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Curcumin Differentially Modulates mRNA Profiles in Jurkat T and Human Peripheral Blood Mononuclear Cells

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Abstract—Curcumin, the yellow pigment of the rhizome of *Curcuma longa* is known to inhibit the transcription factors AP-1, Egr-1, NF-κB, c-myc and several important signaling kinases. We therefore investigated the differential effects of curcumin in concentation between 1.5 and 13.6 μM on gene expression in T Jurkat $CD4^+$ and human peripheral blood mononuclear cells (PBMCs). Relative quantification with reverse transcription real-time PCR (RT-rt-PCR) showed that low concentrations of curcumin significantly down-regulated mitogen-induced granulocyte macrophage colony stimulating factor (GM-CSF) mRNA (3- to 5-fold at 3 μM) in a dose- and time-dependent manner in both cell types. In comparison, the down-regulation of inducible nitric oxide (iNOS) mRNA levels was less pronounced, but interferon gamma (IFN-γ) mRNA was dose-dependently up-regulated with curcumin concentrations up to 8.2 μM. Cyclin D1 mRNA expression was specifically inhibited in Jurkat T cells and stimulated PBMCs. The transcription factors NF-κB and NF-ATc were not affected in PBMCs. Interleukin-2 (IL-2), and-6 (IL-6) mRNAs levels were not influenced markedly by curcumin in stimulated PBMCs, but significantly reduced in stimulated Jurkat T cells. In addition, cytotoxic effects and down-regulation of mRNAs, including p65 and the house-keeping genes could only be measured in Jurkat T cells. These findings confirm previous reports on the anti-neoplastic potential of curcumin and show that this compound differentially modulates the expression profile of Th1 cells and PBMCs.

Introduction

Curcumin [(*E*,*E*)-1,7-bis-(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dion)] or diferuloylmethane is the main bioactive component of the rhizome of the perennial herb *Curcuma longa* L. The plant is used for imparting color and flavor to food, for example as colorant (E 100) or in curry, as well as in Asian medicine. Its dietary intake is believed to play an important role in the prevention of various diseases.[1,2] Curcumin has been shown to exhibit a wide spectrum of pharmacological effects, like anti-inflammatory, anticancer, antioxidant, antihelmintic, and antiviral.[3–12] Of particular interest to this study are the reported anti-inflammatory activities induced by the inhibition of transcription factors. Over recent years, several studies have demonstrated that curcumin inhibits the transcription factors nuclear factor kappa B (NF-κB),[13,14] activated protein 1 (AP-1),[15,16] early growth response-1 gene product (egr-1),[17] and the oncogene c-myc.[18,19] Curcumin has also shown to inhibit phorbol ester induced protein kinase C at higher concentrations.[20,21] Since all of these factors are crucial in the regulation of cytokine gene expression in immune cells it is of interest to investigate whether curcumin differentially modulates cytokine transcription in T Jurkat and in comparison also in polymorphomononuclear blood cells. It has hitherto been shown that curcumin down-regulates the expression of several pro-inflammatory factors in macrophages, such as tumour necrosis factors,[22] interleukin-12,[23] interleukin-1 and-8,[24] and inducible nitric oxide (iNOS).[25,26] The inhibition of iNOS transcription in macrophages by curcumin has recently been investigated in some detail and shown to correlate with the blockage of relA (p65) translocation, inhibition of IκB kinase (IKK)[24] and hence the inhibition of IκB degradation.[27] Despite these investigations there is still little evidence that curcumin directly and specifically interferes with the regulation of gene transcription in CD4 T cells where NF-κB plays a pivotal role. We were therefore prompted to investigate the action of curcumin on Th1 specific gene transcription in stimulated Jurkat T and human mononuclear blood cells. A TaqMan based reverse transcription real-time polymerase chain reaction (RT-rt-PCR) assay was employed, which allowed accurate relative quantification of mRNA levels (Table 1).[28] Differential effects of curcumin on the mRNA levels of cytokines transcription factors, and other important regulatory genes in mitogen-activated Jurkat T cells were examined.

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Table 1. Primer and probe sequences used for RT-rt-PCR[a]

	Primer and probe sequences
IL-6 (GenBank™ M54894):	**FP.** 5′-GCTGAAAAAGATGGATGCTTCC-3′
	RP. 5′-AACTCCAAAAGACCAGTGATGATTT-3′
	Probe. 5′-TCTGGATTCAATGAGGAGACTTGCCTGGT-3′
IFN-γ (GenBank™ XM 006883):	**FP.** 5′-GTTTTGGGTTCTCTTGGCTGTT-3′
	RP. 5′-CATCTGAATGACCTGCATTAAAATATTT-3′
	Probe. 5′-CTGCCAGGACCCATATGTAAAAGAAGCAGA-3′
iNOS (GenBank™ L09210)	**FP.** 5′-CCAAGAGAAGAGAGATTCCATTGAA-3′
	RP. 5′-TGATTTTCCTGTCTCTGTCGCA-3′
	Probe. 5′-CTTGGTCAAAGCTGTGCTCTTTGCCTGTA-3′

[a]Forward primers (FP), reverse primers (RP), and TaqMan probes are listed with the corresponding GenBank™ accession number.

Results and Discussion

Curcumin differentially modulates PMA-induced cytokine transcription in Jurkat T cells and blood mononuclear cells

The mRNA expression profiles of IL-2, IL-6, GM-CSF, iNOS, and IFN-γ were determined in the absence of PMA stimulation. Although IL-2, IL-6, IFN-γ and iNOS mRNAs were shown to be expressed constitutively in non-activated Jurkat and PBMCs, they were up-regulated drastically upon PMA stimulation in a time-dependent fashion (Table 2). For the PMA stimulation experiments we found a correlation between IFN-γ and iNOS mRNA levels in both activated and non-activated cells. With our method, GM-CSF mRNA expression was not detectable in non-activated Jurkat cells and only weakly in PBMCs (Table 2). Stimulation with PMA (2 μg/mL) resulted in a rapid increase of GM-CSF mRNA, which was also found to be time-dependent with peak levels after 18 h (data not shown).

Table 2. mRNA expression in non-stimulated versus stimulated Jurkat T and human peripheral blood mononuclear cells (PBMCs)

Cell type	mRNA	ΔC_T no stimulation	ΔC_T 2.5 h stimulation	ΔC_T 20 h stimulation
PBMCs	IL-2	31.43	25.22	24.81
Jurkat	IL-2	32.72	27.39	26.88
PBMCs	IL-6	29.65	27.90	27.75
Jurkat	IL-6	30.23	28.01	27.67
PBMCs	IFN-γ	29.27	26.95	26.33
Jurkat	IFN-γ	31.11	27.48	26.91
PBMCs	GM-CSF	32.82	29.30	29.00
Jurkat	GM-CSF	34.49	29.93	27.95
PBMCs	iNOS	28.48	24.85	24.08
Jurkat	iNOS	29.20	26.72	26.22
PBMCs	NF-ATc	24.54	24.33	22.01
Jurkat	NF-ATc	20.52	20.48	20.02
PBMCs	p65	27.13	23.08	22.97
Jurkat	p65	22.98	16.35	16.88
PBMCs	I-κBα	30.19	28.02	27.51
Jurkat	I-κBα	24.73	23.38	22.97
PBMCs	cyclin D1	32.87	26.46	25.03
Jurkat	cyclin D1	21.55	21.01	19.93
PBMCs	β-actin	15.47	15.49	15.95
Jurkat	β-actin	15.88	16.20	16.59
PBMCs	GAP-DH	17.49	17.91	17.99
Jurkat	GAP-DH	15.95	16.07	16.16

Cells were stimulated with phorbol 12-myristate 13-acetate (PMA) (2 μg/mL). ΔC_T values represent mean values ($n = 4$).

Low concentrations (2 μM) of curcumin significantly down-regulated the mRNA levels of GM-CSF in both Jurkat and PBMCs, and had only little effect on the other genes (Fig. 1a and b). 8.2 μM of curcumin completely abolished the presence of PMA-induced GM-CSF transcripts in Jurkat cells and significantly down-regulated these transcripts in PBMCs (Fig. 1a). In Jurkat T cells also IL-2 and IL-6 mRNA levels were down-regulated more than 2-fold after 2.5 h, and 3-fold after 20 h at concentrations below 10 μM. These effects were however not found in PBMCs. The expression of iNOS mRNA was modulated weakly in both cell types, and weakly up-regulated below 3 μM (Fig. 1b). Interestingly, after 20 h PMA stimulation, IFN-γ mRNA was up-regulated more than 2-fold at curcumin concentrations as low as 8.2 μM (Fig. 1a).

To check whether mRNA levels are correlated with protein products we have chosen the cytokine IL-2 as control, the expression of which is crucial for leukemia T cell propagation. ELISA quantification of released IL-2 protein product in the medium confirmed the down-regulation of IL-2 mRNA. The decreased immunoreactivity (Fig. 2) was dose and time dependent in a similar range as IL-2 mRNA levels were down-regulated by curcumin.

Curcumin targets cyclin D1 gene transcription in Jurkat cells and PMA-stimulated PBMCs

To differentiate between specific reduction of certain mRNA levels and an overall down-regulation of cytokine expression due to cytotoxic effects or apoptosis, we also monitored mRNA levels of the two house-keeping genes GAP-DH and β-actin. In addition, the mRNA levels of the cell cycle regulator cyclin D1 were measured. Cyclin D1 mRNA expression in Jurkat cells was not modulated by PMA alone (Table 2), but incubation with different concentrations of curcumin resulted in a time and dose dependent down-regulation of this transcript (Fig. 1b). Curcumin thus down-regulated cyclin D1 mRNA levels earlier and much more strongly than the house-keeping mRNAs. Only concentrations higher than 10 μM curcumin significantly reduced the mRNA levels of the house-keeping genes, and also inhibited IFN-γ transcription after 2.5 and 20 h (Fig. 1a). Cyclin D1 mRNA is not detectable in non-stimulated PBMCs. However, we found that PMA strongly induced cyclin D1 mRNA expression in PBMCs in a dose- and

time-dependent manner (Table 2). Curcumin also inhibited the PMA-induced cyclin D1 mRNA up-regulation at concentrations where the house-keeping genes were not affected.

Baseline mRNA expression of NF-κB and NF-ATc in PBMCs is up-regulated by PMA but not influenced by curcumin

NF-κB subunits p65, I-κBα and NF-ATc, mRNAs were upregulated in PBMCs after PMA stimulation. NF-ATc was up-regulated 5-fold in PMBCs after 20 h stimulation, but in Jurkat T cells this factor was not induced (Table 2). P65 was up-regulated time-dependently in both, PMBCs and Jurkat T cells by more than 10-fold after 20 h PMA stimulation. In tendency, this is also true for I-κBα, which is however up-regulated to a lower degree. As shown in Fig. 1b curcumin concentrations above 8 μM down-regulated p65 mRNA in Jurkat T cells more than 2-fold whereas I-κBα was not modulated. In PBMCs neither NF-ATc nor the NF-κB units were influenced by curcumin.

Overall down-regulation of mRNAs correlates with cell viability

Cell viability testing was not possible with a colorimetic assay, such as WST-1, due to the strong yellow color of curcumin. Traditional trypan blue staining showed that curcumin induces cell death in Jurkat cells with increasing concentrations. Concentrations higher than 8 μM resulted in time-dependent decrease in Jurkat T cell

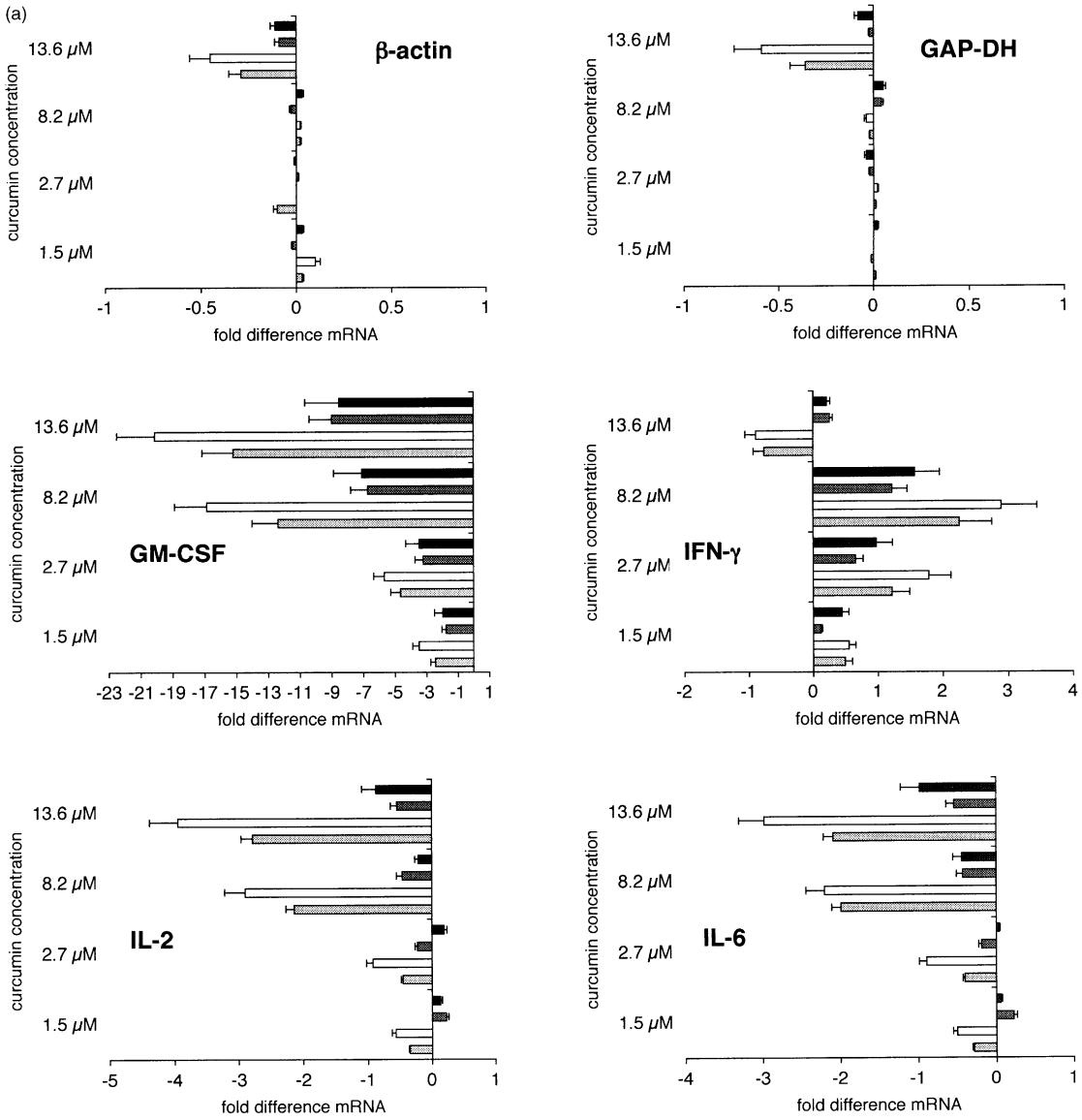

Figure 1. (a) Dose-dependent inhibition of mRNA expression induced by curcumin in 2 μg/mL PMA-activated Jurkat cells. Relative fold modulation of β-actin, GAP-DH, GM-CSF, IFNγ, IL-2 and IL-6 mRNA. Light grey: Jurkat T cells 2.5 h PMA stimulation, dark grey: PBMCs 2.5 h PMA stimulation, white: Jurkat T cells 20 h PMA stimulation, black: PBMCs 20 h PMA stimulation. Data represent mean ± SE of at least four independent experiments. (b) Dose-dependent inhibition of mRNA expression induced by curcumin in 2 μg/mL PMA-activated Jurkat T cells. Relative fold modulation of I-κBα, p65, NF-ATc, cyclin D1, iNOS mRNA. Light grey: Jurkat T cells 2.5 h PMA stimulation, dark grey: PBMCs 2.5 h PMA stimulation, white: Jurkat T cells 20 h PMA stimulation, black: PBMCs 20 h PMA stimulation. Data represent mean ± SE of at least four independent experiments.

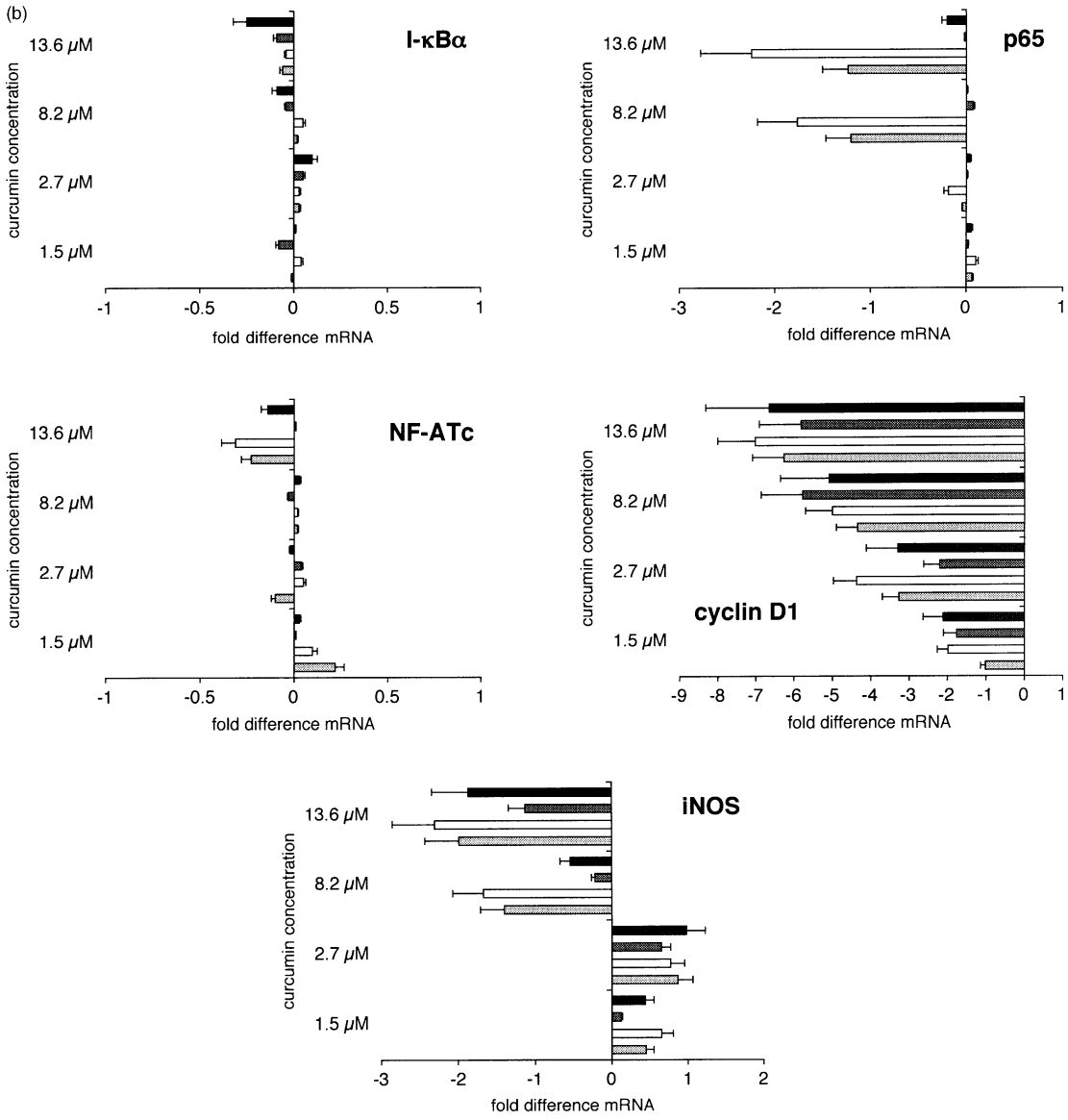

Figure 1. (*continued*).

viability (Fig. 3). The cytotoxic effects of curcumin were less pronounced in PBMCs and only detectable above 12 μM (data not shown). Because dead cells rapidly inhibit and degrade certain mRNA types, the RT-rt-PCR experiments performed in this concentration range need to be interpreted accordingly. The overall down-regulation of the gene transcripts measured in Jurkat T cells therefore also correlated with the increasing amount of dead cells in the population.

In the present study, we have confirmed the immunomodulatory potential of curcumin at the gene transcription level. Our results for the first time provide evidence that curcumin inhibits GM-CSF, IL-2 and IL-6 transcription in $CD4^+$ T cells at concentrations lower than 8 μM. Because GM-CSF is also strongly inhibited in PBMCs it is likely to be a transcription-specific effect. In order to explain this specific inhibition of GM-CSF transcription in both cell cultures, further studies will have to evaluate which target or cascade is modulated at such low curcumin concentrations. Our data are along the same line with previous data by Kobayashi et al.,[29] where it was shown that curcumin reduces GM-CSF and IL-2 protein products in lymphocytes from bronchial asthmatics. We show that the inhibition of PMA-induced IL-2 and IL-6 gene transcription in Jurkat cells is specific to leukemia cells because it was not detected in PBMCs. Since IL-2 production is essential for the survival of leukemia cells, the inhibition of IL-2 gene expression, as shown at the transcription and protein level, might be one reason for the significantly higher cytotoxicity of curcumin in leukemia cells. Interestingly, curcumin had a stimulatory effect on IFN-γ transcription in Jurkat cells and PBMCs. In both cell types, IFN-γ mRNA was up-regulated significantly but more pronounced in Jurkat T cells. These results are opposed to the findings of Kang et al.,[23] where it was shown that IFN-γ production was inhibited in macrophages. Curcumin has previously been shown to modulate NO production[25] by inhibiting iNOS expression in vitro.[26,27] In

our experiments with Jurkat T cells the inhibition of PMA-induced iNOS mRNA was however not very marked when compared to the cytokines. This might be partly explained by the fact that we used lower curcumin concentrations. Pan et al.[27] used 10 μM curcumin to show that iNOS mRNA induction by LPS was potently inhibited. In our system 10 μM curcumin already had cytotoxic effects, leading to an overall down-regulation of mRNAs including the house-keeping genes. Accordingly, the results of the cell viability test revealed that curcumin concentration above 8 μM showed a time-dependent cytotoxicity (Fig. 3), whereas lower concentrations did not signicantly influence the cell viability for a time period of 48 h. Our results further suggest that the differential effects of curcumin on cytokine expression cannot solely be explained by its inhibition of individual transcription factors, such as AP-1 or NF-κB. Moreover, we conclude that curcumin targets a variety of factors and that the differential impact on gene transcription is the sum of all modulations with a certain concentration and time. Since curcumin is often used in pharmacological experiments as a typical inhibitor of AP-1 or NF-κB,[30,31] the interpretation of such results could thus be misleading, especially without analyses of transcriptome data and cell viability.

Curcumin also affected cyclin D1, which is the regulatory subunit of certain protein kinases, such as cdk5,

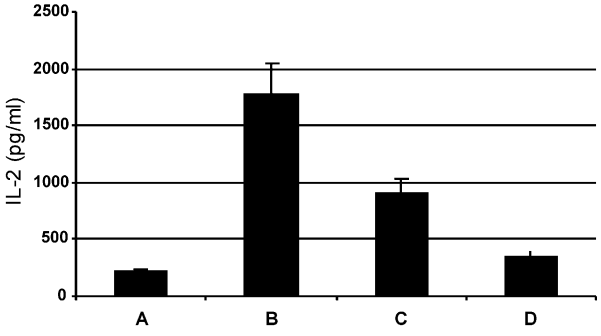

Figure 2. IL-2 specific ELISA. Serum control (A), 24 h PMA stimulation (2 μg/mL) (B), 24 h PMA stimulation (2 μg/mL) and 8.2 μM curcumin, (C), PMA stimulation (2 μg/mL) and13.6 μM curcumin (D). Data represent means of three experiments ± SE.

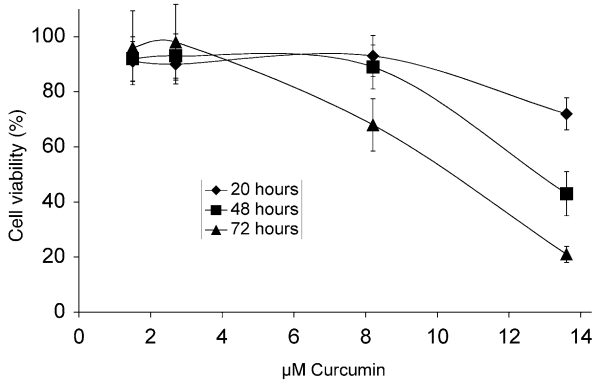

Figure 3. Cell viability with trypan blue exclusion after treatment with different concentrations of curcumin. 100% cell viability was detected for untreated controls. The data are expressed as mean values (% death) ± SE of three experiments.

which advances the G1 phase of the cell cycle. Deregulated cyclin D1 expression has been implicated in several human neoplasms, including leukemia cells[32] and this gene is therefore a possible target for anticancer drugs. Curcumin down-regulated cyclin D1 mRNA levels earlier and more strongly than the house-keeping mRNAs, pointing to a likely anti-neoplastic effects of this compound. Our results with cyclin D1 are therefore comparable to recent results obtained with curcumin in gastric and colon cancer cells, which also abundantly express cyclin D1.[33] There it was suggested that the down-regulation of cyclin D and E observed might lead to cell cycle arrest and then lead to apoptosis. This is also confirmed by our results with human mononuclear blood cells, where PMA-induced cyclin D1 mRNA expression is inhibited by curcumin. To our knowledge, the induction of cyclin D1 upon PMA treatment of PBMCs has not been shown before but coincides with the known tumor promoting effects of phorbol esters.[34] We also measured the mRNA levels of the transcription factors NF-κB and NF-ATc. Non-cytotoxic concentrations of curcumin only significantly down-regulated the mRNA level of the NF-κB subunit p65. Whereas PMA did not influence the NF-ATc mRNA baseline level in Jurkat cells, we could show that this important factor was up-regulated in PBMCs upon PMA stimulation. Curcumin did however not influence PMA induced NF-ATc mRNA expression. The down-regulation of the house-keeping mRNAs at higher curcumin concentrations correlated with an overall down-regulation of mRNAs due to cell death and can therefore be used as an internal factor to control the specificity of the RT-rt-PCR results.[28]

Conclusion

In conclusion, we show that curcumin not only modulates $CD4^+$ T cells via modulation of gene expression in macrophages as previously suggested[23] but that it directly modulates gene transcription in CD4 T cells. The potent down-regulation of GM-CSF mRNAs observed with low concentrations of curcumin might suggest a possible application of curcumin in the treatment of diseases related to increased GM-CSF, such as chronic inflammation or asthma.[35,36]

Experimental

Chemicals

Pure curcumin was isolated from commercially available curcumin (diferuloylmethane), M_r 368.39 (purity 99%) from Fluka AG (Switzerland). This curcumin was found to also contain other curcuminoids in higher amounts and it was therefore necessary to employ a straight-forward isolation and purification method. Low yields of curcumin (purity >99%) were isolated with preparative thin layer chromatography (TLC) on silica gel, using chloroform/ethanol/glacial acetic acid (94:5:1) as solvent. A ^1H NMR spectrum (Bruker, AMX-300) was taken to confirm identity and TLC was used to cover the purity of the isolate.

Cell culture and incubation conditions

$CD4^+$ Jurkat human leukemia T cells (ATCC TIB-152) or freshly isolated PBMCs were cultured in RPMI 1640 medium [(Gibco, Life Technologies) supplemented with 10% fetal bovine serum (FBS), 1 μg/mL fungizone (amphotericin B), 100 units/mL penicillin, 100 μg/mL streptomycin and 2 mM L-glutamine (all from Gibco, Life Technologies, Switzerland)] at 37 °C and 5% CO_2 in 50 mL culture flasks (TPP). 5×10^5 cells from a log-culture were suspended with 1 mL of fresh medium in a 24-well plate and left to rest for 3 h. Curcumin was dissolved in 96% ethanol at a concentration of 4 mg/mL and diluted (1:1) in sterile water to provide the stock solution. Curcumin was then added to the cells and left for 2 h before stimulation. Depending on the experiment, between 1.5 μM (0.55 μg/mL) and 13.6 μM (5 μg/mL) curcumin was added to the culture medium. For mitogen activation, to each well containing 1 mL cell suspension, 2 μg phorbol 12-myristate 13-acetate (PMA) (ICN, Switzerland) was added. The total solvent part never exceeded 0.5% and no effects were detected with a solvent control only. To diminish variability and pipetting errors, three wells were finally pooled for RNA extraction as one experiment with 1.5×10^6 cells. 2.5 μM cyclosporin A (Fluka) was used as a positive control.[28] The PBMCs were isolated with Polymorphprep (Axis Shield) by following the enclosed manufacturers instructions.

RNA extraction and reverse transcription

Cells were harvested and centrifuged. All pipette tips, plastic tubes and glassware were treated with diethyl pyrocarbonate (DEPC) to destroy ribonucleases. Total cytoplasmatic RNA was isolated from cells, using RLN buffer (Qiagen) and RNeasy spin columns (Qiagen), according to manufacturers instructions, and dissolved in 50 μL deionised and DEPC-treated water. RNA was checked for DNA contamination on an ethidium bromide-stained 2.5% agarose gel. 10 μg total RNA was incubated with random hexamers (Microsynth, Switzerland) and 30 units/mL ribonuclease inhibitor RNasin (Promega) at 60 °C for 5 min and cooled stepwise to 20 °C. Final random hexamer concentration was 3 μM. 100 units/mL reverse transcriptase Omniscript (Qiagen) and dNTPs (10 mM each) (Perkin-Elmer) were added and reverse transcription took place at 37 °C for 60 min in a Gene Amp 2400 thermocycler (Perkin-Elmer). All samples were reverse transcribed under the same conditions and from the same reverse transcription master mix in order to minimize differences in the reaction efficiency.

Design of primers and TaqMan® probes

Primers and probes were designed to the cDNA sequences of the genes investigated, using DNA sequence entries from GenBank™ (see Table 1). Primer and probe sequences were chosen to prevent homologies to other genes. To exclude amplification from possible DNA contamination, either the probes or the forward primers were designed to overlap intron-spanning cDNA regions. Primers and probes were made according to instructions of Primer Express software version 1.5 (PE Biosystems). All probes but GAP-DH (purchased as Pre-Developed Factor from PE Biosystems) were labeled with the fluorescent dyes 5′-FAM (6-carboxy-fluorescein) as reporter and 3′-TAMRA (6-carboxy-tetramethyl-rhodamine) as quencher. GAP-DH was 5′-VIC labeled. The primer and probe sequences for IL-2, GM-CSF, cyclin D1, β-actin, p65, I-κBα, and NF-ATc have been published previously.[28]

Real-time PCR and determination of relative mRNA amounts

Relative mRNA expression profiles were determined without normalizing to any specific gene as equal test cell populations showed very little variability in their transcription rates. Relative mRNA levels could therefore be calculated between treated and non-treated Jurkat populations. The house-keeping genes were used as controls to determine the degree of pharmacological specificity. The TaqMan technology employed for our RT-rt-PCR makes use of labeled oligonucleotide probes and a 5′-nuclease PCR assay. The probe has a reporter dye FAM and a quencher dye TAMRA covalently attached to the 3′-end via a linker group. The reporter generates a fluorescent signal upon cleavage by the Taq polymerase. Quantification is based on the early, linear part of the reaction, and by determining the threshold cycle (C_T), at which fluorescence above background is first detected. 5 μL of each cDNA sample was mixed with Universal PCR Master Mix (PE Biosystems) and depending on the gene between 100 and 300 nM primers and probe was added to obtain a 25 μL reaction. Real time PCR was performed in 96-well reaction plates and optical caps (Biolabo, Switzerland). The amplification efficiency for each specific primer and probe template system was calculated on the ABI PRISM 7700 real time thermocycler (PE Biosystems) by measuring the increase in signal with each cycle. Negative controls with total RNA or no template were performed. Serial dilutions of the RNA were reverse transcribed to provide the standard curve. This was achieved by plotting the dilution factor against the C_T values. At least four independent experiments and eight reverse transcriptions were performed. This gave a normalized value for the mRNA expression level of each gene. To calculate the percentage of inhibition we assumed a theoretical zero amount of cDNA after cycle 34. Relative quantity values were either generated as fold difference with respect to the known dilution differences in fluorescent output, or with respect to the theoretical zero amount at C_T 34. Data analysis was performed with Microsoft Excel.

Measurement of IL-2 release

Total IL-2 concentrations were measured quantitatively with a photometric enzyme-linked immunosorbant assay (ELISA) kit (Roche, Switzerland). Jurkat T cells were incubated without or with curcumin prior to PMA-stimulation. The PMA-induced release of IL-2 into RPMI 1640 medium was quantified after 2.5 and 20 h according to manufacturers instructions.

Measurement of cell survival and cytotoxicity

5×10^5 Jurkat or mononuclear cells were incubated with curcumin. After 20, 48, and 72 h, cell viability was measured by trypan blue dye exclusion and counting of the viable cell population in a Neubauer hemocytometer. The assay was performed in triplicate, and for each condition, at least 400 cells were counted. The percentage of dead cells was calculated with respect to an untreated cell population. The data are expressed as mean values (% death)$\pm$SE.

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