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An in-vitro screening assay for the detection of inhibitors of proinflammatory cytokine synthesis: a useful tool for the development of new antiarthritic and disease modifying drugs

S. Laufer*, C. Greim* and T. Bertsche†

**Institute of Pharmacy, Department of Pharmaceutical and Medicinal Chemistry, Eberhard-Karls-University Tübingen, A. d. Morgenstelle 8, D-72076 Tübingen, Germany; †Institute of Pharmacy, Department of Pharmacology, Eberhard-Karls-University Tübingen, A. d. Morgenstelle 8, D-72076 Tübingen, Germany*

Summary

Objective: This work targets the development of a new tool to help develop new anticytokine drugs that prevent or reduce the progression of arthritic diseases. The specific aim of our study was to establish a fast and reliable *in vitro* screening assay of cytokine synthesis inhibitors (TNFα, IL-1β) which shows better correlation with enzyme assays than previously reported *in vitro* assays. The test system should be able to detect p38-MAP kinase inhibitors.

Material and methods: Human peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll density gradient centrifugation from human EDTA-potassium whole blood. Cells were adjusted at 1×10^6 cells/ml. PBMCs were stimulated with lipopolysaccharide (LPS; *E. coli* serotype 026:B6: 1 μg/ml) in the presence of test compound (10^{-5}–10^{-8} M) for 4 h at 37°C in a 5% CO_2-incubator. Induced TNFα and IL-1β protein were measured by ELISA.

Results: The following are representative examples of inhibitors which effect cytokine synthesis. Corticoid Dexamethasone inhibits IL-1β and TNFα synthesis at IC_{50} of 38 nM and 25 nM, respectively. ERK1/ERK2 inhibitor U0126 effects cytokine synthesis at IC_{50} of 0.34 μM for IL-1β production and 0.26 μM for TNFα synthesis.

p38-MAP kinase inhibitor SB 203580 inhibits IL-1β- and TNF-α-synthesis (IC_{50}sof 0.052 μM and 0.46 μM) in the same degree as p38-MAP kinase activity (IC_{50}: 0.34 μM). Same results could be shown for SB 210313, which had same efficacy on IL-1β and TNFα biosynthesis (IC_{50}'s: 1.88 μM and 1.01 μM) and on p38-MAP kinase (IC_{50}: 6.85 μM). Also for SB 202190 this correlation in inhibition of IL-1β and TNFα synthesis (IC_{50}'s: 0.055 μM and 1.01 μM) and p38-MAP kinase inhibition (IC_{50}: 0.088 μM) could be shown.

Conclusion: This study shows the screening assay using PBMCs stimulated with LPS for IL-1β and TNFα synthesis is a reliable test system for the quantification of the effectiveness of new drugs modulating IL-1β and TNFα synthesis which is mainly mediated by p38-MAP Kinase. These assay allows fast detection of IL-1β and TNFα synthesis inhibitors with different modes of action, including p38-MAP kinase inhibitors. The results obtained with our in-vitro screening assay show good correlation with results from enzyme assays. © 2002 OsteoArthritis Research Society International. Published by Elsevier Science Ltd. All rights reserved.

Key words: Cytokine synthesis, p38-MAP kinase, *In vitro* test system.

Introduction

Proinflammatory cytokines such as IL-1β and TNFα play a central role in the pathogenesis of chronic inflammatory diseases like osteoarthritis (OA) and rheumatoid arthritis (RA). Both cytokines are produced in macrophages and monocytes in response to stimuli like endotoxine, UV-light, stress or other cytokines. p38-MAP kinase is a pivotal enzyme in the biosynthesis of IL-1β and TNFα. p38-MAP kinase regulates cytokine production both at the transcription[1] and the translation level[2], and stabilizes cytokine specific mRNA[3,4].

The degradation of arthritic cartilage and other joints is related to a complex interaction of mechanical and biochemical factors[5–7]. Among the latter, a number of catabolic factors, including proinflammatory cytokines and proteases have been demonstrated to play major roles[5,8]. Among the different signaling systems activated by proinflammatory cytokines, MAP kinase pathways are believed to be the key ones[9–14]. The most predominant ones are p38, MEK1/2 and Jun kinases, which have been demonstrated in a number of studies to be involved as key factors in the synthesis of catabolic factors responsible for inducing the structural changes seen in arthritic diseases[9]. p38-MAP kinase has also been shown to be involved in the synthesis of proinflammatory cytokines.

A recent study has demonstrated that treatment with specific a p38-MAP kinase inhibitor in an inflammatory rat model of arthritis was found to reduce the progression of structural damage at the same time as it reduced the synthesis of proinflammatory cytokines[15].

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Address correspondence to: Stefan Laufer, Institute of Pharmacy, Department of Pharmaceutical and Medicinal Chemistry, Eberhard-Karls-University Tübingen, A. d. Morgenstelle 8, D-72076 Tübingen, Germany. Fax: +49/70 71/29 78 788; E-mail: Stefan.Laufer@uni-tuebingen.de

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After the introduction of anticytokine therapy for treatment in chronic inflammatory diseases, such as arthritis, p38-MAP kinase became a valid target for many drug discovery programs.

In the past, several test models designed to study p38-MAP kinase inhibitors have been reported. For example, Hartman *et al.*[16] investigated the effects of drugs on IL-1β and TNFα synthesis in human whole blood. Wadsworth *et al.*[17] studied p38-MAP kinase inhibitors in PBMCs as well as in an isolated p38-MAP kinase enzyme assay.

These test systems are useful tools for the investigation of novel p38-MAP kinase inhibitors but have two disadvantages: (1) most isolated p38-MAP kinase enzyme assays work with [γ^{32}-P]ATP; (2) the screening assays based on human whole blood or PBMCs are time consuming and IC_{50}-values show only low correlation with IC_{50}-values obtained from enzyme assays.

To allow best possible correlation with human whole blood assays and to the situation in man freshly isolated human PBMCs should be used rather than cell lines. As interindividual variability is a general problem cells from at least four donors should be used.

The aim of our study was to develop a test system using freshly isolated, pooled human mononuclear cells with the advantages of short incubation times, no radioactivity, reproducible results and good correlation with IC_{50}-values obtained with the isolated p38-MAP kinase enzyme assay.

Materials and methods

MATERIALS

All the following drugs and solutions were of analytical grade: Macrophagen-SFM-Medium® (Life Technologies, Eggenstein, Germany); Histopaque® 1.077-solution, LPS (*E. coli* serotype 026:B6), trypan blue solution 0.4%, gentamicin sulfate (Sigma-Aldrich Chemie, Steinheim, Germany); salts for Dulbecco's phosphate buffered saline (DPBS), DMSO (Merck, Darmstadt, Germany); Cremophor EL® (Fluka, Buchs, Switzerland); Ethanol (Merck, Darmstadt, Germany); adenosine-5′-triphosphate disodium salt hydrate, 4-nitrophenyl phosphate disodium salt, β-glycerolphosphate disodium salt pentahydrate (Fluka, Buchs, Switzerland); dithiothreitol, sodium orthovanadate, bovine serum albumin (BSA) (Sigma-Aldrich Chemie, Steinheim, Germany); $MgCl_2 \times 6\ H_2O$, $NaHCO_3$, HCl (Merck, Darmstadt, Germany); polyclonal rabbit antibody detecting ATF-2 phosphorylated on Thr71, polyclonal rabbit antibody detecting p38-MAP kinase phosphorylated on Thr180/Tyr182, alkaline phosphatase-linked antirabbit IgG antibody (New England Biolabs, Beverly, MA, U.S.A.); alkaline phosphatase-linked goat antirabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.); polystyrene 96-well microtiter plates (Immunolon 4, Dynex Technologies, Frankfurt a.M., Germany).

SUBJECTS

Human whole blood from four healthy donors (male or female, 20 ml each) was removed by venopuncture and collected in EDTA-potassium-monovettes (Sarstedt, Nümbrecht, Germany).

ISOLATION OF PBMCS FROM HUMAN WHOLE BLOOD

Human whole blood was diluted 1:3 with 0.9% NaCl-solution. 8 ml diluted blood was layered on top of 3 ml Histopaque® 1.077-solution, placed in 15 ml conical tubes (Falcon 2096, Oxnard, California). After centrifugation at 400×***g*** for 25 min at room temperature, mononuclear cells were taken off and washed three times in DPBS (136.7 mM sodium chloride, 8.1 mM sodium phosphate dibasic, 1.5 mM potassium phosphate monobasic, 2.7 mM potassium chloride, 5.5 mM D(+)-Glucose anhydrous). PBMCs were resuspended at 1×10^6 cells/ml in Macrophagen-SFM medium®. Cell viability was determined by trypan blue exclusion.

LPS INDUCTION

390 µl cell suspension aliquots were preincubated with 10 µl gentamicin sulfate (50 µg/ml) 15 min at 37°C in a 5% CO_2-incubator before cells were incubated with LPS (100 µl, *E. coli* serotype 026:B6: 1 µg/ml) or Macrophagen-SFM medium® at 37°C for 4 h in a 5% CO_2-incubator.

TNFα and IL-1β formation was stopped by placing samples on ice and addition of ice-cold DPBS. After centrifugation for 12 min at 16 400×***g*** at 2°C supernatants were removed and stored at −20°C.

TNFα and IL-1β were quantified by ELISA (Beckman Coulter, Krefeld, Germany).

QUANTIFICATION OF IL-1β AND TNFα mRNA LEVELS

PBMCs (5×10^6 cells/ml) were stimulated with LPS (IL-1β: 5 µg/ml; TNFα: 10 µg/ml) from 0 h to 4 h at 37°C in a 5% CO_2-incubator. RNA from PBMCs was isolated by Rneasy® (Qiagen, Hilden, Germany) according to the manufacturers protocol. mRNA levels were determined using Quantikine® mRNA (R&D Systems, Minneapolis, MN, U.S.A.) for quantification of IL-1β- and TNFα-mRNA.

CHARACTERIZATION OF p38-MAP KINASE ACTIVITY

PBMCs (1×10^7 cells/ml) were treated with LPS (100 µg/ml) from 0 min to 35 min at 37°C in a 5% CO_2-incubator. Cell lysates were used for the kinase reaction. 50 µl cell lysate and 50 µl of ATP-mixture (50 mM HCl, 10 mM $MgCl_2$, 10 mM β-glycerolphosphate, 100 µg/ml BSA, 1 mM DTT, 1 mM ATP, 0.1 mM Na_3VO_4) were added in a ATF-2 coated microtiter plate and incubated at 37°C for 1 h. After being washed three times, the plate was incubated with phosphoATF-2 antibody (1:2000) for 1 h at 37°C. After three washes, alkaline phosphatase labeled goat antirabbit IgG (1:2000) was added for 1 h at 37°C. After a next washing step alkaline phosphatase substrate solution (3 mM 4-NPP, 50 mM $NaHCO_3$, 50 mM $MgCl_2$) was added for 1.5 h at 37°C. The formation of 4-nitrophenolate was measured at 405 nm using a microtiter plate reader.

WESTERN BLOT ANALYSIS18

PBMCs (1×10^7 cells/ml) were treated with LPS (100 µg/ml) from 0 min to 35 min at 37°C in a 5% CO_2-incubator. To cell lysates, 5×concentrated SDS-electrophoresis sample buffer (final concentrations: 0.4% SDS, 2.9% 2-mercaptoethanol, 5% glycerol and 0.1% BPB in 12 mM

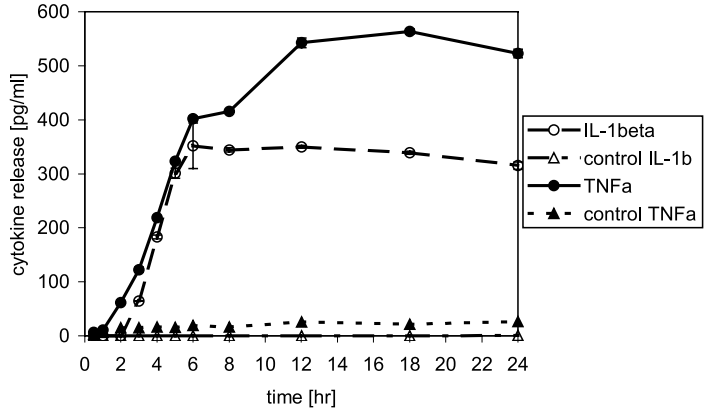

Fig. 1. Time course of IL-1β and TNFα production by PBMCs. PBMCs were isolated from human whole blood. Cell suspension was adjusted to 1×10^6 cells/ml and incubated with LPS (1 μg/ml) or DPBS at 37°C for 0 h to 24 h in a 5% CO_2-incubator. The reactions were terminated by placing samples on ice and centrifuging (16 400×***g***/2°C). IL-1β and TNFα were assayed in supernatants by ELISA. Data shown are representative of at least three independent experiments.

Tris buffer, pH 6.8) was added and samples were heated at 95°C for 5 min. Proteins were electrophoresed on 10% SDS-polyacrylamide gels and then transferred to PVDF membranes. The membranes were probed with dual-phosphospecific (Thr180/Tyr182) rabbit anti-p38-MAP kinase antibody and goat alkaline phosphatase-linked anti-rabbit IgG antibody (1:1000) (New England Biolabs Inc., Beverly, MA, U.S.A.).

DETERMINATION OF INHIBITORY POTENCY OF TEST CANDIDATES OF p38-MAP KINASE

The activity was measured using method of Forrer *et al.*[19]. Microtiter plates were coated with 50 μl ATF-2-solution (10 μg/ml) 1 h at 37°C. Plates were washed three times and 50 μl kinase mixture (50 mM Tris–HCl, 10 mM $MgCl_2$, 10 mM β-glycerol phosphate, 100 μg/ml BSA, 1 mM DTT, 100 μM ATP, 100 μM Na_3VO_4, 10 ng p38α activated) with or without inhibitor was added to the wells and incubated for 1 h at 37°C. After three washes, plates were incubated with phospho-ATF-2 antibody (1:2000) for 1 h at 37°C. After washing plates three times, alkaline phosphatase labeled goat antirabbit IgG (1:2000) was added for 1 h at 37°C (to capture the antibody phosphorylated-protein-substrate complex). After three washes, alkaline phosphatase substrate solution (3 mM 4-NPP, 50 mM $NaHCO_3$, 50 mM $MgCl_2$, 100 μl/well) was added for 1.5 h at 37°C. The formation of 4-nitrophenolate was measured at 405 nm using a microtiter plate reader (Molecular Devices, Ismaning, Germany). IC_{50}-values were calculated.

STANDARD TEST CONDITIONS TO EVALUATE CYTOKINE SYNTHESIS INHIBITORS

PBMCs were prepared from human whole blood (potassium-EDTA) used within 4 h after being drawn from healthy donors as described above. After washing cells three times in DPBS, PBMCs were suspended at a concentration of 1×10^6 cells/ml in Macrophagen-SFM medium. 390 μl cell suspension was preincubated with 5 μl gentamicin sulfate (50 μg/ml) and 5 μl test compound (10^{-5}–10^{-8} M, 1% DMSO) for 15 min at 37°C in a 5% CO_2-incubator. PBMCs were stimulated with 100 μl LPS (*E. coli* serotype 026:B6, 1 μg/ml) for 4 h at 37°C in a 5% CO_2-incubator for cytokine production. The reaction was terminated by placing samples in a ice bath and centrifuging for 12 min at 16 400×***g*** at 2°C.

Supernatants were used to quantify TNFα and IL-1β by ELISA (Beckman Coulter, Krefeld, Germany).

Potency of the test compounds (IC_{50}-values) was calculated by blotting percentage inhibition of IL-1β and TNFα synthesis vs concentration on a semi-logarithmic scale.

Potency of test compounds can also be determined in human whole blood. Human whole blood (potassium-EDTA) was used within 4 h after being drawn from healthy donors. 400 μl whole blood was preincubated with 50 μl test compounds (10^{-4}–10^{-7} M, 1% Cremophor EL®/Ethanol (66.6%/33.3%), 50 μg/ml gentamicin sulfate) 15 min at 37°C in a 5% CO_2-incubator. Blood was stimulated for 4 h with 50 μl LPS (*E. coli* serotype 026:B6; 1 μg/ml) at 37°C in a 5% CO_2-incubator. The reaction was terminated by placing samples on ice and centrifuging for 15 min at 1000×***g*** at 4°C. Plasma samples were used for quantification of IL-1β and TNFα by ELISA.

Results

LPS-INDUCES IL-1β AND TNFα SYNTHESIS

The time course of IL-1β and TNFα synthesis from PBMCs after treating with LPS (1 μg/ml) for 24 h are shown in Fig. 1.

IL-1β and TNFα were measurable 3 h and 1 h after LPS treatment respectively. IL-1β reached a maximum concentration after 6 h of incubation and TNFα a maximum concentration after 12 h. There was no additional change in IL-1β and TNFα levels up to 24 h. In unstimulated PBMCs, cytokine synthesis was very low, indication that there is no substantial stimulation by pooling cells from different donors at least during 24 h.

QUANTIFICATION OF IL-1β AND TNFα mRNA

To demonstrate the expression of IL-1β and TNFα during LPS treatment over a period of 4 h, the formation of IL-1β and TNFα mRNA was quantified by Quantikine® for IL-1β and TNFα mRNA. PBMCs were incubated with

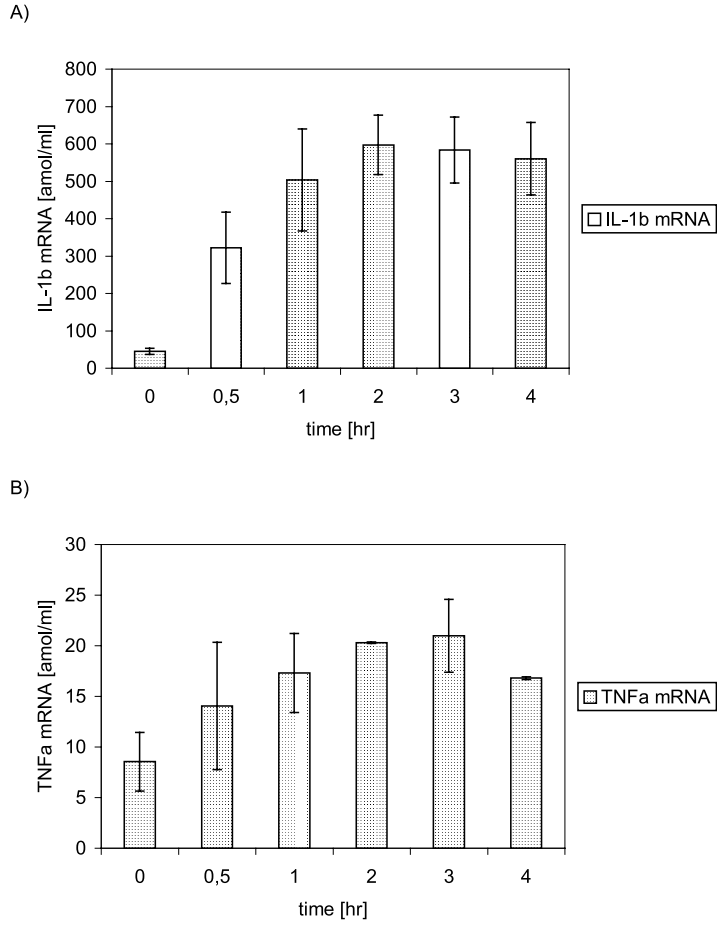

Fig. 2. Time course of IL-1β(A) and TNFα (B) mRNA production by PBMCs. PBMCs were isolated from human whole blood. Cell suspension was adjusted to 5×10^6 cells/ml and incubated with LPS (IL-1β: 5 μg/ml; TNFα: 10 μg/ml) at 37°C for 0 h to 4 h in a 5% CO_2-incubator. The reactions were terminated by placing samples on ice. RNA was isolated with Rneasy® and mRNA was quantified by Quantikine® for IL-1β or TNFα mRNA. Results represent the means and standard errors of four independent experiments.

Macrophagen-SFM-Medium® or LPS (IL-1β: 5 μg/ml; TNFα: 10 μg/ml) from 0 h to 4 h.

The mRNA quantification indicated that there were only very low levels of mRNA for both cytokines in unstimulated samples. LPS-treated cells showed an IL-1β and TNFα mRNA increase after 0.5 h, with a peak at 2–3 h post LPS-challenge. mRNA levels decreased after 3 h LPS treatment. Fig. 2

CHARACTERIZATION OF p38-MAP KINASE ACTIVITY

To demonstrate that the signal transduction pathway via p38-MAP kinase leads to the expression of IL-1β and TNFα in PBMCs after LPS treatment, we examined the phosphorylation of ATF-2 through cell lysate and the formation of dual phosphorylated p38-MAP kinase by Western blot. The measurement of ATF-2 phosphorylation through cell lysate from LPS-treated PBMCs was perfomed because ATF-2 is a major substrate of p38-MAP kinase in cells and ATF-2 phosphorylation is a good indicator of p38-MAP kinase activity.

For both assays, PBMCs (1×10^7 cells/ml) were incubated with Macrophagen-SFM-Medium® (control sample) or LPS (100 μg/ml) from 0 min to 35 min. The analyses of ATF-2 phosphorylation (Fig. 3) showed that after treating cells with LPS the amount of ATF-2 phosphorylated by cell lysate increases about 85% within 25 min. After 25 min the phosphorylation activity decreases.

The next step was to establish that the increase in phosphorylation activity was associated with an increase of phosphorylated p38-MAP kinase. Western blot analyses were conducted with a di-phospho-p38-MAP kinase (Thr180/Tyr182) polyclonal antibody. Only very low levels of phosphorylated p38-MAP kinase were detected (Fig. 4, lane 1) in unstimulated samples. The stimulation of PBMCs with LPS led to an increase of phosphorylated p38-MAP kinase (lanes 2 and 3).

These facts show that IL-1β and TNFα synthesis from LPS treated PBMCs in our *in vitro* test system is based on a de-novo synthesis of both cytokines. This was demonstrated by an increase in cytokine specific mRNA after LPS stimulation of PBMCs. The increase in IL-1β and TNFα mRNA results from the upregulation of the p38-MAP kinase signal transduction pathway that occurs when PBMCs are stimulated by LPS.

EFFECTS OF IL-1β AND TNFα SYNTHESIS INHIBITORS

Four different p38-MAP kinase inhibitors, the Corticoid Dexamethasone and ERK1/ERK2 inhibitor U0126 were tested on isolated human mononuclear cells (pooled from four donors, male and female).

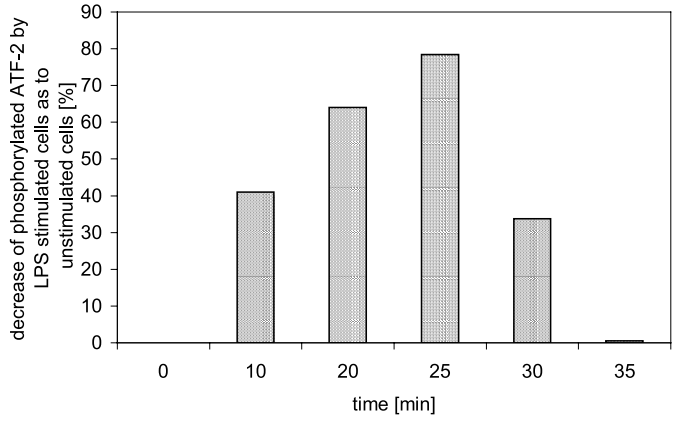

Fig. 3. Time course of p38-MAP kinase activation by LPS. PBMCs were isolated from human whole blood, cell suspension was adjusted to 1×10^7 cells/ml and incubated with LPS (100 μg/ml) at 37°C for 0 min to 35 min in a 5% CO_2-incubator. The reactions were terminated by placing samples on ice. Kinase assays were performed with cell lysates using ATF-2 as substrate. Results represent the means of three independent experiments.

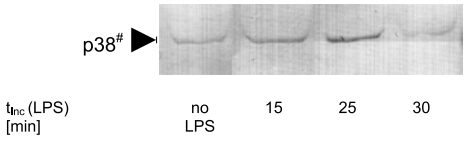

Fig. 4. Western blot analysis of activated p38-MAP kinase in PBMCs treated with LPS (100 μg/ml) at 37°C for 0 to 35 min in a 5% CO_2-incubator. Activated p38-MAP kinase was established with polyclonal antibody detecting p38-MAP kinase phosphorylated on Thr180/Tyr182. Data shown are representative of at least two independent experiments.

For tests with PBMCs, test compounds in concentrations from 10^{-5}–10^{-8} M were added to the cell suspension. Samples were incubated for 4 h at 37°C in a 5% CO_2-incubator with LPS (1 μg/ml). p38-MAP kinase inhibitor activities for SB 203580, SB 210313, SB 202190 and SK&F 86002 were determined according to Forrer *et al.*[19]. Results are shown in Table I.

Discussion

Proinflammatory cytokines like IL-1β and TNFα are major catabolic factors on cartilage metabolism. Comparably view small molecule drug candidates targets the inhibition of these cytokines. Here we describe a cell based screening assay which is fast and easy to handle to determine IC_{50}-values of proinflammatory cytokine synthesis inhibitors. Such assays are a major prerequisite for efficient drug discovery programs.

The model allows the broad detection of inhibitors with very different modes of action, e.g. corticoids, ERK1/2 inhibitors and especially but not only p38-MAP kinase inhibitors. For a first drug screening this is a valuable asset. However positive hits have to be followed up by more specific secondary tests, e.g. isolated enzyme assays.

Examples for inhibition data are: Dexamethasone, a Corticoid, inhibits IL-1β and TNFα synthesis at nanomolar range (IC_{50}'s: 38 nM and 26 nM). The ERK1/ERK2 inhibitor U0126 effect inhibition of IL-1β and TNFα synthesis at micromolar concentrations, IC_{50}sare for IL-1β inhibition 0.34 μM and for TNFα inhibition 0.62 μM. p38 MAP kinase inhibitors like SB 203580, SB 210313, SB 202190 or SK&F 86002 reduce IL-1β and TNFα synthesis at micromolar concentrations, too. For example SB203580 inhibits IL-1β synthesis with IC_{50}-value of 0.052 μM and TNFα production with IC_{50}-value of 0.46 μM.

These data agree with several previously reported IC_{50}-values for these cytokine synthesis inhibitors[20–22].

Monocytes and macrophages are major sources for proinflammatory cytokines. Their role as cytokine producing cells is well established in the pathophysiology of inflammation. They stimulate important inflammatory cells like synoviocytes, endothelial cells and condrocytes. Our test systems target these most important cell types which are not the predominant target for known antiinflammatory drugs.

Possible advantages over whole blood assays are less variability due to pooled cells from at least four donors. Pooling did not prestimulate the cells in the short incubation time of 4 h. A certain disadvantage is the lack of cross-talk between different cell types, which is well reflected in whole blood assays.

Compared with cell lines, freshly isolated PBMCs have the clear advantage of being much closer to physiologic conditions, which is also an advantage over assays using isolated enzymes. Other pros compared with known enzyme tests are absence of radiolabeled reagents and coverage of cell penetration properties of the test candidates.

One weakness of several published cell-based test systems is the poor correlation with enzyme assays for p38-MAP kinase inhibition. Therefore we tried to monitor the whole process of cytokine synthesis after LPS stimulation, e.g. p38-MAP kinase activation, ATF-2 phosphorylation and mRNA increase and finally IL-1β and TNFα synthesis.

We showed that IL-1β and TNFα mRNA synthesis is induced and p38-MAP kinase signal transduction pathway is activated after treating cells with LPS. By comparing our *in vitro* screening assay with isolated enzyme assay we found IC_{50}-values obtained with our cell-based screening assay correlated well with IC_{50}-values calculated with isolated enzyme assay. For SB 203580, SB 210313, SK&F 86002 and other known p38-MAP-Kinase inhibitors obtained IC_{50}-values for IL-1β and TNFα synthesis were closed to IC_{50}-values measured with isolated enzyme assay. For example IC_{50} of p38-MAP kinase inhibition for SB 203580 is 0.34 μM, while IL-1β and TNFα biosynthesis is inhibited with IC_{50}sat 0.052 μM and 0.46 μM. Also SB 210313 inhibits IL-1β and TNFα biosynthesis (IC_{50}sof

Table I
Inhibition of IL-1β and TNFα synthesis in PBMCs of cytokine synthesis inhibitors and inhibition of p38-MAP kinase by p38-MAP kinase inhibitors

Inhibitor	IC_{50}-values [μM]±S.E.M.				
	IL-1β		TNFα		p38-MAP kinase
SB 203580	*N*=22	0.052±0.009	*N*=22	0.46±0.11	0.34
SB 202190	*N*=2	0.055±0.007	*N*=2	1.01±0.17	0.088
SB 210313	*N*=2	1.88±0.37	*N*=2	4.08±1.2	6.85
SK&F 86002	*N*=2	0.52±0.028	*N*=2	5.5±0.35	1
Dexamethasone	*N*=4	0.038±0.0047	*N*=4	0.026±0.0038	—
U0126	*N*=2	0.34±0.06	*N*=2	0.62±0.05	—

Mean IL-1β and TNFα synthesis in LPS stimulated PBMCs: IL-1β: 189.1±34.6 pg/ml (*N*=22), TNFα: 654.9±100.4 pg/ml (*N*=22); mean IL-1β and TNFα synthesis in unstimulated PBMCs: IL-1β: 5.6±5.6 pg/ml (*N*=22), TNFα: 18.7±12.5 pg/ml (*N*=22).

1.88 μM and 4.08 μM) in the same range as p38-MAP kinase activity is inhibited (IC_{50} of 6.85 μM).

LPS concentrations used in most studies varies between 1 ng/ml and 100 μg/ml depending on type and number of cells and serotype of LPS used. Following kinetic pre-experiments (data not shown) 1 μg/ml proofed to be optimal in terms of cytokine concentrations obtained. Only for ATF-2 phosphorylation and p38-MAP kinase activation experiments 100 μg/ml was used but with 1×10^7 cells/ml instead of 1×10^6 cells/ml. This experimental setting was necessary as we worked with cell lysates and not with immunoprecipitated p38-MAP kinase protein[23]. This relatively high concentrations is tolerated with no signs of cytotoxicity (data not shown).

In conclusion, our PBMC based screening assay met our objectives to develop a fast and reliable method to screen cytokine synthesis inhibitors including p38-MAP kinase inhibitors.

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