The Chemopreventive Polyphenol Curcumin Prevents Hematogenous Breast Cancer Metastases in Immunodeficient Mice

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Breast Cancer • Metastasis in vivo • Matrix Metalloproteinase • Apoptosis • NFκB

Abstract
Dissemination of metastatic cells probably occurs long before diagnosis of the primary tumor. Metastasis during early phases of carcinogenesis in high risk patients is therefore a potential prevention target. The plant polyphenol Curcumin has been proposed for dietary prevention of cancer. We therefore examined its effects on the human breast cancer cell line MDA-MB-231 in vitro and in a mouse metastasis model. Curcumin strongly induces apoptosis in MDA-MB-231 cells in correlation with reduced activation of the survival pathway NFκB, as a consequence of diminished IkB and p65 phosphorylation. Curcumin also reduces the expression of major matrix metalloproteinases (MMPs) due to reduced NFκB activity and transcriptional downregulation of AP-1. NFκB/p65 silencing is sufficient to downregulate c-Jun and MMP expression. Reduced NFκB/AP-1 activity and MMP expression lead to diminished invasion through a reconstituted basement membrane and to a significantly lower number of lung metastases in immunodeficient mice after intercardiac injection of 231 cells (p=0.0035). 68% of Curcumin treated but only 17% of untreated animals showed no or very few lung metastases, most likely as a consequence of down-regulation of NFκB/AP-1 dependent MMP expression and direct apoptotic effects on circulating tumor cells but not on established metastases. Dietary chemoprevention of metastases appears therefore feasible.

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**Introduction**

Curcumin (Diferuloylmethane), a polyphenol derived from the plant turmeric (*Curcuma longa*) is commonly used as a spice component. In certain countries, Curcumin has traditionally been applied to treat various inflammatory disorders [1, 2]. Curcumin exerts anti-proliferative and pro-apoptotic effects against diverse tumors *in vitro* [3-5] and *in vivo*, as it has been found to suppress carcinogenesis of the breast [6] and other organs [7-9].

Curcumin acts at least in part through inhibition of the translocation of the transcription factor nuclear factor kappaB (NFκB) [10]. In the majority of cells, NFκB exists in an inactive form in the cytoplasm bound to the inhibitory proteins referred to as IκB. NFκB activity is associated with anti-proliferative effects, as well as with the induction of apoptosis [11, 12]. As a consequence, activation of NFκB constitutes a crucial step in tumor promotion and progression, angiogenesis, inflammation, invasion, and metastasis [13]. Constitutive NFκB expression correlates with the metastatic potential of breast tumors and has been proposed as both, a prognostic marker and a drug target [14].

Yet, the role of NFκB in cancer progression is not limited to its anti-apoptotic potential. NFκB also induces expression and activation of matrix metalloproteinases (MMPs) that enable invasive growth through the degradation of the peritumoral matrix [15]. We and others have shown that the expression of major MMPs correlates with the growth behavior of tumor cells *in vivo*. Tumor cell models of malignant keratinocytes [16-18] and breast carcinoma cells [19-21] showed increased MMP expression levels along with increasing aggressiveness of tumor cell growth and metastatic potential. The control of MMP synthesis and activation can therefore be regarded as an important target for the prevention of tumor progression.

The promoter regions of the genes encoding MMP-1, -2, -3, -7, -9, -12 and -13 have been analyzed. All of them contain a proximal activating-protein-1 (AP-1) binding site approximately 70°bp 5’ to the transcription start [22, 23] with the exception of the gene encoding MMP-2 whose promoter contains a modified AP-1 binding site approximately 70°bp 5’ to the transcription start [24, 25]. Moreover, other transcription factors are known to influence MMP expression as the promoter regions of these enzymes contain NFκB-like elements as e.g in the case of MMP-1 or MMP-13 [26-27].

Therefore natural substances like Curcumin that down-regulate transcription of matrix degrading enzymes are of outstanding interest for cancer prevention and treatment especially as they have negligible side-effects. The molecular mechanisms of the anti-tumor properties of Curcumin are only partially understood, particularly in the context of breast cancer. Hence the aim of our study was to examine in a first step the pathways that are activated or suppressed by Curcumin *in vitro* in highly invasive and metastatic breast cancer cells MDA-MB-231. The second step was to investigate the anti-metastatic potential of the polyphenol *in vivo* in a model of immunodeficient nude mice.

In the present study, we demonstrate that Curcumin induces apoptosis and inhibits expression and activity of the transcription factors AP-1 and NFκB leading to diminished expression and activity of several MMPs. We are the first to show that Curcumin significantly reduces the number of metastases formed from intracardially injected breast cancer cells. Down-regulation of the matrix degrading enzymes by Curcumin through the inhibition of NFκB/AP-1 mediated transcription can explain reduced invasion *in vitro* and metastasis *in vivo*.

**Materials and Methods**

**Cell Types and Culture Conditions**

The human breast cancer cell line used in this study was MDA-MB-231 (ATCC) and is referred to in the text as 231 cells. The cell line is ER-negative and was isolated from the pleural effusion of a patient with breast carcinoma [28, 29]. The cell line is commonly used for breast cancer studies and is well-defined in its growth, invasive, and metastatic characteristics. MDA-MB-231 cells injected into the mammary fat pad of nude mice result in the formation of tumors and distant metastases in lungs, brain, and lymph nodes of some mice [30].

The cell line was cultured at 37°C in a humidified atmosphere of 5% CO₂. The cells were grown in MEM (Eagle’s) with Earle’s salts supplemented with 5% heat inactivated fetal calf serum, 1% L-glutamine solution (200mM), 1% sodium pyruvate solution (100mM), non-essential amino acids and vitamins. Medium was changed every 2d. For invasion assays and zymography serum-free media were used.

**Curcumin** and **TNFα. Treatment of Cells**

Curcumin with a purity of 95% was purchased from Fluka (Buchs, Switzerland), dissolved in 0.5M NaOH as a 25mM stock solution and stored at -20°C. For the use in cell culture a 2.5mM solution in sterile PBS was prepared. Curcumin was applied at an end concentration of 25µM for all assays. TNFα was obtained from SIGMA (Milan, Italy) and used at a concentration of 10ng/ml.
**Gene Silencing**

Small interfering RNAs [r(GAU CAA UGG CUACACAGG A) d(TT) and r(UCC UGU GAU GCC AUU GAU C)d(TT)] targeted to NFκB, were synthesized and annealed (Qiagen, Hilden, Germany). A non-silencing fluorescein labeled siRNA (Qiagen, Hilden, Germany) was used as control for transfection efficiency as well as for monitoring the effect of silencing NFκB during all experiments. Cell cultures with at least 90% transfection efficiency were used for further studies. Transfection of MDA-MB-231 cells (40% confluency) with siRNA was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the recommendations of the manufacturer. Briefly, the transfection reagent was pre-incubated with the siRNA Oligos either targeted to NFκB or to an irrelevant control 30min prior to the application to the cells. For RT-PCR, cells were harvested 6h and 24h after transfection. For zymography, the media were replaced with serum-free media 24h after transfection and after additional 24h, the conditioned media were used for analysis.

**Preparation of Conditioned Media**

Serum containing media was removed and cells were washed 3 times with PBS, media without serum were added to the cells and after 24h the conditioned media were harvested and pelleted by centrifugation. The supernatants were concentrated 50 fold by centrifugation in spin columns (Amicon, 10kDa exclusion limit).

**Protein Determination**

Protein concentrations were determined by the BCA protein assay (Pierce, Oud-Beijerland, Netherlands) with bovine serum albumin as the standard. To cross check that equal amounts of protein in Curcumin treated conditioned media (yellow colored) were applied for Zymography and Western Blot assays, SDS-PAGE was performed. Subsequent silver staining of the proteins served as a loading control.

**RNA-Extraction and Real-time PCR**

Total RNA was extracted from cells according to the method of Chomczynski and Sacchi [31] using TRI Reagent (Sigma, Deisenhofen, Germany). Thereafter, oligo dT primed cDNAs were synthesized using the First Strand cDNA Synthesis Kit (Pharmacia Biotech, Freiburg, Germany) following the manufacturer’s instructions. Quantitative RT-PCR was performed on a Light-Cycler (Roche, Mannheim, Germany) using the QuantiTect™ SYBR® Green PCR kit (Qiagen, Hilden, Germany). Primers for the MMP-1, -2, -3, -9, NFκB, and c-jun target genes as well as for the housekeeping gene RPII were designed using the Primer3 program and purchased from TibMolbiol (Berlin, Germany). PCR conditions were set according to the manufacturer’s instructions provided with the QuantiTect™ SYBR® Green PCR kit. All experiments were carried out in duplicates as recommended for the use of the LightCycler. The specificity of the RT-PCR products was proven by the appropriate melting curves (specific melting temperature) and by the expected size of the PCR products (data not shown). Expression data were normalized on house keeping genes as indicated in the results section.

**Gel Zymography**

Gelatin zymography was essentially performed as previously described in detail [16, 17]. MMP-1, -2 and -9 controls were purchased at Calbiochem (Merck Biosciences, Germany). A molecular mass standard (Biorad, Germany) was used in all experiments. These experiments were repeated three times.

**Chemo-Invasion Assays**

The chemo-invasion assays were performed as previously described [32, 33]. Cells were treated for 16h in complete medium with Curcumin and harvested with trypsin, washed with PBS, counted, centrifuged and suspended in serum free medium. Subsequently, equal cell numbers were placed in the upper compartment of the Boyden chambers (1.5 x 10^5 cells in 800µl). The lower compartment was filled with serum-free conditioned media derived from NIH-3T3 cells. Curcumin was added directly to the cells in the upper compartment. The two compartments of the Boyden chambers were separated by a 12µm-pore size polycarbonate filter (Corning Costar, Acton, MA) coated with 15µg/filter Matrigel (a reconstituted basement membrane [34]). Chambers were incubated at 37 °C in 5% CO₂, for 6h and 16h. At the end of the incubation period, cells remaining at the upper surface of the filter were removed by wiping them with a cotton swab. Cells on the lower surface were fixed in ethanol, stained (touli dine blue) and counted in a microscope (5 fields per filter). Assays were performed in triplicate.

**Western Blots**

Western blots of conditioned media were performed as previously described [17]. Equal amounts of protein as determined by BCA protein assay and SDS-PAGE with silverstaining of the Curcumin treated and non-treated cells were subjected to SDS-PAGE and analyzed for MMP-1, -2, -3, and -9 (kindly provided by Dr. R. Lichtinghagen, Medical School Hannover, Germany).

**Electrophoretic Mobility Shift Assay**

Cells were seeded onto 150cm² culture dishes with 25ml culture medium and incubated until the cells reached either subconfluent (30-50% confluency) or confluent stages. Nuclear extracts were prepared as described previously [35]. Oligonucleotides corresponding to the consensus sequences (AP-1 site: 5’-GAT CTG TGA TCTG GCC GCAG-3’; NFκB site: 5’-GGTT AGT GGA GCC TTT CTT CCC AGG-3’) were labeled with [α-32P]dATP (3000Ci/mM) and Klenow enzyme and were incubated with 10µg of nuclear protein in 20µl of 7mM Hepes-KOH (pH 7.9), 100mM KCl, 3.6mM MgCl₂, and 10% glycerol on ice for 20min. Poly(dI-dC) (0.05mg/ml) was added as an unspecific competitor. The samples were run on a 5% nondenaturing polyacrylamide gel in a buffer containing 25mM Tris-HCl (pH 8.0), 190mM glycerine, and 1mM EDTA. Gels were dried and analyzed by autoradiography. In order to prove the specificity of the probes, a 50 fold excess of unlabeled probe was incubated with the binding reaction mixture for 45 min on ice before adding the radiolabeled DNA fragment.
60% subconfluent cells treated with or without 25µM Curcumin phosphorylation, total cellular extracts were prepared from 50- or phosphorylated IκBα dehydrogenase) kinetic assay. The cells (50% confluency and absorption of MTT-formazan was measured at 570nm. Incubation at 37°C, 50µl isopropanol was added. The solution (1mg/ml in PBS) was added to each well. After 2h incubation (0, 2, 4, 24h). The cells were washed with PBS and 50µl MTT and incubated for 24h at 37°C. Curcumin was added to each sample. Data were analyzed using a Flow-Cytometer kit from Roche, Germany) according to the recommendations of the manufacturer. Ten thousand events were counted for each sample. Data were analyzed using a Flow-Cytometer (Beckman Coulter XL-MCL, Software: System II).

Flow Cytometric Analysis

The breast cell lines were grown to subconfluent and confluent growth stages in a 24 well-plate and incubated with 25µM Curcumin for various time intervals (2, 4, 24, 28h). The cells were harvested and then treated with (FITC)-conjugated annexin V and propidium iodide (Annexin-V-FLUOS Staining kit from Roche, Germany) according to the recommendations of the manufacturer. Ten thousand events were counted for each sample. Data were analyzed using a Flow-Cytometer (Beckman Coulter XL-MCL, Software: System II).

MTT Assay

The antiproliferative effects of Curcumin on different breast carcinoma cell lines were determined by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) dye uptake method as described earlier [37]. Briefly, the cells were seeded in triplicate at a density of 10⁶ or 5x10⁵ respectively per well in a 96-well plate in a final volume of 0.2ml complete media and incubated for 24h at 37°C. Curcumin was added to each well and incubation was continued for various time intervals (0, 2, 4, 24h). The cells were washed with PBS and 50µl MTT solution (1mg/ml in PBS) was added to each well. After 2h incubation at 37°C, 50µl isopropanol was added. The absorbance of MTT-formazan was measured at 570nm.

Cytotoxicity Assay

The cytotoxic effects of Curcumin in different breast carcinoma cell lines were determined by LDH (lactate dehydrogenase) kinetic assay. The cells (50% confluency and 95% confluency) were seeded in triplicates in a 24-well plate in a final volume of 1ml complete media for 24h at 37°C. Cells were treated with 25µM Curcumin for different time intervals (0, 2, 4, and 24h) and media were harvested and assayed. Cytotoxicity induced by Curcumin was evaluated by measuring specific activity of LDH secreted by the cells. LDH activity is measured by an enzymatic assay that results in the conversion of a tetrazolium salt into a red formazan product detected at 340nm.

Hematogenous Metastases in Immunodeficient Mice

Animal studies and research protocols were reviewed and approved by the institutional ethics committee with and were conducted in accordance with the national current regulations and guidelines for the care and use of laboratory animals (D.L. 27/01/1992, n. 116). Five-week-old CD-1 Foxn1nu female mice were obtained from Charles River Laboratories and were maintained under specific pathogen-free conditions and given sterile food and water ad libitum. Before intracardiac tumor inoculation, mice were anaesthetized with i.p. mixture of ketamine (50mg/kg) and xylazine (5mg/kg). Animal health and survival rate was observed until their euthanasia due to one of the following medical reasons: severe weights loss (exceeding 20% of original body mass), hyperventilation, paralysis, or bone fracture.

MDA-MB-231 cells were collected by trypsinization and washed and resuspended in PBS. 5 x 10⁵ cells were injected into the heart of the mice. Mice were fed with Teklad 2019 (Harlan Winkelmann GmbH; Borchen, Germany) containing 1% Casein (controls) or 1% Curcumin (Fluka, Buchs, Switzerland). On day 35 after inoculation, mice were humanely sacrificed. Following sacrifice of the animals all internal organs, including the brain, the vertebral column, humeri, and femora were removed and immersed for 24-36h in buffered formaldehyde, pH 7.4. Subsequently, all material was dissected and completely embedded into paraffin as routinely performed. Samples containing bone were first decalcified in 0.1M EDTA until complete decalcification. Sections from all resulting tissue blocks were stained for H&E. In addition, particular attention was paid to the dissection of the lungs resulting in all samples in a cross-section through the largest diameter of the organs. Thereby, the pulmonary work up was standardized so that comparably sized cross-sections through all lung samples were achieved. Following embedding into paraffin, serial sections were prepared from the lungs which were stained by immunohistochemistry in addition to the H&E stain.

The immunostainings comprised the localization of (human) cytokeratin (pan-keratin), p53 protein and the proliferation marker Ki-67 (all antibodies DAKO, Hamburg, Germany).

All organ sections were analyzed by light microscopy and the presence and number of tumor cells/tumor cell aggregates was recorded. In the lung samples, all tumor cells/tumor cell aggregates present within the pulmonary parenchyma (intrapulmonary) were distinguished from those present at the pleural surface and/or seen in mediastinal soft tissue (peripulmonary). Metastases were identified by typical morphology, positive reaction for (human type) cytokeratin and enhanced proliferative capacity (Ki-67) or enhanced positivity of tumor cell nuclei for p53 protein. All metastases were counted irrespective of the metastasis size or the number of tumor cells...
Fig. 1. Cell Viability and Apoptosis. a) Curcumin reduces cell viability measured by MTT assay (left panel) to approximately 50% after 2h in MDA-MB-231 cells. Prolonged treatment of up to 24h does not result in a significant further reduction of viability in these cells. LDH release into the conditioned media of both cell lines can be observed after 24h treatment, where it reaches considerable levels (right panel). Reduced mitochondrial activity (left) and cytotoxicity (right) appear to be more pronounced in subconfluent than in confluent cells. b) Fluorescence micrographs of annexin V and propidium iodide stained Curcumin treated cells show clear evidence for early apoptosis already after 2h as evidenced by annexin V staining (green). Most of the early apoptotic MDA-MB-231 cells reach a late apoptotic, propidium iodide positive stage (red) after 24h.

Table 1. Flow Cytometry Analysis of Cellular Apoptosis. Cells treated for up to 28h with 25µM Curcumin showed clear evidence of early apoptosis from early time points on. More than 70% of MDA-MB-231 cells entered the suicide program within 4h of treatment. Most of the early apoptotic cells reached a late apoptotic, propidium iodide positive stage at 24 to 28h.

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<th>Flow Cytometric Analysis of Cellular Apoptosis</th>
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<td>72.9</td>
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Results

Cell Viability and Apoptosis

We examined the effect of Curcumin on MDA-MB-231 cell viability using two complementary tests. The MTT assay measures cellular integrity in terms of mitochondrial activity, while the LDH (lactate dehydrogenase) assay indicates enzyme activities released by lysed cells into the culture media. Figure 1a (left panel) shows that the cells responded with a rapidly reduced mitochondrial energy metabolism that dropped to approximately 50% after only 2h treatment. Prolonged treatment for up to 24h did not result in a significant further reduction of viability in these cells. The analysis of cytotoxicity, however, showed that the early drop in mitochondrial activity is not due to a direct toxic effect since relevant present. The resulting metastasis frequencies were statistically evaluated.
LDH release could be observed only after 24h treatment (Fig. 1a, right panel). Reduced mitochondrial activity and cytotoxicity appeared to be more pronounced in subconfluent cells than in confluent ones.

To clarify whether the LDH release correlated with induction of apoptosis, we treated the cells for up to 28h with 25µM Curcumin. From early time points on (2 and 4h, Table 1) the cells showed clear evidence of early

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Fig. 2. Effects of Curcumin on NFκB. a) Binding of 32P-labeled NFκB specific oligonucleotides to the cognate transcription factor present in nuclear extracts of MDA-MB-231 cells was monitored by EMSA. Curcumin treatment of MDA-MB-231 cells for different time intervals (left panel: lane 2: 2h; lane 3: 4h; lane 4: 6h) led to a reduced binding of NFκB to its response element as compared to untreated cells (left panel: lane 1). The specificity of the binding was assessed for all three oligos by addition of a 50x molar excess of cold oligonucleotides (lanes 5). Experiments were repeated at least three times. b) IkBα expression and phosphorylation was analyzed by Western blots using total cellular extracts and specific antibodies against phosphorylated IkB (pIkB). Untreated control cells contain low amounts of phospho-IkB. IkB phosphorylation is strongly induced by treating the cells with the NFκB activator TNF-α for one hour. When Curcumin is added to the cells in the absence of TNF-α for only 30min, a slight reduction in phospho-IkB as compared to basal levels is observed. In Curcumin treated cells that are also stimulated with TNF-α, the TNF-α dependent induction of phospho-IkB is strongly reverted. Phosphorylation of the p65 subunit of NFκB is induced by TNF-α, which can be reduced by Curcumin (bottom panel). Protein amounts loaded onto the gels are controlled by concomitant staining with antibodies against tubulin. c) Immunofluorescent NFκB translocation assays using specific antibodies against the p65 unit (left panels) show that NFκB in untreated control cells is located almost exclusively in the nucleus of the cells (DAPI stain, right panels). Upon treatment of the cells with Curcumin, fluorescence increases in the cytoplasm and the nuclear signal decreases indicating that more NFκB is retained in the cytoplasm as shown here after 4h of treatment with the polyphenol.
Fig. 3. Crosstalk between NFκB and AP-1. a) Reduced binding of nuclear proteins from Curcumin treated cells to the AP-1 consensus sequence (see Material and Methods) could be shown by mobility shift experiments which were performed for treated (lanes 2: 2h; lanes 3: 4h; lanes 4: 6h) and untreated (lanes 1) MDA-MB-231 cells using 10µg of nuclear protein incubated with the 32P-labeled oligonucleotide. Cells of both lines treated with Curcumin show a clear reduction in the specific bands for the transcription factor AP-1 in comparison to untreated cells (lanes 1). The specificity of the binding was assessed for all three oligos by addition of a 50x molar excess of cold oligonucleotides (lanes 5). Experiments were repeated at least three times. b) mRNA expression of the AP-1 subunit c-jun was reduced in MDA-MB-231 cells upon treatment from 1h to 20h with the polyphenol as evidenced by Real Time RT-PCR. A statistically significant 50% depletion of c-jun expression was reached after 2h of Curcumin treatment. Experiments were performed in triplicates. c) NFκB silencing of the p65 subunit resulted in a more than twofold depletion of p65 mRNAs 6 and 24h after transfection with p65 siRNAs. The effects on mRNA expression were similar for c-jun which was reduced in p65 silenced MDA-MB-231 cells (left panel). *= p<0.05 (unpaired t-test). Experiments were repeated at least three times.

apoptosis in terms of translocation of phospholipid phosphatidylserine from the inner to the outer leaflet of the plasma membrane, where it becomes accessible to annexin V staining as confirmed by flow cytometry analysis. More than 70% of cells entered the suicide program within 4h treatment. Most of the early apoptotic cells reached a late apoptotic, propidium iodide positive stage after 24 to 28h. Figure 1b shows fluorescence micrographs of annexin V and propidium iodide staining in Curcumin treated cells. Induction of apoptosis in 231 cells was consistent with the observed effect on cell viability. The raise in LDH release, which we observed in these cells, appeared simultaneously with propidium iodide stained late apoptotic cells. The release of LDH may therefore be attributed to apoptotic disruption of the cell membrane.

Effects of Curcumin on IκB Phosphorylation and NFκB Activation

Nuclear extracts from Curcumin treated and untreated cells were applied to electrophoretic mobility shift assays (EMSA) where the binding of transcription factors is revealed by the retarded electrophoretic migration of radioactively labeled oligonucleotides of the
specific binding sequence (Fig. 2a). The specificity of the binding was assessed by addition of a 50fold excess of cold oligonucleotides that abolished the band shifts observed (Fig. 2a; lane 5). Addition of an unrelated mutant oligonucleotide had no effect on NFκB binding (data not shown). Curcumin treatment for 2, 4, and 6h of MDA-MB-231 cells (Fig. 2a, lanes 2, 3, and 4, respectively) show a clear reduction in the specific bands for the transcription factor NFκB in comparison to untreated cells (lane 1).

As a prerequisite to p65 activity inside the nucleus, we have followed IκB phosphorylation by Western Blots of total cellular extracts using specific antibodies that recognize IκBα only in its phosphorylated form (Fig. 2b). In untreated control cells, only few phospho-IκBα is present. IκBα phosphorylation is strongly induced by treating the cells with the NFκB activator TNF-α for one hour. When Curcumin was added to the cells, in the absence of TNF-α, for only 30 min, the phospho-IκBα band was reduced as compared to basal levels. In Curcumin treated cells that were also stimulated with TNF-α, the TNF-α dependent induction of phospho-IκBα was strongly reverted (Fig. 2b). Phosphorylation of the p65 subunit of NFκB followed a similar pattern: the induction by TNF-α was inhibited by the polyphenol (Fig. 2b, bottom panel).

Reduced NFκB activitation led to a diminished NFκB translocation to the nucleus as monitored by immunofluorescence analyses using specific antibodies against the p65 unit of NFκB (Fig. 2c). In untreated MDA-MB-231 control cells, NFκB was mainly located in the nucleus with a minor fluorescent signal detectable in the cytoplasm of the cells (Fig. 2c, upper panel). Upon 4h treatment of the 231 cells with Curcumin, fluorescence in the cytoplasm increased and the nuclear signal decreased indicating that NFκB was retained in the cytoplasm (Fig. 2c, lower panel). The results of the EMSA, the translocation assays and Western blots concordantly show an effect of Curcumin on NFκB activation in these breast cancer cells.

**Effects of Curcumin on AP-1 and NFκB/AP-1 Cross-Talk**

EMSA of nuclear extracts of the cells (Fig. 3a) showed a clear reduction of AP-1 binding upon treatment with Curcumin for 2, 4 and 6h (lanes 2, 3, and 4 respectively) when compared to untreated cells (lane 1). The specificity of the binding was assessed by addition of a 50fold excess of cold oligonucleotides that abolished the band shifts observed (Fig. 3a; lane 5). Addition of an unrelated mutant oligonucleotide had no effect on AP-1 binding (data not shown).

Reduced AP-1 binding to its cognate response element can be attributed to reduced expression of the AP-1 factor, c-jun, as evidenced by Real Time RT-PCR analyses of MDA-MB-231 cells (Fig. 3b). After only 1h of Curcumin treatment, c-jun expression was reduced with further decrease up to 20h. This effect is statistically significant.

We wished to test the hypothesis that reduced AP-1 activity is due to an effect of NFκB on c-jun mRNA abundance and therefore, we performed silencing experiments using small interfering oligonucleotides specific for NFκB/p65 that we transfected into MDA-MB-231 cells. The effects on transcription of p65 itself and on c-jun were analyzed by Real Time RT-PCR (Fig. 3c). A more than twofold depletion of p65 mRNAs at 6 and 24h after transfection with anti-p65 siRNAs that was paralleled by a three- to fourfold reduction of c-jun mRNA expression at 6 and 24h, respectively, was observed in 231 cells. We are therefore confident that the effect of Curcumin observed depends, at least partially, on reduced NFκB activity.

**Matrix Metalloproteinase Expression**

We measured MMP mRNA expression by quantitative Real Time RT-PCR (Fig. 4a) and normalized expression values on those obtained for the housekeeping gene RNA-polymerase II. The normalization also monitors the general transcriptional activity of the cells in a way to exclude any influence of late apoptotic cells on the results. Previous analyses showed that the major MMPs expressed by 231 cells were MMP-1, -2, -3 and -9, although the latter is present only in very small amounts [19]. MMP-2 protein is expressed at considerable levels (see below) yet only low levels of the appropriate transcripts were detectable, probably due to unstable mRNAs. Curcumin treatment of 231 cells resulted in strongly diminished levels of MMP-1 and MMP-2 mRNA already after 2h leading to 3.45 fold (MMP-1) and 2.5 fold (MMP-2) reduction of expression after 20h. MMP-3 and -9, on the other hand, showed no considerable alteration of expression (Fig. 4a).

Well in line with the results obtained from Real Time RT-PCR, Western blot analyses of cell supernatants (Fig. 4b) revealed a strong down-regulation of MMP proteins upon treatment with the polyphenol. Accumulation of newly released MMPs secreted into fresh serum-free medium added at the beginning of the time course, was monitored in the presence (+) or in the absence (-) of...
**Fig. 4.** Effect of Curcumin on MMP Expression. a) Quantitation of mRNA by real time RT-PCR of breast cancer cells treated with 25µM Curcumin for 2 and 20h revealed a significant downregulation of MMP-1 and -2 in MDA-MB-231 already after 2h with ongoing effects up to 20h treatment. Experiments were performed in triplicate; error bars indicate SD; *=p<0.05 (one way Anova with Bonferroni’s post test). b) Western blot analysis of supernatants of cells treated for several time intervals with Curcumin revealed reduced amounts of MMP-1 and MMP-2. c) Zymography of gelatinolytic (e.g. MMP-2 and -9) and caseinolytic enzymes (e.g. MMP-1) showed a reduction of proteolytic activities present in conditioned media from the cells treated for different time intervals with Curcumin as compared to those of untreated cells. MMPs were identified according to their migration behavior as compared to a molecular mass standard (not shown) and to MMP-1, -2 and -9 controls (lanes indicated with S and the according MMP control). The faint gelatinolytic band visible in supernatants of MDA-MB-231 cells below the bands identified as MMP-2 most probably corresponds to MMP-1 activity which is detectable also in gelatin zymographies. The caseinolytic band at about 89 kDa (molecular mass standard not shown) which could be detected above the lytic bands of MMP-1 was not yet identified (marked in Figure 4c by an asterisk). d) P65 expression is reduced to 40% and 25% after 6 and 16h of silencing, respectively, by means of transfection of siRNAs specific for p65. Silencing of the NFκB subunit led to a significantly reduced expression of mRNAs (real time PCR, left panel; *=p<0.05, unpaired t-test) and proteins (Western blot, right panel) of MMPs 1, and 2 in MDA-MB-231 cells.

25µM Curcumin. We found that MMP-1 and -2 protein were released into the supernatants of 231 cell cultures. The amount of MMP-1 protein secreted into the media of non-treated cells was almost completely abrogated already after 3h incubation in the presence of Curcumin. The effect on MMP-2 was similar and reached a plateau.
after 15h. Even after 24h, MMP-1 and -2 proteins were not detectable in supernatants of Curcumin treated cells. The Western blot analyses thus show that MMP expression was also regulated post transcriptionally: MMP-1 and -2 levels appeared to be more drastically regulated at the protein level (Fig. 4b) than at the mRNA level (Fig. 4a).

The Western blot data were further confirmed by zymography analyses on substrate containing gels visualizing the enzymatic activities present in cell culture supernatants. The electrophoretically separated proteases were detected as translucent bands on the Coomassie brilliant blue stained substrate background (gelatin for MMP-2 and -9, casein for MMP-1). The accumulation of MMP activity released into fresh medium was monitored in comparison to purified control MMPs (figure 4c, lanes labeled “S”). 231 cells responded to the Curcumin treatment with a transient reduction in MMP-2 and -9 activities, comigrating with the respective controls, detected on gelatin gels (Fig. 4c, upper panel). A persistent suppression of MMP-1 activity, comigrating with a MMP-1 control, was observed on casein containing gels (Fig. 4c, lower panel).

The faint gelatinolytic band visible at 51kDa (molecular mass standard not shown; below the MMP-2 bands; Fig. 4c, upper panel) most probably corresponds to MMP-1 activity which is detectable, though weaker, also in gelatin zymographies. The caseinolytic band at about 89kDa (molecular mass standard not shown) which could be detected above the lytic bands of MMP-1 was not yet identified (marked in Fig. 4c, lower panel by an asterisk).

We then asked whether the reduced NFκB activation operated by Curcumin and the effect of the polyphenol on MMP expression are causally linked or just coincident. For this purpose we silenced the expression of the NFκB subunit, p65, by means of transfecting the cells with p65 siRNAs. The siRNAs reduced p65 expression to 40% and 25% as compared to cells transfected with non-specific siRNAs after 6 and 16h, respectively. In the silenced cells, expression of MMP-1 and -2mRNA (Fig. 4d; left panel) and protein (Fig. 4d; right panel) was significantly reduced. The effect of Curcumin on NFκB thus explains the diminished MMP expression.

**Invasion**

Breast cancer cells revealed a diminished expression of major MMPs upon Curcumin treatment and we therefore investigated the influence of the polyphenol on the invasive capacity through a reconstituted basement membrane. If the filter is covered with a reconstituted basement membrane (Matrigel) active invasion and proteolytic digestion of the matrix is required for the cells to respond to the stimulus (Fig. 5). Invasion was significantly reduced by Curcumin after only 6h of treatment with the polyphenol. This establishes a functional consequence of decreased MMP production.

**Mouse Metastasis of Breast Cancer Xenografts**

2 x 10⁵ subconfluent 231 cells were injected into the heart of nude mice that were subsequently divided into two groups that received casein or casein-Curcumin diets. This route of administration, though leading to suboptimal absorption of the polyphenol that is mainly excreted with the faeces, was chosen in consideration of the probable route of administration for dietary chemopreventive agents. Mice were observed for 5 weeks within which the two groups did not show any differences, especially, Curcumin treated mice did not reveal any side effects of the treatment, and the weights of the animals of the two groups were comparable.

After five weeks, mice were sacrificed and all internal organs including the brain, the vertebral column, and both humeri and femora were collected and examined. Brain, Humeri, femura and vertebral column were free

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**Fig. 5. Invasion.** Treatment of MDA-MB-231 cells with 25µM Curcumin for 6h led to a significant decrease of the invasive capacity as compared to untreated cells. The reduced number of invading cells after only 6h indicates that reduced invasion cannot be attributed to cell death. Fibroblast conditioned medium was used as chemoattractant and Matrigel as an extracellular matrix equivalent. Experiments were performed in triplicate; error bars indicate the standard error. ***= p<0.001 (unpaired t-test).
Fig. 6. Hematogenous Lung Metastases. 5 x 10^5 MDA-MB-231 cells were injected into the heart of immunodeficient mice. Animals were untreated (control) or treated with Curcumin in the diet and sacrificed after 5 weeks. Lungs were removed fixed and four sections for each animal were analyzed for metastases. a) histology of metastases formed. The immunolocalization of (human) cytokeratin in the periphery of a mouse lung specimen reveals multiple selectively and positively stained tumor cells and small tumor cell aggregates (brown color). Immunohistochemical staining for p53 protein of a mouse lung specimen also shows multiple positively labeled cell nuclei (brown color) suggesting implanted breast cancer metastases. Localization of the cell proliferation antigen Ki-67 with positive labeling of isolated tumor cell groups (brown color) indicates cell proliferation of the transplanted tumor cells. The overview of lung tissue 5 weeks after intracardiac tumor cell injection shows small groups of cells with typical morphological features of tumor cells (polymorphism, atypia, mitotic activity). (A-D original magnification x 400; A: anti-human cytokeratin; B: anti-human p53 Protein; C: anti-human Ki-67 antigen (MIB-1); D: H&E) b) Number of metastases formed. The scattergrams report the numbers of metastases found in each of the untreated (control, n=18) and Curcumin treated (Curcumin; n=19) animals (mean of 4 sections analyzed). Mean number of metastases per animal are reduced in Curcumin treated animals. Note the high number of animals with very few metastases in treated animals (Mann-Whitney p=0.0035).

Table 2. Curcumin Inhibits Lung Metastases. Curcumin treated animals had less lung metastases. Four controls and three treated animals had high total metastases counts (>10) and the number of animals with very few metastases (<3) was higher in treated (13 of 19 animals; 68%) than in untreated animals (3 of 18 animals; 17%). Four Curcumin treated and none of the control animals remained metastases free.

<table>
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<th># metastases</th>
<th>Effects of Curcumin on Lung Metastases</th>
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<td></td>
<td># (%) Control animals</td>
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<td>n=18</td>
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<td>&gt;10</td>
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Curcumin Prevents Hematogenous Breast Cancer Metastases

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of metastases. Lung metastases were counted after preparation of eosin-hematoxilin stained paraffin sections. In both study groups, tumor cells/ cell aggregates were seen in the intrapulmonary and the peripulmonary compartment, though to different numbers. Peripulmonary metastases are more likely to be derived from direct dissemination during the intercardiac injection. We therefore limited our analysis to intrapulmonary metastases that are of hematogenous origin. The tumor cell morphology showed characteristic atypia, significant expression of human p53 protein was observed only in tumor cells. Vitality of the tumor cells was confirmed by
a high number of proliferating Ki-67 positive cells thus excluding tumor cell dormancy (Fig. 6a). Tumors of treated and untreated animals were similar in dimension, morphology and histology.

Figure 6b demonstrates that Curcumin prevented lung metastases in a highly significant manner (Mann-Whitney test, \( p=0.0035 \)). The effect of Curcumin on lung metastasis becomes also evident from Table 2 that shows the mice subdivided in ranks with no, very few (<3), several (3-10) and many (>10) metastases. The number of animals with very few metastases (<3) was dramatically increased in treated (13 of 19 animals; 68%) as compared to untreated animals (3 of 18 animals; 17%). Four Curcumin treated and none of the control animals remained metastases free. 4 controls and 3 treated animals had high total metastases counts (>10). Metastases of other tissues were rare in both groups.

**Discussion**

Chemopreventive agents such as Curcumin are natural or synthetic substances that suppress or slow down tumor formation [38]. Most of them are plant extracts and have negligible side effects. Chemoprevention has two targets: (i) avoidance of neoplastic cell transformation and (ii) inhibition or retardation of tumor promotion and progression. Tumor metastasis is the major determinant of mortality of cancer patients. Yet most of the cells dispersed from the primary tumor never form colonies because they fail to reach the target tissue or do not grow once they have reached it. All steps in metastasis, from invasion of the tumor surrounding stroma and distribution through the blood flow to extravasation from capillaries and growth in the target tissue, rely on host factors [39-41]. In an ideal setting, chemoprevention would therefore target both the actual tumor cells and the host.

NF\( \kappa \B \) comes close to an ideal target as it acts in the tumor cell itself, where it controls survival pathways [42] as well as in the host, where it regulates the expression of genes involved in inflammatory processes and stress responsiveness [42-44] that may promote tumor progression and metastasis through the induction of angiogenesis and through the expression of tethering molecules on the surface of activated endothelium [45]. NF\( \kappa \B \) has been shown to be constitutively activated in breast cancer cells [38], where it mediates a wide variety of cellular processes involving growth, differentiation, and apoptosis. Increasing evidence of deregulation of pathways that converge on NF\( \kappa \B \) in various human breast cancer cell lines and primary tumors supports a role for NF\( \kappa \B \) in mammary carcinoma progression [38, 46]. As a consequence, inhibition of NF\( \kappa \B \) could prevent tumor progression and some chemopreventive polyphenols act at least in part through the inhibition of NF\( \kappa \B \) [47, 48].

Here, we establish an anti-metastatic role of the strong NF\( \kappa \B \) inhibitor Curcumin and outline the molecular pathways that lead to a reduced metastatic potential of mammary carcinoma cells in an animal model of hematogenous metastasis. We have analyzed the effects of the polyphenol on the highly invasive and metastatic breast cancer cell line MDA-MB-231. We chose to use a concentration of 25\( \mu \)M Curcumin since dose dependent effects in the range of 1-50\( \mu \)M had been reported for breast cancer cell lines with strongest effects between 20 and 50\( \mu \)M [49].

Several studies have shown anti-metastatic properties of Curcumin in vivo [50-54]. However, it is important to distinguish between effects on the formation versus growth of metastases. Most studies analyzed metastases formed from a primary tumor of subcutaneously injected cells. Effects on primary tumor growth are most likely to also affect the number of metastases formed and no distinction between the two processes is therefore possible [50]. The molecular effects of Curcumin on the NF\( \kappa \B \) pathway are well established [55-57] yet the correlation between these effects and breast cancer invasion and metastasis is not clear. The \textit{in vitro} results presented here clearly proof that Curcumin treatment reduces the viability and induces apoptosis in breast cancer cells as a consequence of disruption of the NF\( \kappa \B \) activation pathway. The intrusion probably occurs at a step before Ik\( \B \) phosphorylation as we could demonstrate that Curcumin treatment of MDA-MB-231 cells inhibits Ik\( \B \)a phosphorylation which in turn reduces nuclear translocation and activation of p65/NF\( \kappa \B \). Aggarwal and coworkers have recently reported similar observations on U937 (human myeloid leukemia) and A293 (human embryonic kidney) cells [57].

Furthermore, our \textit{in vitro} data confirm that the role of NF\( \kappa \B \) in tumor progression is not restricted to its influence on cell death but comprises the transcriptional regulation of gene products related to invasion and metastasis such as the matrix degrading enzymes MMPs which exhibit functional NF\( \kappa \B \) binding sites in their promoter regions [58, 59].

In addition to NF\( \kappa \B \), another transcription factor - AP-1 - is also known to be affected by Curcumin [60]. This is likely to be due to the inhibition of NF\( \kappa \B \) activation
since gene silencing of p65 in MDA-MB-231 cells also reduced the messenger RNA levels of the AP-1 subunit c-jun. Park et al. [61] recently showed that curcuminoids suppress the formation of DNA-Jun-Fos complexes. This is due at least in part to reduced c-jun transcription similar to what we observe here, yet Park et al. observed this effect at much higher, millimolar concentrations. A direct cross-talk between NFκB and AP-1 becomes even more likely given the presence of a perfect NFκB binding element 994 nucleotides upstream of the transcription start site in the c-jun promoter.

Both transcription factors - NFκB and AP-1 - are known to regulate MMP expression [23, 25, 59, 62] and enhanced production of MMPs is associated with a more aggressive tumor growth, a higher metastatic potential, and poor clinical outcome of malignant tumors [63-66].

The effect on NFκB and AP-1 mediated expression of MMPs probably explains the significantly decreased metastatic potential of breast cancer cells that we observed in vivo. The number of hematogenous metastases obtained through intracardial injection of MDA-MB-231 cells was significantly lower in Curcumin treated animals but size and morphology of the metastases formed are indistinguishable from those seen in control animals. Decreased formation of metastases in Curcumin treated animals could be due to effects of the polyphenol on the tumor environment, inflammation or angiogenesis. The histological examination of the metastases from treated and untreated animals does, however, not reveal any such difference. We therefore suggest that a concerted action of MMP inhibition and reduction of survival of single cells, probably due to the repression of an anti-anoikis effect of NFκB, may explain why Curcumin has an effect on the formation of metastases rather than on their growth once they are established. Formation of metastases from prostate carcinoma xenografts has also been reported to be affected by Curcumin [50]. The authors correlated reduced metastases to the direct action of Curcumin on MMP-9 activity. However, MMP-9 is expressed at very low levels in the breast cancer cells used in our study and metastasis formation crucially relies on collagen I degradation operated by MMP-1 expressed by MDA-MB-231 cells and not by the gelatinase MMP-9 that cannot degrade the ECM compound native collagen and therefore promotes only local tumor growth. Mammary carcinoma cells show highest levels of MMP expression in sparse cultures or at the tumor periphery, where cells grow in a less compact pattern and three-dimensional cultures of these cells have an elevated proteolytic activity in regions of low cellular density [67-69]. Well in line with our previous observations, we show that the effects of Curcumin on cell growth and viability are more pronounced in subconfluent cells and could thus have a particular impact on single metastasizing cells.

It is difficult to establish whether the effects on the NFκB pathway observed in vitro persist in vivo, or if they are limited to single cells present in the blood flow. The few metastases formed in Curcumin treated animals probably have managed to escape the drug effects and are therefore not expected to show any alteration in NFκB dependent cell survival or MMP regulation.

Aggarwal et al. very recently established that Curcumin can overcome chemoresistance in breast cancer cells through the inhibition of taxol induced activation of NFκB [70]. These data are consistent with ours and we extend this concept to constitutive, therapy independent activation of NFκB, and thus establish that Curcumin is also suited for the prevention of metastasis.

The present work makes one step in this direction since we establish for the first time Curcumin effects on the formation of metastases through the inhibition of NFκB/AP-1 mediated expression of MMPs. Prevention of breast cancer in subjects at elevated risk using Curcumin is therefore conceivable. Our data indicate, however, that beneficial effects of primary prevention with Curcumin will be limited to patients whose tumors have an intact IKK/IκB signaling, an information that is not available unless the tumor has formed and can be analyzed. In similar way, the functionality of IKK/IκB of the primary tumor should be monitored if Curcumin is to be added to chemotherapy as proposed by Aggarwal et al. [70].

Curcumin is consumed at considerable quantities in regions where it belongs to the normal diet [71]. It appears, however, unlikely that the substance could become a supplement for primary cancer prevention in its original form, in particular because of its poor bioavailability. We are convinced that the effects on the formation of metastases in vivo described here could be obtained by much lower doses of a more water soluble analog. NFκB inhibitors like Curcumin may therefore be considered lead compounds for the development for properly targeted chemopreventive agents.

**Abbreviations**

AP-1 (activating protein 1); CPB (cyclophilin B); G-6-PDH (glucose-6-phosphate dehydrogenase); GAPDH (glyceraldehydes-3-phosphate dehydrogenase);
EMSA (electrophoretic mobility shift assay); HPRT (hypoxanthine guanine phosphoribosyltransferase); IxB (Inhibitor of xB); IKK (IkappaB kinase); LDH (lactate dehydrogenase); MMP (matrix metalloproteinase); MTT [3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide]; NFκB (nuclear factor kappaB); PBGD (porphobilinogen deaminase); RPII (RNA polymerase II); siRNA (small interfering RNA oligonucleotides); TNF-α (tumor necrosis factor alpha).

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