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Curcumin Inhibits the Growth, Induces Apoptosis and Modulates the Function of Pituitary Folliculostellate Cells

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Key Words
Curcumin · Folliculostellate pituitary cells · Proliferation · Cell cycle · Apoptosis · Vascular endothelial growth factor · Interleukin-6 · Tlr4 · Nuclear factor-κB

Abstract
The polyphenol curcumin (diferuloylmethane) is the active componenet of the spice plant *Curcuma longa* and has been shown to exert multiple actions on mammalian cells. We have studied its effect on folliculostellate (FS) TtT/GF mouse pituitary cells, representative of a multifunctional, endocrine inactive cell type of the anterior pituitary. Proliferation of TtT/GF cells was inhibited by curcumin in a monolayer cell culture and in the colony formation assay in soft agar. Fluorescence-activated cell-sorting (FACS) analysis demonstrated curcumin-induced cell cycle arrest at G_2/M accompanied by inhibition of cyclin D_1 protein expression. Curcumin had a small effect on necrosis of TtT/GF cells, but it mainly stimulated apoptosis as demonstrated by FACS analysis (Annexin V-fluorescein isothiocyannate/7-aminoactinomycin D staining). Curcumin-induced apoptosis involved suppression of Bcl-2, stimulation of cleaved caspase-3 and induction of DNA fragmentation. Functional studies on FS cell-derived compounds showed that curcumin inhibited mRNA synthesis and release of angiogenic vascular endothelial growth factor-A (VEGF-A). Immune-like functions of FS cells were impaired since curcumin downregulated Toll-like receptor 4, reduced nuclear factor-κB expression and suppressed bacterial endotoxin-induced interleukin-6 (IL-6) secretion. The inhibitory action of curcumin on VEGF-A and IL-6 production was also found in primary rat pituitary cell cultures, in which FS cells are the only source of these proteins. The observed effects of curcumin on FS cell growth, apoptosis and functions may have therapeutic consequences for the intrapituitary regulation of hormone production and release as well as for pituitary tumor pathogenesis.

Introduction

The anterior pituitary is not only composed of organotypic endocrine cells but contains another pituitary-specific cell type, the so-called folliculostellate (FS) cells, which have a stellar morphology, are able to form small intrapituitary follicles and are immunopositive for S-100 protein and glial fibrillary acidic protein [1–3]. FS cells do

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not produce hormones and represent about 5% of all anterior pituitary cells [2, 3]. They form an intrapituitary network with their long cellular processes [4, 5] and are connected to each other by gap junctions and to endocrine cells, suggesting a direct cell-to-cell communication between FS and hormone-producing cells [6–8]. FS cells have also been shown to phagocytose intrapituitary cell debris [9, 10]. The origin of FS cells is still unclear, and it is still a matter of debate whether they might derive from dendritic immune cells, astrocytes, or the recently identified hypophyseal progenitor cells [12, 13]. FS cells are thought to play regulatory and coordinative roles for the endocrine pituitary cells since they contain receptors for many cytokines, neuropeptides and hormones and because they secrete a number of substances, among them vascular endothelial growth factor-A (VEGF-A) [13] and interleukin-6 (IL-6) [14, 15]. VEGF-A is needed to maintain blood vessel integrity, regulates vascular permeability and is one of the most important stimulators of angiogenesis [16]. FS cell-derived IL-6 is an important intrapituitary regulator of anterior pituitary hormone secretion [15]. FS cells also express the Toll-like receptor 4 (TLR4) through which bacterial endotoxin (lipopolysaccharide, LPS) induces intrapituitary IL-6 secretion during infectious or inflammatory processes [17, 18].

Plants contain polyphenolic substances, some of which are used as spices and/or as drugs in traditional herbal medicine. During recent years, a growing number of these drugs have been tested preclinically and clinically for their efficacy in a broad range of diseases. One of the most promising compounds is curcumin (diferuloylmethane), a biphenolic natural product and the active component of turmeric *(Curcuma longa),* which gives Indian curry its yellow color and distinctive taste [19]. Recent preclinical studies have shown that curcumin has antiproliferative, proapoptotic, antiangiogenic, antioxidative, anti-inflammatory and neuroprotective actions [20–24]. Several clinical trials are currently running to test the efficacy of this drug in the treatment of colon and pancreatic cancer as well as in inflammation-linked disorders like psoriasis and Alzheimer's disease [20, 25–27]. Regarding its mechanism of action, curcumin was shown to act at multiple levels. Curcumin affects gene expression by modifying histone acetylation [28], influences cyclin and cyclin-dependent kinases, modulates PI3 kinase/Akt and MAP kinase signaling pathways, induces proapoptotic proteins (Bcl-2, Bax), reduces secretion of angiogenic factors and modifies the expression of transcription factors (e.g. down-regulates nuclear factor-κB, NF-κB) [23, 29–31].

In a first study in the pituitary, it has recently been shown that curcumin inhibits proliferation, induces apoptosis and suppresses hormone secretion in the lactosomatotroph GH3 and lactotroph MMQ pituitary tumor cell lines [32]. In the present work, we show that curcumin affects growth, apoptosis and function of FS pituitary cells.

Materials and Methods

Materials

Cell culture materials and reagents were obtained from Life Technologies, Inc. (Karlsruhe, Germany), Falcon (Heidelberg, Germany), Nunc (Wiesbaden, Germany), Seromed (Berlin, Germany), Flow Cytometry Standards Corp. (Meckenheim, Germany), and Sigma (St. Louis, Mo., USA). Curcumin (95%, turmeric powder) and dimethylsulfoxide (DMSO) were purchased from Sigma. Due to nonavailability of pure curcumin preparations, the 95% curcumin powder is widely used throughout for in vitro and in vivo studies. The components of the remaining 5% of the powder are not known, but are supposed to be salts, amino acids, sugars and soluble cell wall fragments without specific effects on mammalian cells.

Cell Culture and Curcumin Stimulation

FS TtT/GF mouse pituitary tumor cells [33] were cultured at 5% CO_2 and 37°C in Dulbecco's Modified Eagle's Medium (DMEM; pH 7.3) supplemented with 10% fetal calf serum, 2.2 g/l $NaHCO_3$, 10 mM HEPES, and 2 mM glutamine, 2.5 mg/l amphotericin B, 10^5 U/liter penicillin-streptomycin, 5 mg/l insulin, 5 mg/l transferrin, 20 μg/l sodium selenite and 30 pM triiodothyronine (Henning, Berlin, Germany).

For primary rat pituitary cell culture, pituitaries of adult male Sprague-Dawley rats (150–180 g) were mechanically and enzymatically dispersed as previously described [34]. Dispersed cells were cultured under the same conditions and in the same medium as TtT/GF cells. Experiments were performed after an initial attachment period of 48 h.

For in vitro experiments with curcumin, a stock solution of 10 mM curcumin (prepared in DMSO) was diluted with cell culture medium to obtain concentrations of 0.5–30 μM curcumin for cell treatments. During the handling of curcumin, exposure to light was reduced as much as possible. Since the solution with the highest curcumin concentration contained 0.3% DMSO, corresponding controls with this DMSO concentration were performed in each experiment to exclude effects of this compound. Depending on the experimental protocol, cells were treated with curcumin for various periods of time as indicated.

In vitro Proliferation Assays

Three methods were applied to study TtT/GF cell growth. For ^{3}H-thymidine incorporation experiments, TtT/GF cells were treated with curcumin for 24, 48 and 72 h with ^{3}H-thymidine being present during the last 3 h of the treatment period. Incorporated ^{3}H-thymidine was determined as previously described [34]. In addition to this, the effect of curcumin on TtT/GF cell growth was confirmed by direct cell counting using an adapted Coulter counter.

The soft-agar colony-forming assay was used to assess the growth-inhibitory effect of curcumin on TtT/GF cells in a 3-dimensional in vitro cell culture model. Native and curcumin-pretreated (24 h, 30 μM) TtT/GF cells were seeded in soft agar. To this end, cells were suspended in 0.3% agar (Noble agar, Sigma, Germany) solution consisting of 4,5 ml 2× DMEM medium (Invitrogen, Germany), 4.5 ml 1× DMEM medium and 2.25 ml fetal calf serum. The cells were plated in triplicate in 6-well plates (Nunc), coated with 0.5% agar at a density of 8,000 cells per well and maintained at 37°C for 14–21 days. Curcumin solution (30 μM, 1 ml per well) was added to native and pretreated cells on top of agar on the first day after seeding, and was then renewed every 4 days. During the whole treatment period, cell plates were covered with aluminum foil for maximum protection from light. At the end of the treatment period, colonies were stained with 0.05% thiazolyl blue tetrazolium bromide (Sigma) solution in 0.01 M PBS. Photographs were made with a Nikon D80 camera with a Nikkor 60-mm lens. Colony counting was performed with a special analyzing tool from S.CO LifeScience GmbH, Garching, Germany.

Fluorescence-Activated Cell-Sorting Analysis

Fluorescence-activated cell-sorting (FACS) analysis was performed to determine the effect of curcumin on TtT/GF cell cycle arrest and apoptosis/necrosis. For studies on cell cycle arrest, cells were treated for 24 h, harvested by trypsinization and fixed in 70% ethanol at 4°C overnight. Samples of fixed cells were resuspended and stained in PBS containing 20 μg/ml propidium iodine (PI) and 10 μg/ml RNAse A. FACS analysis of PI emission at 630 nm was performed using a Beckman Coulter (Krefeld, Germany) XL flow cytometer. After gating out doublets and clumps, cell cycle analysis of DNA histograms was done using the Multicycle software (Phoenix Flow Systems, San Diego, Calif., USA).

Curcumin-induced apoptosis and necrosis were assessed by FACS analysis after treatment of TtT/GF cells with 30 μM curcumin for various periods of time measuring Annexin V-fluorescein isothiocyanate (FITC)/7-aminoactinomycin (7-AAD) staining with a commercially available kit (Beckman Coulter) according to the manufacturer's instructions. Excitation wavelength was 488 nm, FITC fluorescence was recorded on a fluorescence 1 (FL1) sensor (515 nm-545 nm BP), and 7-AAD fluorescence was recorded on a fluorescence 4 (FL4) sensor (600 nm LP). Data analysis was performed with Coulter XL™ software EXPO 32™.

Detection of Apoptosis by DNA Fragmentation

In addition to FACS analysis, DNA fragmentation as an indicator of apoptosis was measured by cell death detection ELISA (DNA Fragmentation Kit; Roche, Penzberg, Germany). After seeding and 24-hour treatment with 5–30 μM curcumin, TtT/GF cells were harvested using lysis buffer provided with the kit, and DNA fragmentation activity was quantified in triplicate according to the manufacturer's instructions.

Western Blot Analysis

Western blot was performed as previously described [35]. After treatment of TtT/GF cell cultures with 5, 10, 20 and 30 μM curcumin for different periods of time, cells were washed once with PBS (pH 7.0), and proteins were extracted in a commercially available protease inhibitor cocktail (Sigma). According to the manufacturer's instructions, 50 mg protein mixture obtained from each sample were separated using a precast Tris-glycine 12% gel (Anamed, Darmstadt, Germany) in an Invitrogen electrophoresis apparatus, and then transferred on a nitrocellulose membrane (Hybond ECL). After blocking for 2 h at room temperature in a 1× TBS/0, 1% Tween solution (TBS-T) containing 5% milk powder, the membrane was then incubated overnight at 4°C with the primary antibody diluted in 2.5% milk powder/TBS-T. Subsequently, the membrane was washed and incubated with horseradish peroxidase (HRP)-conjugated secondary antibody for 1 h at room temperature. The membrane was then washed and protein bands were visualized using a commercially available chemiluminescence kit (Roche) according to the manufacturer's instructions. Primary antibodies against Bcl-2, caspase-3, cyclin D_1 (all from Cell Signaling Technology, Beverly, Mass., USA) and NF-κB/p65 (Santa Cruz Biotechnology, Santa Cruz, Calif., USA) were made in rabbits and anti-β-actin antibody (Millipore Corp., Billerica, Mass., USA) was made in mice. HRP-conjugated secondary antibodies against rabbits and mice, respectively, were all obtained from Santa Cruz Biotechnology.

Measurement of VEGF and IL-6

Mouse and rat VEGF and IL-6 were measured in cell culture supernatants using species-specific ELISAs (R&D Systems, Wiesbaden, Germany) according to the manufacturer's instructions.

Real-Time PCR

For quantitative analysis of TLR4 and VEGF-A mRNA, TtT/GF cells were treated for 24 h with the indicated concentrations of curcumin. Variable amounts of total RNA were isolated using the NucleoSpin RNA II Kit (Macherey-Nagel, Düren, Germany) but only 1 μg of total RNA was reverse transcribed with Superscript II (Invitrogen) after DNAse treatment. All steps required for reverse transcription were performed according to the manufacturer's protocol. For quality control, a small aliquot of cDNA was analyzed on an agarose gel.

PCR amplifications were performed in duplicates using the Lightcycler®2.0 instrument (Roche Diagnostics, Mannheim, Germany) and QuantiFast SYBR Green RT-PCR Kit (Qiagen, Hilden, Germany) under the following PCR conditions: initial denaturation at 95°C for 5 min, 40 cycles of denaturation (95°C for 10 s), annealing and elongation (60°C for 30 s). At the end of every run, a melting curve (50–95°C with 0.1°C/s) was generated to ensure the quality of the PCR product. The primers for quantification of TLR4 (NCBI gene accession No. NM_021297.2), VEGF-A (NM_001025250) and HPRT (NM_013556) were 5′-ACAGCAGAGGAGAAAGCATC-3′ (TLR4, forward) and 5′-GGGGCACTCCTTCTTCTAAA-3′ (TLR4, reverse), 5′-GCAGACCAAAGAAAGACAGAA-3′ (VEGF-A, forward) and 5′-GTTTAACTCAAGCTGCCTCG-3′ (VEGF-A, reverse), 5′-ACCTCTCGAAGTGTTGGATACAGG-3′ (HPRT, forward) and 5′-CTTGCGCTCATCTTAGGCTTTG-3′ (HPRT, reverse). Crossing points were calculated with the LightCycler®Software 4.0 (Roche Diagnostics) using the absolute quantification fit point method. Threshold and noise band were set to the same level in all compared runs. Relative gene expression was determined by the $2^{-\Delta\Delta CT}$ method [20] using the real PCR efficiency calculated from an external standard curve. Quantification was carried out using a dilution standard from pooled sample probes. The crossing point was normalized to the housekeeping genes HPRT and GAPDH. As there was no difference between HPRT and GAPDH, the following measurements were done with HPRT as housekeeping gene.

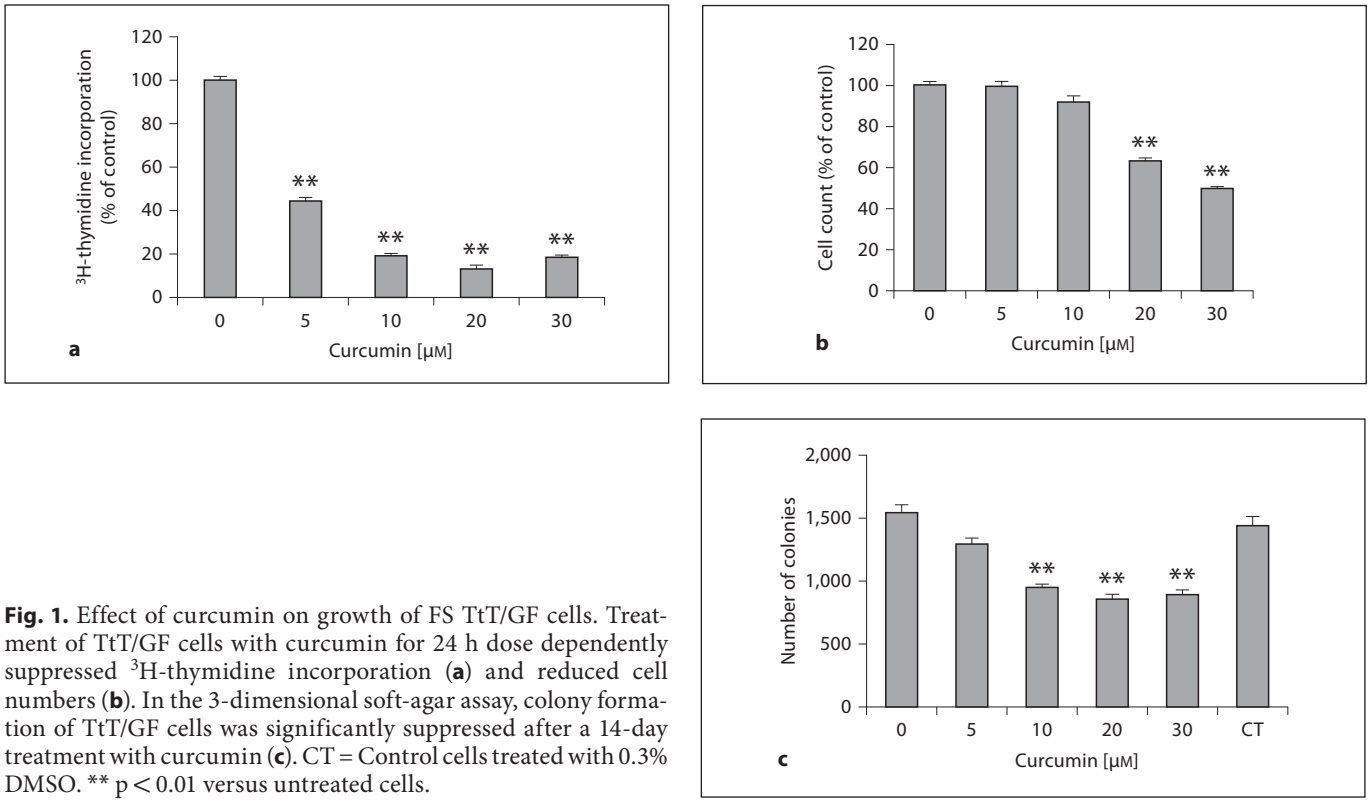


Fig. 1. Effect of curcumin on growth of FS TtT/GF cells. Treatment of TtT/GF cells with curcumin for 24 h dose dependently suppressed ^{3}H-thymidine incorporation (**a**) and reduced cell numbers (**b**). In the 3-dimensional soft-agar assay, colony formation of TtT/GF cells was significantly suppressed after a 14-day treatment with curcumin (**c**). CT = Control cells treated with 0.3% DMSO. ** $p < 0.01$ versus untreated cells.

Statistics

Each of the experiments was repeated at least 3 times. The individual experiments were performed with quadruplicate wells. Results were analyzed by one-factorial univariate analyses of variance (ANOVA) and followed by univariate F tests where appropriate. $p = 0.05$ was accepted as the nominal level of significance; it was corrected (according to the Bonferroni correction procedure) for all post-hoc tests in order to keep the type I error less or equal to 0.05. Data are given as mean ± standard error of the mean (SEM).

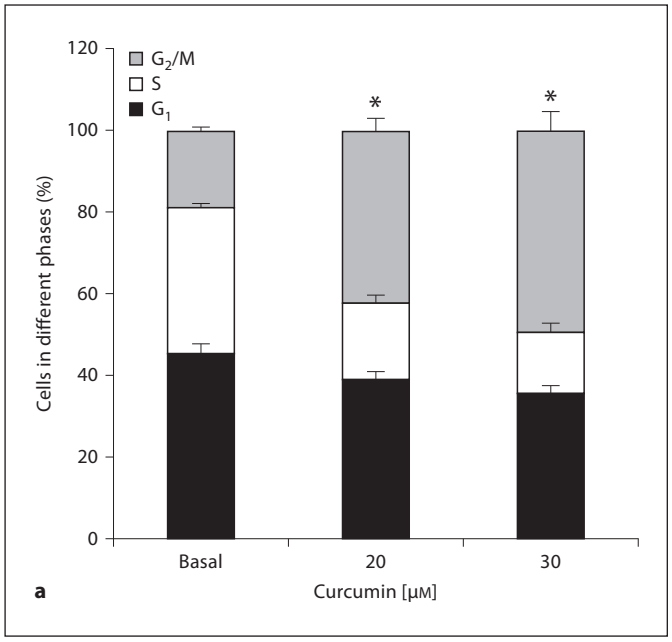
Results

Effect of Curcumin on TtT/GF Cell Proliferation

In TtT/GF monolayer cell cultures, curcumin dose dependently inhibited both ^{3}H-thymidine incorporation and the increase in cell number. Significant growth inhibition was obtained with 5–30 μM curcumin for more sensitive ^{3}H-thymidine incorporation and with 20 and 30 μM curcumin for cell numbers. The inhibitory effect was observed after treatment for 24 h (fig. 1a, b) and persisted after 48- and 72-hour treatment periods (not shown). In order to test whether the growth-inhibitory effect of curcumin was not only restricted to monolayer cell cultures, the antiproliferative effect of curcumin on TtT/GF cells was also studied in a 3-dimensional soft agar cell culture model. In one experiment, TtT/GF cells were pretreated with curcumin for 24 h before seeding in soft agar, and treatment with curcumin was then continued. In the other experiment, cells were only treated with curcumin after seeding. Curcumin significantly reduced the number of colonies formed in soft agar of both native (not shown) and pretreated (fig. 1c) TtT/GF cells during the treatment period.

Influence of Curcumin on TtT/GF Cell Cycle

FACS analysis was performed to determine at which phase of the cell cycle curcumin induced growth arrest in TtT/GF cells. In parallel, the expression of cyclin D_1, a key protein regulating the cell cycle, was measured by Western immunoblotting in extracts of curcumin-treated cells at the indicated time periods (fig. 2). A 24-hour curcumin treatment induced a dose-dependent reduction in TtT/GF cell number at G_1 accompanied by an increase in cell number at the G_2/M phase (fig. 2a), suggesting cell cycle arrest in the G_2/M phase. In extracts of TtT/GF cells, 24 h of treatment with curcumin led to a downregulation of cyclin D_1 (fig. 2b), which is needed for cell promotion through the initial phase of G_1.



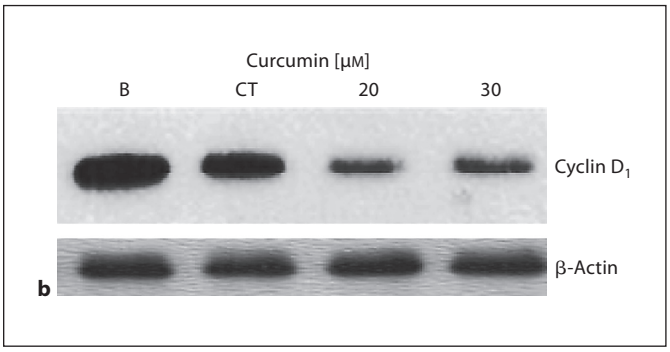


Fig. 2. Influence of curcumin on the cell cycle of TtT/GF cells. Treatment of TtT/GF cells for 24 h with 20 and 30 μM curcumin induced significant accumulation of cells in G_2/M phase as demonstrated by FACS analysis (**a**). The expression of cyclin D_1 protein, which promotes cells to pass through early G_1 phase, was reduced by 24-hour curcumin treatment (**b**). * $p < 0.05$ versus basal.

Curcumin Induced Apoptosis in TtT/GF Cells

The observed decrease in TtT/GF cell numbers after curcumin treatment might not only be a consequence of suppression of proliferation, but may also result from induction of cell death by apoptosis. Therefore, alterations in the expression of apoptosis-regulating proteins were studied; two different methods were applied to detect apoptosis. The apoptosis-associated shift of membrane serine phospholipids was determined by Annexin V-FITC followed by FACS analysis, and apoptotic DNA degradation was measured by a specific ELISA.

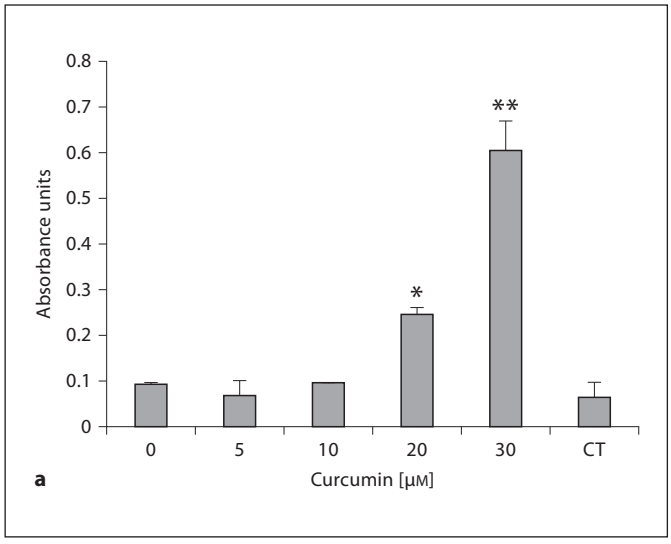
Western immunoblot analysis showed a curcumin-induced, dose-dependent reduction of anti-apoptotic-acting Bcl-2 and an increase of apoptosis-promoting cleaved caspase-3 (fig. 3b). Curcumin induced a dose-dependent increase in apoptotic DNA fragmentation as measured by ELISA (fig. 3a). FACS analysis for 7-AAD staining showed a small increase in necrotic cell number, which indicates that curcumin has some unspecific cytotoxic effects on FS TtT/GF cells (fig. 3c). Thus, curcumin not only suppressed proliferation of TtT/GF cells but also induced apoptosis.

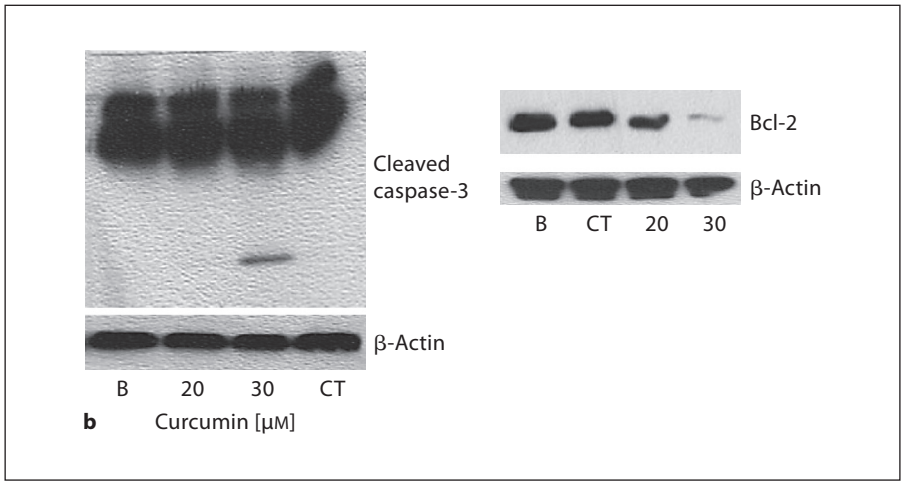
Suppression of Angiogenic VEGF-A by Curcumin

Because curcumin has been shown to act as an antiangiogenic substance, we studied its effect on mRNA synthesis and release of the angiogenic factor VEGF-A, known to be produced by TtT/GF cells. Treatment of the latter with curcumin for 24 h resulted in a dose-dependent inhibition of VEGF-A mRNA synthesis as determined by quantitative RT-PCR (fig. 4a). Consequently, the release of VEGF-A protein by TtT/GF cells was also dose dependently suppressed by curcumin (fig. 4b).

Influence of Curcumin on Immune-Like Components of TtT/GF Cells

FS cells are often considered as a local, intrapituitary immune cell type due to their ability to respond to circulating compounds produced by activated immune cells and to produce IL-6 [15]. We have tested the effect of curcumin on IL-6 production by TtT/GF cells and have found that curcumin had no significant effect on basal IL-6 secretion after 24 h of treatment (not shown), but it strongly and dose dependently inhibited the LPS-induced IL-6 release (fig. 5a). We have observed by quantitative real-time PCR that curcumin reduced the synthesis of mRNA for TLR4, a membrane-bound receptor mediating LPS effects (fig. 5b). Moreover, Western blot analysis showed that curcumin suppressed the production of NF-κB, a transcription factor mediating the LPS-induced IL-6 production downstream of TLR4 (fig. 5c). Thus curcumin-induced downregulation of TLR4 and suppression of NF-κB may explain the specific suppressive action of curcumin on LPS-stimulated but not on basal IL-6 release.





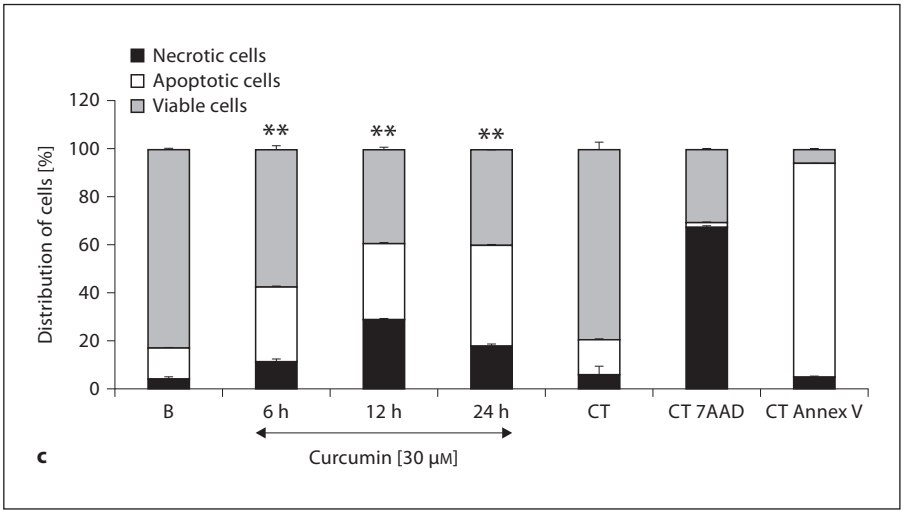


Fig. 3. Induction of apoptosis by curcumin in TtT/GF cells. 24-hour treatment with curcumin induced apoptosis in TtT/GF cells in a dose-dependent manner as determined by Cell Death ELISA (**a**). Cleaved caspase-3 was upregulated whereas Bcl-2 protein was suppressed by curcumin (**b**). A time-dependent increase in the proportion of apoptotic cells was observed by FACS analysis after treatment of TtT/GF cells with 30 μM curcumin (**c**). b = Basal; CT = control with 0.3% DMSO; CT 7-AAD = control for induction of necrosis; CT Annex V = control for induction of apoptosis. **c** Black columns represent the proportion of necrotic cells, white columns are apoptotic cells and gray columns are viable cells. * $p < 0.05$; ** $p < 0.01$ versus basal.

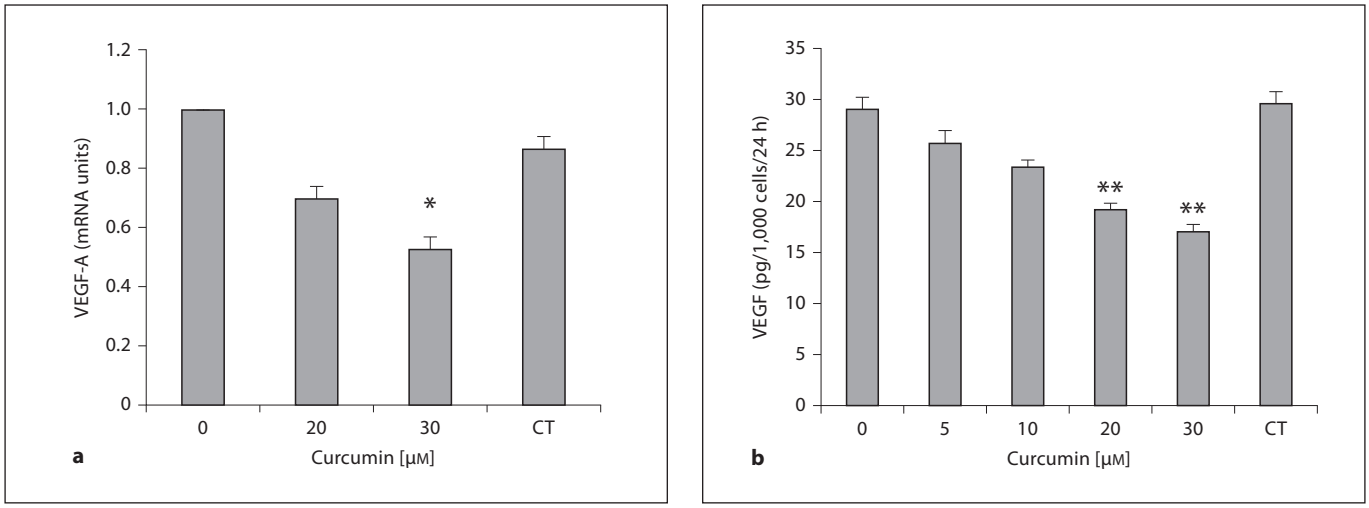


Fig. 4. Suppression of VEGF-A production by curcumin in TtT/GF cells. Treatment of TtT/GF cells with curcumin for 24 h induced a dose-dependent suppression of VEGF-A mRNA synthesis (measured by real-time PCR) (**a**) and VEGF-A release into the cell culture supernatant (determined by ELISA) (**b**). * $p < 0.05$; ** $p < 0.01$ versus basal.

Effects of Curcumin on Primary Rat Pituitary Cell Cultures

In order to prove the relevance of the findings obtained with the TtT/GF pituitary tumor cell line for normal FS cells, the effect of curcumin was studied in primary rat pituitary cell cultures, in which FS cells predominantly produce IL-6 and VEGF-A [15, 35]. Curcumin dose dependently inhibited VEGF-A secretion in rat pituitary cell cultures (fig. 6a). The low basal IL-6 secretion rate was not affected by curcumin whereas the LPS-stimulated IL-6 release was strongly suppressed (fig. 6b). Thus, regarding VEGF-A and IL-6 secretion, curcumin had identical effects in FS TtT/GF pituitary tumor cells and normal FS pituitary cells. At the end of the experiments, the viability of curcumin-treated and untreated cells was controlled by acridine orange/ethidium bromide staining of some control wells [34] to ensure that the effects of curcumin on VEGF-A and IL-6 secretion were not a consequence of the induction of cell death.

Discussion

The polyphenol curcumin is currently being tested for its beneficial therapeutic effects in various diseases ranging from cancer to inflammatory disorders. In the present study, we have shown for the first time that curcumin exerts an antitumor action on FS pituitary tumor cells by suppressing growth, inducing apoptosis and inhibiting VEGF-A and IL-6 production. Interestingly, VEGF-A and IL-6 suppression by curcumin was also found in normal FS cells, suggesting a pharmacological modulation of normal pituitary physiology. The latter observation raises concerns about the supposed disease-specific action of curcumin and thus, the safety of this drug.

The anterior pituitary has been described as a lifelong, slowly expanding endocrine gland. Therefore, in the adult organ, a very low proliferation index and a slightly lower apoptotic index have been measured [36, 37]. Mitotic and apoptotic activities of pituitary cells change depending on the physiological conditions, but only under certain conditions, e.g. during pregnancy and lactation [37]. It is therefore assumed that most FS cells in the normal pituitary are growth arrested and hardly undergo apoptosis. Thus our findings about antiproliferative and pro-apoptotic actions of curcumin in the rapidly growing FS TtT/GF tumor cell line suggest that the combination of both effects may suppress the expansion of FS cell tumors as discussed below, whereas in normal hypophyseal FS cells, the suppressive effect of curcumin on the production of IL-6 and VEGF-A cells comes to the fore.

IL-6 is an important mediator of immune-endocrine interactions, and FS cells are involved in these processes [15, 38, 39]. Membranes of FS cells contain TLR4, through which LPS, a cell wall component of Gram-negative bacteria, stimulates IL-6 secretion by FS cells during bacte-

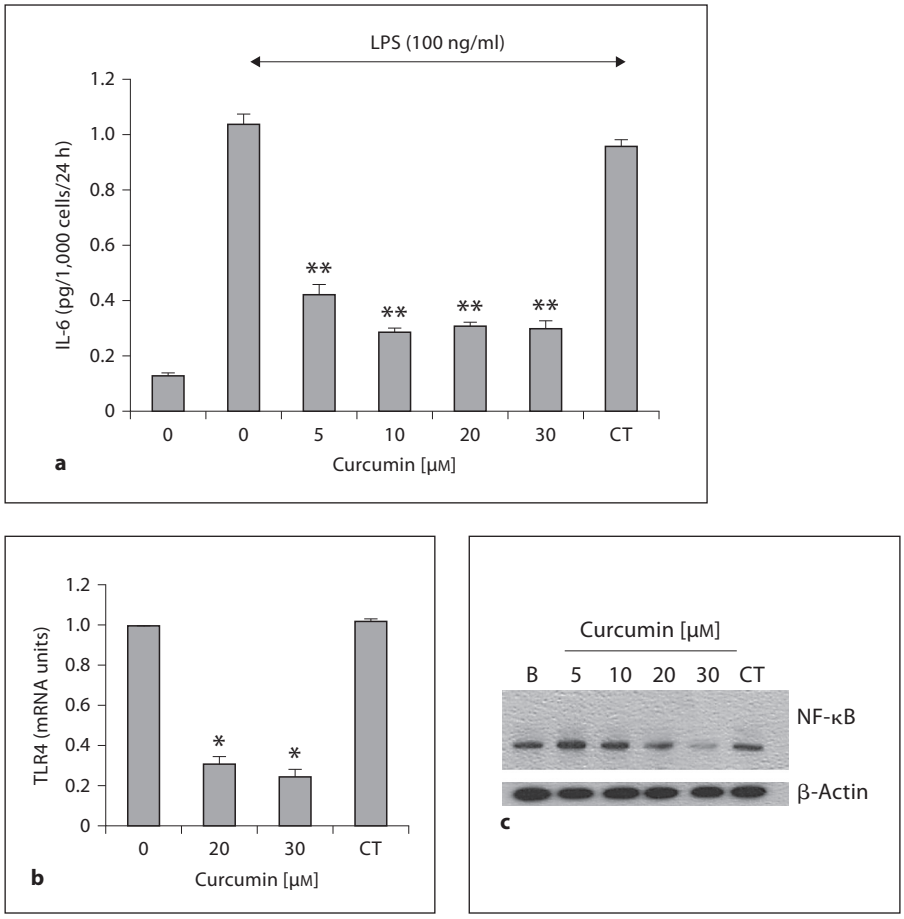


Fig. 5. Influence of curcumin on immune function-related components of TtT/GF cells. Curcumin strongly suppressed LPS-stimulated IL-6 secretion by TtT/GF cells after a 24-hour treatment period (**a**) but had no effect on the low basal IL-6 release (not shown). A strong reduction in TLR4 mRNA synthesis by 24-hour curcumin treatment was observed by real-time PCR (**b**). The protein expression of NF-κB was also reduced by curcumin (**c**). * $p < 0.05$; ** $p < 0.01$ versus untreated cells. CT = Control cells treated with 0.3% DMSO.

rial infections/inflammations [17]. The enhanced intrapituitary IL-6 production, in which NF-κB is critically involved, stimulates the hypophyseal ACTH secretion in a paracrine manner [17, 18]. Thus elevated serum IL-6 levels would act in concert with enhanced intrapituitary IL-6 production to activate anti-inflammatory glucocorticoids. It has already been shown that curcumin acts as an anti-inflammatory agent [40] and that part of its action is mediated by suppression of IL-6, TLR4 and NF-κB in immune cells [40, 41]. We have shown in the present study that curcumin has no effect on basal IL-6 secretion, but that it inhibited LPS-induced IL-6 secretion, both in rat TtT/GF cells and in primary pituitary cell culture. This may be explained by the suppressive action of curcumin on the synthesis of LPS-binding TLR4. Subsequently, through a reduction in the number of membrane-bound TLR4 molecules, LPS would less effectively initiate IL-6 production and release whereas the TLR4-independent basal IL-6 secretion is not affected. Since curcumin also downregulated NF-κB, which mediates effects of LPS on IL-6 production at the transcriptional level, suppression of NF-κB may also participate in the inhibitory effect of curcumin on IL-6 production. However, since NF-κB and TLR4 downregulation occurs at higher curcumin concentrations than needed for suppression of LPS-stimulated IL-6 secretion, curcumin may suppress other, not yet identified curcumin-sensitive components of the complex LPS signaling pathway. Reduction in intrapituitary IL-6 levels would result in reduced paracrine stimulation of ACTH [18] by this cytokine and would finally lead to a less efficient ACTH-induced glucocorticoid production. Thus the intrapituitary effects of curcumin would represent an additional way through which curcumin treatment may modulate the complex endocrine-immune interactions during bacterial infection/inflammation.

VEGF-A, probably the most important angiogenic factor, plays a major role in tumor neovascularization [13, 16, 42]. In normal tissues, VEGF-A is needed for the maintenance of the vascular system and stimulates vessel

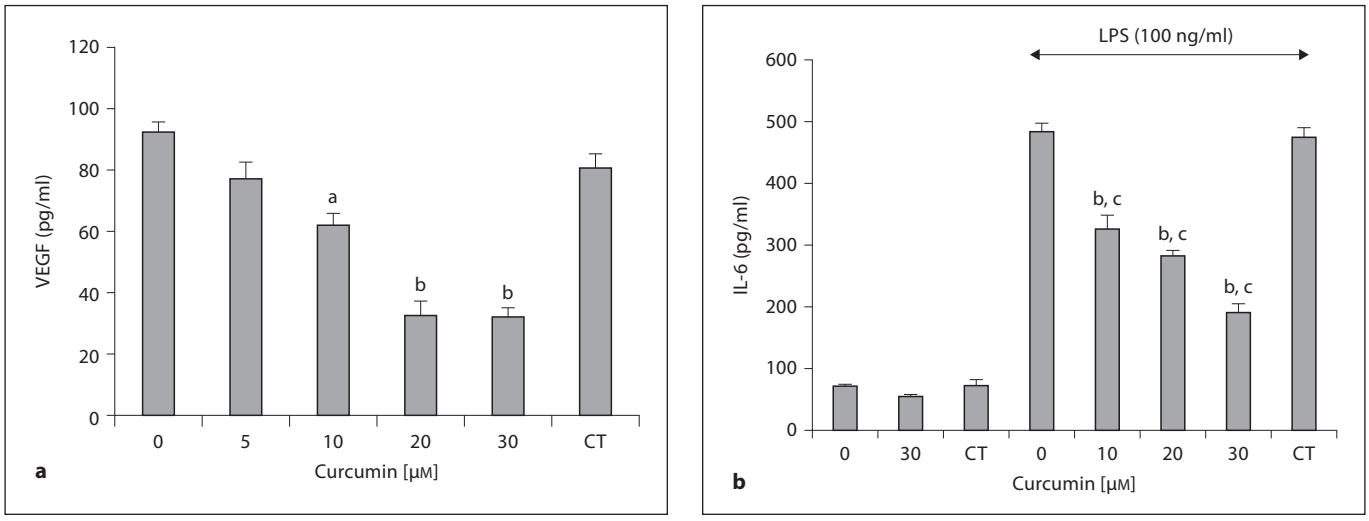


Fig. 6. Effect of curcumin on VEGF and IL-6 secretion in rat pituitary cell cultures. Treatment of primary rat pituitary cell cultures with curcumin for 24 h suppressed VEGF-A secretion in a dose-dependent manner (**a**). The basal IL-6 release was not significantly affected by 24-hour curcumin treatment whereas the LPS-stimulated IL-6 production was dose dependently inhibited (**b**). In contrast to figures 4b and 5a, VEGF-A and IL-6 production is expressed as picograms per milliliter since it is impossible to count the FS cells in a mixed anterior pituitary cell population. However, since there was no change in viability (determined by acridin orange/ethidium bromide staining) in cells in primary rat pituitary cell culture, and because the volume of the cell culture supernatant is constant, curcumin-induced changes in VEGF-A and IL-6 production can be expressed this way. [a] $p < 0.05$; [b] $p < 0.01$ versus untreated cells. [c] $p < 0.01$ versus cells treated with LPS alone.

permeability [16]. The normal anterior pituitary is characterized by an extremely dense vascular network [35], which is needed for optimal regulation of pituitary hormone secretion by hypothalamic or peripheral factors and for rapid release of hypophyseal hormones into the blood stream. FS cells represent the major intrapituitary source of VEGF-A, and its continuous production may be essential for maintaining the dense vascular network needed for optimal function of this central endocrine organ. However, it has also been speculated that hypophyseal VEGF-A may regulate vascular permeability [43] and therefore, stimulation or inhibition of intrapituitary VEGF-A secretion may represent a way to alter pituitary hormone release by modulating intrapituitary vessel permeability. Based on this concept, curcumin would reduce intrapituitary vessel permeability by downregulating VEGF-A production and would thus hamper hypophysiotropic-hypophyseal hormone exchange. However, more work is needed to prove this hypothesis.

In pituitary tumors, IL-6 not only stimulates hormone production, but also stimulates tumor cell proliferation, and VEGF-A does the same, in addition to its stimulatory effects on pituitary tumor neovacularization [15, 35, 44]. FS cell tumors represent an extremely rare pituitary adenoma entity and only few cases have been reported worldwide [45–48]. In this rare tumor type, the effects of curcumin presented here, such as arresting cell cycle, inducing apoptosis and blocking growth-stimulating IL-6 and angiogenic-acting VEGF-A, would have direct antitumorigenic consequences. In other types of pituitary adenoma, the role played by FS cells in tumor formation and progression is still a matter of debate. Earlier reports had postulated that most pituitary adenomas are devoid of FS cells, but this was not confirmed in more recent studies [47–50]. It has also been shown that at the borderline between normal pituitary and pituitary tumor there is a transition zone in which FS cells accumulate [49]. Therefore, it has been hypothesized that intratumoral FS cells or those of the transition zone might produce factors, among them IL-6 and VEGF-A, supporting pituitary tumor progression. Therefore, curcumin would suppress pituitary tumor development by eliminating FS cells through induction of apoptosis at the borderline and inside the tumor and by inhibiting IL-6 and VEGF-A production in the remaining ones. In addition to this, curcumin might have direct suppressive actions on human pituitary tumor cells as has recently been shown for lactotroph MMQ and lactosomatotroph GH3 rat pituitary

tumor cell lines [32]. Thus, there is strong evidence that curcumin acts antitumorigenically in all types of pituitary adenomas through different mechanisms, which may involve suppression of FS cell growth and function, but complementary studies – which are currently in progress in our laboratory – are needed to confirm this hypothesis in human pituitary tumors.

In summary, we have shown that curcumin suppressed FS TtT/GF mouse pituitary tumor cell growth by inducing cell cycle arrest at the G_2/M-G_1 interface. In addition to this, curcumin induced apoptosis in TtT/GF cells. Moreover, curcumin-mediated changes in tumoral and normal FS cell functions, such as VEGF-A and IL-6 suppression, have been demonstrated; these observation may have therapeutic relevance.

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