI3K). Accordingly, pre-incubation with LY 294002, an inhibitor of the lipid kinase activity of PI3K, blocked the observed inhibition of ROS production by Rap1 in RasV12+RapV12 co-transfection experiments. In addition we report that Rap1 activation in T cells after activation with CD3 or PMA proved to be redox sensitive as shown by pretreatment of T cells with N-acetylcysteine and BSO. Furthermore hydrogen peroxide was capable of Rap1 activation in T cells, and pre-incubation with diphenylene iodonium (a flavoprotein inhibitor) completely blocked Rap1 activation.

Conclusion We define a role for ROS in T cell signalling pathways downstream from Ras and we propose a possible pivotal role for the small GTPase Rap1. Rap1 can be activated by ROS and antagonises the intracellular generated ROS in signalling pathways.

OP0005 LEFLUNOMIDE REDUCES TRANSENDOTHELIAL MIGRATION

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10.1136/annrheumdis-2001.519

Background The disease modifying drug leflunomide is widely used in the therapy of rheumatoid arthritis (RA). Its active metabolite, A771726, leads to inhibition of dihydroorotate dehydrogenase, an enzyme necessary for pyrimidine de-novo synthesis. Since activated lymphocytes expand their pyrimidine pool, A771726 decreases their proliferation. A771726 also suppresses TNF mediated nuclear factor kappaB activation. We were therefore interested if A771726 also would be capable to influence transendothelial migration (TEM) of peripheral mononuclear cells (PBMC).

Objectives

Methods We investigated the TEM of PBMC, which migrated through endothelial cell monolayers in an in-vitro model. Human umbilical vein endothelial cells (EC) were cultured to confluence on collagen gels and then incubated with human PBMC of healthy blood donors. PBMC were recollected in three groups: 1) cells that did not adhere to the endothelium, 2) cells