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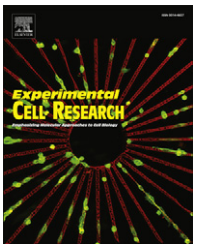

Research Article

Curcumin targets fibroblast–tumor cell interactions in oral squamous cell carcinoma

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ABSTRACT

Co-culture of periodontal ligament fibroblasts (PDLs) and SCC-25 oral squamous carcinoma cells (OSCC) results in conversion of PDLs into carcinoma-associated fibroblasts (CAFs) and induces epithelial-to mesenchymal transition (EMT) of OSCC tumor cells. We hypothesized that Curcumin targets this dynamic mutual interaction between CAFs and tumor cells. Normal and 2 μM Curcumin-treated co-culture were performed for 4 days, followed by analysis of tumor cell invasivity, mRNA/protein expression of EMT-markers and mediators, activity measure of matrix metalloproteinase 9 (MMP-9), and western blot analysis of signal transduction in tumor cells and fibroblasts. In Curcumin-treated co-culture, in tumor cells, the levels of nuclear factor κB (NFκBα) and early response kinase (ERK)—decreased, in fibroblasts, integrin αv protein synthesis decreased compared to corresponding cells in normal co-culture. The signal modulatory changes induced by Curcumin caused decreased release of EMT-mediators in CAFs and reversal of EMT in tumor cells, which was associated with decreased invasion. These data confirm the palliative potential of Curcumin in clinical application.

Introduction

Recent data suggest a pivotal role of collaborative interactions between neoplastic cancer cells and their supporting stroma in tumor growth, progression and treatment resilience of most cancers [1]. In this study, we focus on cells of head and neck squamous cell carcinoma (HNSCC), a common cancer form in men. Our recent investigations indicate that invasive HNSCC

Abbreviations: AKT, another kinase of transcription; AP-1, activator protein-1; bcl2, B-cell lymphoma-2; BDNF, brain-derived neurotrophic factor; CAFs, carcinoma-associated fibroblasts; COX-2, prostaglandin-endoperoxide synthase 2; DMSO, dimethyl sulfoxide; ECM, extracellular matrix; EMT, epithelial-to-mesenchymal transition; ERK, early response kinase; FBS, fetal bovine serum; HNSCC, head and neck squamous cell carcinoma; IL, interleukin; ITGA5, integrin αV; MMP, matrix metalloproteinase; MTT, 3-(4,5-dimethylthiazol-2-yl)2,5diphenyltetrazolium bromide; NFkBα, nuclear factor kBα; OSCC, oral squamous cell carcinoma; PDLs, periodontal ligament (PDL) fibroblasts; TGF-β1, transforming growth factor-β1; TrkB, neurotrophin receptor B

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tumor cells undergo epithelial-to-mesenchymal transition (EMT), which leads to gain of mesenchymal characteristics in epithelial cells. This enables them to migrate across the basal membrane and initiate metastasis formation [2–4]. This phenotype of tumor cells is a consequence of a communication between tumor cells and carcinoma associated fibroblasts (CAFs), which is orchestrated by cytokine–chemokine interactions [2,3,5].

In a co-culture model of periodontal ligament fibroblasts (PDLs) and SCC-25 oral squamous carcinoma cells (OSCC), we investigated the conversion of normal fibroblasts into CAFs and EMT in SCC-25 cells [2]. We observed that SCC-25 cells produce active, processed IL-1β, and PDLs possess receptor for it, whose expression is increased in the presence of SCC-25 tumor cells. Upon interaction with SCC-25 cells active IL-1β signaling occurs in co-cultured fibroblasts leading to induction of several genes involved in tumor progression, including interleukin-6 (IL-6) and prostaglandin-endoperoxide synthase 2 (COX-2) [3]. In fact, dynamic interaction between CAFs and tumor cells dictate gene expression changes in the interacting cells, which covers major events of tumor progression. We have previously reported that fibroblasts might facilitate the invasion of SCC-25 cells by expressing matrix metalloproteinases (MMPs) on their own, in response to tumor-cell-produced cytokines (i. e. IL-1β), to TGF-β)1 or to integrin–fibronectin interactions [5]. At the same time, the tumor cells are capable of activating pro-MMPs [5]. For instance pro-MMP-2 is higher expressed in fibroblasts, but its activation only occurred in the presence of tumor cells. In contrast to pro-MMP-2, pro-MMP-9 is produced by SCC-25 cells, not by PDLs, and pro-MMP-9 is activated on the surface of tumor cells. In fact, the paracrine interaction between CAFs and tumor cells is required for the upregulation of MMP-9 [5]. A dynamic mutual interaction of CAFs and tumor cells is a prerequisite for tumor progression and it represents a significant therapeutic target.

Curcumin (diferuloylmethane) is a polyphenol derived from the *Curcuma longa* plant, commonly known as turmeric. Curcumin has been used extensively in Ayurvedic medicine for centuries, as it is nontoxic and has a variety of therapeutic properties including anti-oxidant, analgesic, anti-inflammatory and antiseptic activity. Curcumin has been found to possess anticancer activities via its effect on a variety of biological pathways involved in mutagenesis, oncogene expression, cell cycle regulation, apoptosis, tumorigenesis and metastasis. Curcumin has shown anti-proliferative effect in multiple cancers, and is an inhibitor of the transcription factor NFκBα and downstream gene products (including c-Myc, Bcl-2, COX-2, nitric oxide synthase (NOS), Cyclin D1, tumor necrosis factor alpha (TNF-α), interleukins and MMP-9) [6]. Studies of Curcumin in various head and neck cancer cell lines have demonstrated decreased cell growth and survival, which was related with inhibition of NFκBα activation [7].

The lipophilic nature of Curcumin and relative insolubility in aqueous solutions, combined with short half life and low bioavailability following oral administration has presented a significant challenge in developing an effective delivery system for its use as a chemotherapeutic agent [8]. In an effort to overcome this obstacle, various strategies are being tried including the development of liposomal, phospholipid and nanoparticulated formulations of the compound to enable intravenous administration [8]. In a pharmacokinetic study performed in rats, following oral administration of the Curcumin–phosphatidyl choline complex, the maximal achieved plasma concentration of Curcumin was around 800 ng/ml [9], which is 2.17 μmol/L. In a human phase 1 trial Curcumin was orally applied for advanced colorectal cancer, patients consumed 3.6 g of Curcumin daily, and up to 2–2.5 μM Curcumin concentrations have been detected in their urine. Temporary stable diseases have been observed in two patients, whereas no response was observed in the other study participants [10]. In an *in vitro* study: Curcumin above 50 μmol/L concentration caused growth suppression of HNSCC cell lines [11], which is much higher than the concentrations achievable *in vivo*.

In the current study we hypothesize that Curcumin targets the dynamic mutual interaction of CAFs and tumor cells in head and neck cancer. We assume that Curcumin achieves this effect even at those low concentration levels that are detected in blood or urine (2 μmol/L) in previous *in vivo* studies. We presume that it modifies the interaction between carcinoma associated fibroblasts and tumor cells, which leads to decrease of the tumor cell invasivity, without growth suppression effects on tumor cells.

Materials and methods

Chemicals

Curcumin powder was purchased from Sigma-Aldrich (Vienna, Austria). Curcumin is a lipophilic polyphenol and thus is insoluble in water, but is readily soluble in organic solvents such as dimethyl sulfoxide (DMSO), acetone and ethanol [12,13]. A stock solution of Curcumin was prepared at 100 mmol/L in DMSO. Chemicals used in the study were purchased from Sigma, from Roth (Karlsruhe, Germany) and from Serva (Heidelberg, Germany).

Cell lines

PDL fibroblasts [2,14] were isolated from periodontal ligament (PDL) and received from Prof. Dr. Miosge (Department of Prosthodontics, Georg-August-University, Göttingen, Germany) [14]. They were routinely cultured in DMEM-low glucose (PAA, Pasching, Austria) supplemented with 10% fetal bovine serum (FBS) (PAA), 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin. SCC-25 cells were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany, ref. ACC 617), and were routinely cultured in DMEM/F12 (PAA) supplemented with 10% FBS (PAA), 2 mM L-glutamine, 1 mM sodium pyruvate, 100 units/ml penicillin, 100 μg/ml streptomycin [2,3,5,15]. BEAS-2B immortalized bronchial epithelial cells [15,16] were purchased from the European Collection Agency of Cell Cultures (Salisbury, UK), were routinely cultured in RPMI-1640 (PAA) supplemented with 10% fetal bovine serum (FBS) (PAA), 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin.

Treatment of cells with Curcumin

The Curcumin treatment of the cells is based on a published Ref. [11]. SCC-25 cells, PDL fibroblasts and BEAS-2B immortalized epithelial cells were plated in 96-well plates, with 10^4 cells per

well, or in 10 cm Petri-dishes at 10^5 cells/ml, 10 ml cell suspension/dish, and were allowed to grow for 24 h. The cells were then serum starved (using 0.3% fetal bovine serum containing medium) [11]. Curcumin was diluted to final concentrations ranging from 2 to 250 μmol/L. To control experiments 2.5 μl DMSO/ml medium was added, representing the highest DMSO content of the treated cells. After 24 h serum starvation treatment was started with several Curcumin concentrations in 10% serum-containing medium for 24 h. After completion of the treatment, cell viability was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT) system [17], and cell counts were determined.

Co-culture treatment with Curcumin

PDL fibroblasts were plated in DMEM-low-glucose/10% FBS at 10^4 cells/ml on cell culture inserts containing a 0.45 μm plastic membrane filter, and SCC-25 cells were cultured separately in DMEM-F12/10% FBS at 4×10^4 cells/ml in wells of six-well plate (Greiner, Kremsmünster, Austria) [2,3,5]. Each experimental system contained controls, which were plated in the same way. After 48 h the cell culture inserts with the fibroblasts were put together with the wells containing SCC-25 cells, the medium was replaced in the inserts and in the wells of six-well plates by DMEM-low-glucose–DMEM-F12/5% FBS. The controls were not co-cultured, but received the same mixed medium. In the controls the fibroblasts or SCC-25 cells were omitted [2,3,5]. Direct contact of the cells was prevented by a 0.45 μm plastic membrane, but there was a continuous communication of soluble substances between the upper and the lower cells. Some of the co-cultures were treated with 2 μM Curcumin for 3 days, by daily medium and Curcumin replacements. Similarly, the medium in the co-cultures and in the controls was changed daily. After 3 days of co-culture and treatments, on the fourth day, the control cells and the cells in co-culture received mixed DMEM-low-glucose–DMEM-F12/0.3% FBS, the treated co-cultures were additionally provided with 2 μM Curcumin for the last 24 h. On the fifth day, after co-culture and treatments, the experiment was terminated; the supernatants were used for ELISA and gelatinase assays, the cells in the inserts and in the wells were used for RNA isolation, or for protein fractionation. This experimental setup was performed in four repeats.

Tumor cell migration and invasion assays

Co-culture and Curcumin treatment of co-culture have been performed as described above, for migration and invasion assays SCC-25 cells were used further. After completion of co-culture with and without Curcumin treatment, control, co-cultured and co-cultured/Curcumin-treated SCC-25 cells were released by trypsinization, and the cells were counted. From all SCC-25 cell samples (control, co-cultured and co-cultured, Curcumin-treated), 2 ml, 2×10^5 cells/ml suspensions were prepared in DMEM/F12–0.3% serum. For cell migration assays 2 ml DMEM/F12–10% fetal bovine serum (FBS) medium was added in 6-well plates under 8 μm inserts. 1 ml cell suspension (in DMEM/F12–0.3% serum) was added into the inserts [18]. For cell invasion assays Matrigel (Sigma) was diluted to 200 μg/ml and applied to the top side of the filter for coating for 1 h [18]. 1 ml cell suspension (in DMEM/F12—0.3% serum) was added into the inserts and 2 ml DMEM/F12—10% fetal bovine serum (FBS) medium was added in 6-well plates under the inserts. After incubation for 24 h at 37 °C for both the migration and the invasion experiments, the cells in the upper surface of the 8 μm inserts were carefully removed with a cotton swab, and migrated or invaded cells were trypsinized and counted from the lower surface of the 8 μm inserts [18].

RNA extraction, reverse transcription and real-time RT-PCR

Total RNA was isolated and reverse transcribed from control and co-cultured cells as described before, real-time RT-PCR was done as referenced [2,19]. All used human PCR primers were published previously: *β-actin* [20], *E-cadherin*, *vimentin* [21], *stoma-derived factor-1* (*SDF-1*) [22], *brain-derived neurotrophic factor* (*BDNF*) [2]; *β-actin* functioned as housekeeping gene [2], and did not show significant changes in co-culture and treated co-culture conditions compared to controls. The relative gene expression was calculated as previously reported [19].

Protein expression and activity measurements

Conditioned media were collected from both the fibroblast and the SCC-25 side of the co-culture, Curcumin-treated co-culture and also from the control cells. From the supernatant (conditioned media) of the control cells and from both sides of the co-cultures 20 μl medium was used from all samples for gelatinase zymogram [5]. Supernatants were always examined at low-serum-containing conditions [5]. Cells after co-culture, Curcumin-treated co-culture and controls were used for non-nuclear and nuclear protein fractionation, as described previously [3,23]. For gelatinase analysis supernatants and cytoplasmatic/membrane fractions were subjected to polyacrylamide gel electrophoresis using gelatin-containing 10% polyacrylamide gels [5,24].

For the analysis of integrin αv, phospho-another kinase of transcription (p-Akt), (phospho)-neurothrophin receptor B (TrkB, p-TrkB), (phospho)-interleukin-1 receptor-associated kinase-1 (IRAK, p-IRAK) and nuclear factor kappa B alpha (NFκBα) non-nuclear protein fractions were subjected to western blot analysis [2] as described previously [2,3,5,15,23]. Identical equal protein containing 20 μl samples were loaded for western blot, which was performed with the JLA20 β-actin antibody (in 1:100 dilution), purchased from Developmental Studies Hybridoma Bank (Iowa City, Iowa, USA) as a loading control [25].

For the analysis of (phospho)-early response kinase (ERK1/2 and p-ERK1/2) and of NFκBα nuclear protein fractions were subjected to western blot analysis [2,3,5,15,23]. Details of the used antibodies are available in Supplementary Table 1.

ELISA

Conditioned media were collected from both the fibroblast and the SCC-25 side of the co-culture, Curcumin-treated co-culture and also from the control cells, as described above. From the supernatant (conditioned media) of the control cells and from both sides of the co-culture 100 μl medium was used from all samples for quantitative TGF-β1, SDF-1 and BDNF detection using RayBio human ELISA kits (RayBiotech, Norcross, GA, USA).

Statistical analysis

Each experiment was performed in four independent sets containing four biological repeats/set. The relative gene-expression results were tested for normal distribution by D'Agostino and Pearson omnibus normality test using the Graphpad Prism 4.03 (Graphpad Software Inc., San Diego, CA, USA). Significance of changes in co-culture vs. controls was tested by non-parametric tests (Mann–Whitney) and Students' *t*-tests depending on the distribution of the data. The independent experimental sets were then compared for reproducibility. Only reproducible changes with a $p<0.05$ level [2,3] were considered as significant.

Results

Determination of growth suppressing concentrations of Curcumin in SCC-25, BEAS-2B cells and in PDL fibroblasts

SCC-25 cells, BEAS-2B cells and PDL fibroblasts were treated with Curcumin as described previously [11]. Curcumin showed significant reduction of MTT-measured metabolic activity in SCC-25 cells (Fig. 1 and Table 1), if it was administered over 2 μM concentration. In addition, at a concentration over 25 μM, Curcumin also caused significant reduction of MTT-measured metabolic activity in BEAS-2B not transformed epithelial cells, and at higher concentrations it reduced the metabolic activity of PDL fibroblasts (Fig. 1 and Table 1). Nevertheless, in tumor cells the effect was clearly stronger. Selectivity of Curcumin has been shown at concentrations up to 60 μM (Fig. 1), by more intensive growth suppression of SCC-25 cells. The MTT results have been also confirmed by determination of the cell numbers after Curcumin treatment (not shown). 2 μM Curcumin concentration caused up to 5% loss of the metabolic capacity of SCC-25 cells, in PDL fibroblasts or BEAS-2B cells this concentration was ineffective. In this regard, at the level of at least 95% viability, we have used 2 μM Curcumin for further experimentation. Interestingly, this concentration level has been reached by previous *in vivo* studies or in clinical trial in blood or in urine [9,10].

Influence of Curcumin on the migration and invasion of SCC-25 tumor cells in co-culture system with PDL fibroblasts

We have recently reported that several days co-culture of SCC-25 tumor cells with PDL fibroblasts leads to EMT of tumor cells [2], which might be associated with increased tumor cell invasivity. Adopting a method described by Sun et al. [18] we have investigated the migratory and invasive capacity of SCC-25 cells after normal or Curcumin-treated co-culture. As previously described, 2 μM Curcumin had no growth suppressive effects either on SCC-25 cells or on PDLs. Migration and invasion tests have been performed with SCC-25 cells immediately after normal or Curcumin-treated co-culture. Since the cell number of SCC-25 cells slightly increased after co-culture compared to control cells (not shown), before performing migration and invasion tests, the number of the cells was adjusted to 2×10^5 cells/ml in all cases. As depicted in Fig. 2A and B: both the migration and invasion capacities of SCC-25 cells increased after co-culture, whereas in 2 μM Curcumin-treated co-culture both of them remained at the control level.

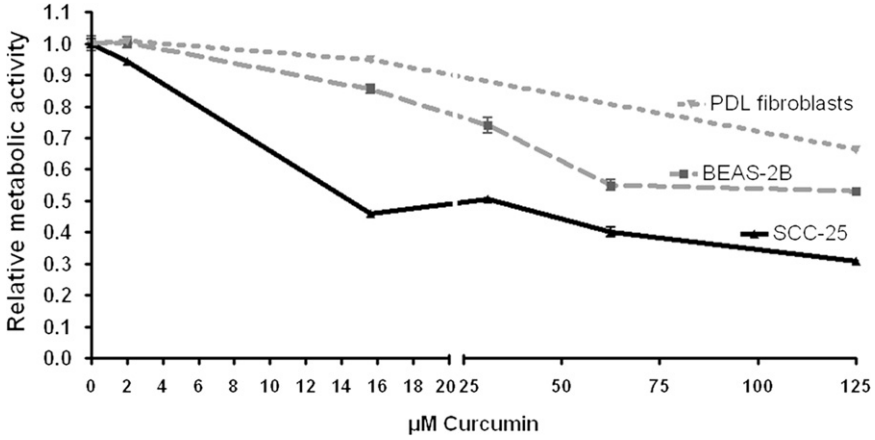

Fig. 1 – Relative metabolic activity measured by MTT-assay in Curcumin-treated SCC25, BEAS-2B and PDL fibroblast cells after Curcumin-treatment related to DMSO-treated control cells.

Table 1 – Metabolic activity measured by MTT-assay in Curcumin-treated SCC25, BEAS-2B and PDL cells after Curcumin-treatment related to DMSO-treated controls. 2 μM Curcumin concentration caused 5% loss of the metabolic capacity of SCC-25 cells, in PDL fibroblasts or BEAS-2B cells this concentration was ineffective. In this regard, at the level of 95% viability, we have used 2 μM Curcumin concentrations for further experimentation. The dose limiting cells were the carcinoma cells.

	Control	2 μM Curcumin	15.62 μM Curcumin	125 μM Curcumin
SCC-25	1.00±0.015	0.95±0.007	0.46±0.005	0.31±0.001
BEAS-2B	1.00±0.02	1.00±0.02	0.85±0.01	0.53±0.007
PDL fibroblasts	1.00±0.01	1.00±0.006	0.95±0.01	0.66±0.02

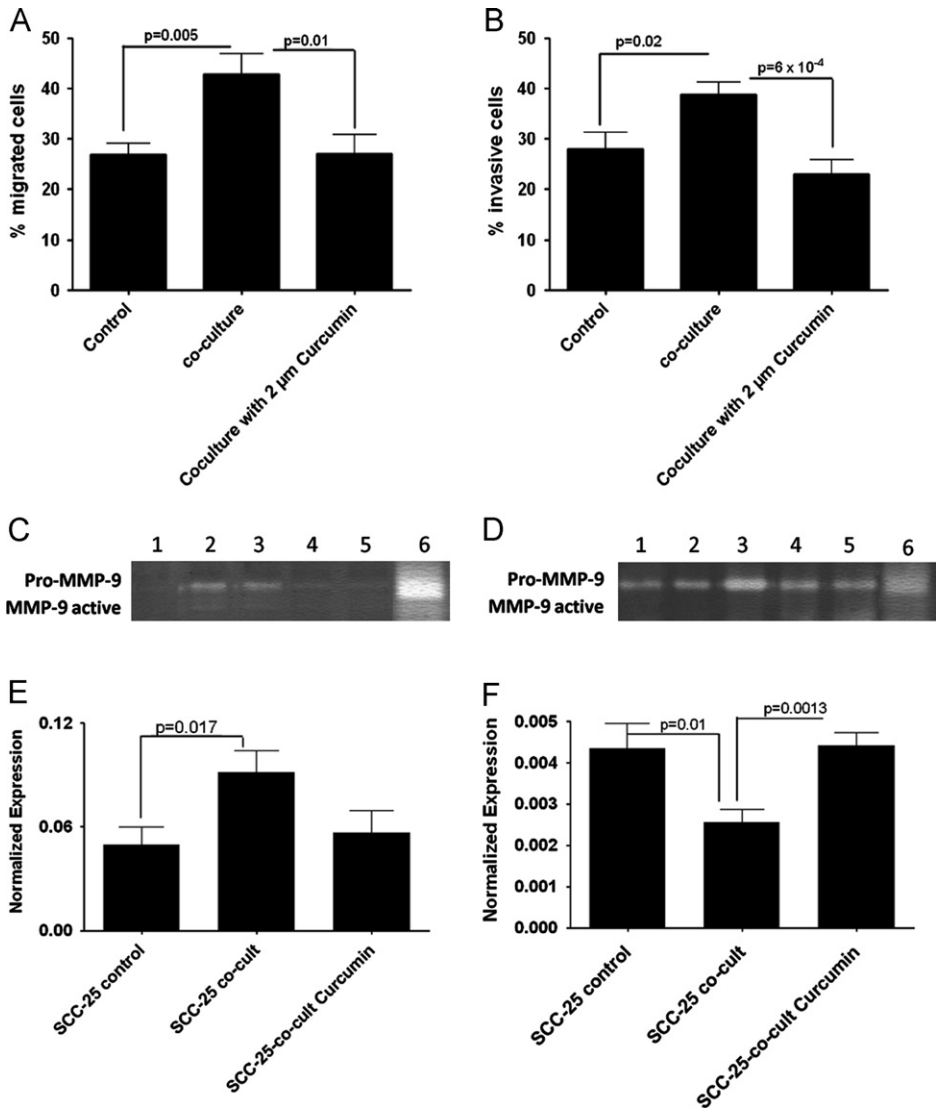

Fig. 2 – Effects of Curcumin on the invasivity and EMT of SCC-25 cells in co-culture. After 4 days of normal or Curcumin-treated co-culture (PDL fibroblasts with SCC-25 cells): migration (A) and invasivity (B) of SCC-25 cells was determined as described in Materials and methods. Co-culture resulted in significant increase of migration (A) and invasivity (B) of SCC-25 cells, which has remained at the control level if the co-culture was treated with 2 μM Curcumin. MMP-9 gelatinase activity in non-cell-nuclear fraction (C) and in conditioned medium of SCC-25 cells (D) in control (1), co-cultured (2 and 3), co-cultured and 2 μM Curcumin-treated (4 and 5) conditions. FBS was used as positive control (6). Relative gene expression of vimentin (E) and E-cadherin (F) in control, co-cultured and 2 μM Curcumin treated-co-cultured SCC-25 cells. Co-culture induced a significant increase of vimentin gene expression in SCC-25 cells (E), which has remained at the control level if the co-culture was treated with 2 μM Curcumin. At the same time, co-culture with PDL cells induced a significant decrease of E-cadherin gene expression in SCC-25 cells (F), which has remained at the control level if the co-culture was treated with 2 μM Curcumin.

The increased invasivity of co-cultured SCC-25 cells might be related to production and activation of matrix metalloproteinases. We have previously reported the increased activity of MMP-9 in co-cultured SCC-25 cells [5], which has been confirmed in the current study as well (Fig. 2C and D). Mostly pro-MMP-9 gelatinase bands increased in non-cell-nuclear protein fractions (Fig. 2C), whereas in the conditioned media (Fig. 2 D) the increase of pro-MMP-9 was less evident, and active MMP-9 was not detectable. Nevertheless, in 2 μM Curcumin-treated, co-cultured SCC-25 cells the MMP-9 gelatinase activity in cell lysates and in conditioned medium remained at the control level (Fig. 2C and D).

Influence of Curcumin on EMT of SCC-25 tumor cells in co-culture system with PDLs

In addition to the achieved invasive phenotype, the co-culture with PDL fibroblasts lead to induction of the mesenchymal marker, *vimentin* gene in SCC-25 cells, as published before [2] (Fig. 2E). This was accompanied by the down-regulation of *E-cadherin* [2] (Fig. 2F). Interestingly, both *vimentin* and *E-cadherin* gene expression have remained at the control level if the co-culture was treated with 2 μM Curcumin (Fig. 2E and F).

Influence of Curcumin on signal transduction of SCC-25 tumor cells in co-culture system with PDL fibroblasts

In order to investigate the signal transduction effects of Curcumin on SCC-25–PDLs interactions, we have fractionated proteins of cytoplasma/cell membrane and nucleus [23] of both SCC-25 cells and PDLs. We have previously published that integrin αv (ITGA5) expressed in SCC-25 cells might contribute to regulation of MMP-9 [5]. Confirming our previous data, in the non-nuclear extracts, ITGA5 was upregulated (up to 153% of the control by densitometry of western blots normalized to beta-actin) in SCC-25 cells in co-culture, whereas, in 2 μM Curcumin-treated co-culture, the ITGA5 protein levels were slightly reduced (to 90±0.3% compared to normal co-cultured SCC-25 cells by densitometry of western blots normalized to beta-actin) (Fig. 3A, top panel). EMT and invasivity in tumor cells of HNSCC might be mediated by the receptor of brain-derived neurotrophic factor (BDNF): TrkB [2]. The active phosphorylated form of TrkB was analyzed in the non-nuclear extracts of SCC-25 cells by western blot in control, co-cultured and Curcumin-treated co-cultured conditions. Its protein levels increased in co-culture (to 171±10% by densitometry of western blots normalized to beta-actin) and returned to control levels in Curcumin-treated co-cultured conditions (to 75±7.5% of the normal co-culture by densitometry of western blots normalized to beta-actin) (Fig. 3A).

Two main signal targets of Curcumin [18] have also been investigated at protein level in co-cultured and 2 μM Curcumin-treated, co-cultured SCC-25 cells, one is Akt and the other is NFκBα. The active phospho-Akt has suffered only minor changes, 92±4% in Curcumin-treated, co-cultured compared to normal co-cultured SCC-25 cells (Fig. 3A). In contrast, cytoplasmatic levels of p65 NFκBα have dramatically decreased to 45±10% (by densitometry normalized to beta-actin) in SCC-25 cells of Curcumin-treated co-culture compared to normal co-culture (Fig. 3A). These changes in the cytoplasma levels of p65 NFκBα have been accompanied by similar changes in the cell nuclear fraction (Fig. 3B top panel). The relation of nuclear NFκBα density to cytoplasmatic density in control, co-cultured and Curcumin-treated co-cultured SCC-25 cells was as follows: 0.92, 0.87, and 1.39. In addition, in cell nuclear extracts the protein levels of (phospho)–ERK1/2 have decreased to 73±3% (by densitometry normalized to beta-actin) in Curcumin-treated, co-cultured compared to normal co-cultured SCC-25 cells (Fig. 3 B middle and lowest panel). The relation of pERK to ERK density was the same in control, co-cultured and Curcumin-treated co-cultured SCC-25 cells: 1.05±0.04.

Influence of Curcumin on expression of EMT-mediators in fibroblasts in co-culture system with SCC-25 cells

As we have previously reported, in co-culture between SCC-25 cells and PDLs, fibroblasts were converted to CAFs, which in return initiated EMT of SCC-25 cells. EMT has been induced in tumor cells due to the release of EMT-mediators in CAFs, such as stroma derived factor (SDF) or brain derived neurotrophic factor (BDNF) [2]. As previously described, in 2 μM Curcumin treated co-culture, vimentin and E-cadherin gene expression levels remained at the control level in SCC-25 cells, which was accompanied with reduced migratory and invasive activity. In this regard, it seems that treatment of SCC-25–PDLs co-culture with Curcumin abrogates EMT in tumor cells. In a further step, it was investigated, if it might be due to the reduced production levels of EMT-mediators in co-cultured fibroblasts.

After 4 days of co-culture and in some experiments Curcumin-treated co-culture, relative gene expression (Fig. 4A and B) and protein synthesis (Fig. 4C and D) of SDF-1α (Fig. 4A and C) and

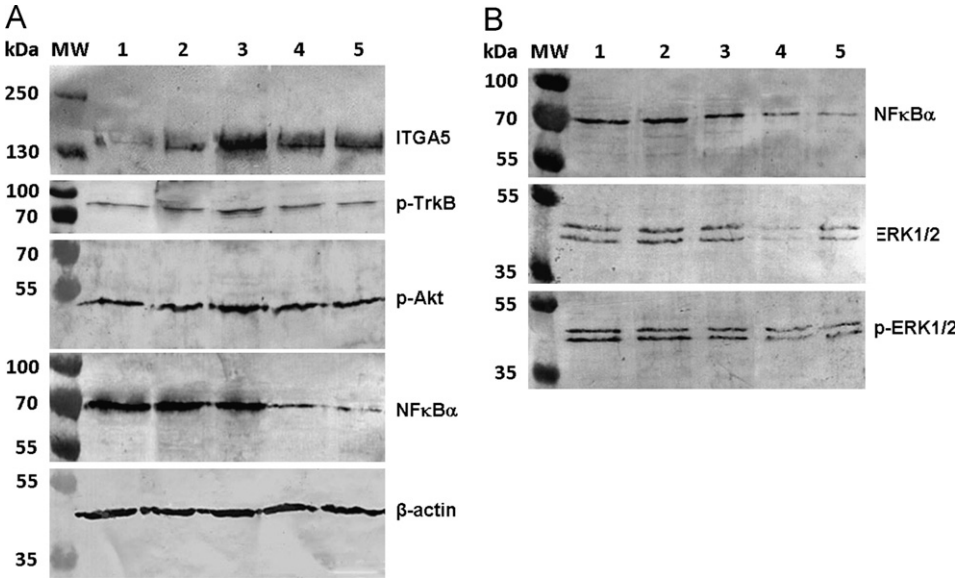

Fig. 3 – Effects of Curcumin on the signal transduction of SCC-25 cells in co-culture. After 4 days of co-culture (PDL fibroblasts with SCC-25 cells) and in some experiments treatment of the co-culture with 2 μM Curcumin non-nuclear (A) and cell nuclear (B) fractions of SCC-25 cells were analyzed by western blot using β-actin as loading control. In non-nuclear protein fraction: integrin αv (ITGA5), p-TrkB, p-Akt, NFκBα, in cell nuclear fraction: NFκBα, ERK1/2 and p-ERK1/2 have been analyzed. Samples loaded on polyacrylamide gels: MW: molecular weight marker, control (1), co-cultured (2 and 3), co-cultured and 2 μM Curcumin-treated (4 and 5) SCC-25 cells.

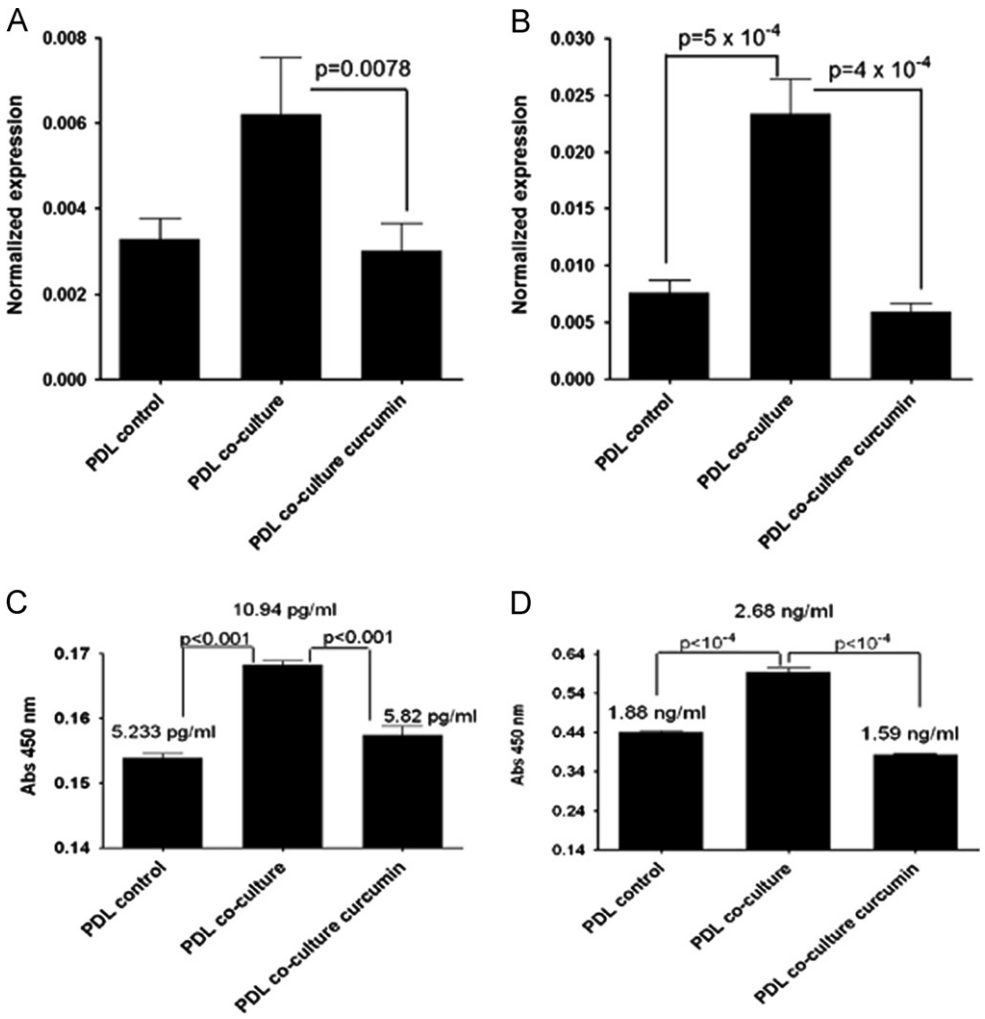

Fig. 4 – Effects of Curcumin on gene expression and production of EMT-mediators in PDL fibroblasts in co-culture. After 4 days of co-culture (PDL fibroblasts with SCC-25 cells) and in some experiments treatment of co-culture with 2 μM Curcumin, relative gene expression (A and B) and protein synthesis (measured by ELISA, shown as absorption at 450 nm) (C and D) of SDF-1α (A and C) and BDNF (B and D) have been analyzed in control, co-cultured and 2 μM Curcumin treated-co-cultured fibroblasts. Co-culture induced a significant increase of SDF-1α and BDNF gene expression and protein synthesis in fibroblasts, both of which has remained at the control level if the co-culture was treated with Curcumin.

BDNF (Fig. 4B and D) have been analyzed in control, co-cultured and 2 μM Curcumin treated-co-cultured PDLs. Co-culture induced a significant increase of SDF-1α and BDNF gene expression and protein synthesis in PDLs (Fig. 4), both of which has remained at the control level if the co-culture was treated with Curcumin.

Influence of Curcumin on signal transduction of PDLs in co-culture system with SCC-25 cells

Similarly to the SCC-25 cells, also in PDL fibroblasts, signal transduction effects of Curcumin on SCC-25–PDLs interactions were investigated in non-nuclear and nuclear fractions [23]. In the non-nuclear extracts, ITGA5 was slightly upregulated (up to 110% by densitometry normalized to beta-actin) in co-cultured PDLs similarly to SCC-25 cells, whereas, in 2 μM Curcumin-treated co-culture, the ITGA5 protein levels were reduced (until 60% of the normal co-culture by densitometry normalized to beta-actin) (Fig. 5A, top panel) in them. The interaction between SCC-25 cells and PDLs is partly mediated by IL-1β [3], across activation of interleukin-1 receptor-associated kinase-1 (IRAK). The density relation of p-IRAK to IRAK in control cells was 0.64, in co-cultured PDLs: 1.13 in curcumin-treated co-cultured PDLs: 1.21. Compared to control cells IRAK phosphorylation increased in co-cultured PDL fibroblasts (to 127±9.4% by densitometry of western blots normalized to beta-actin), and remained increased in 2 μM Curcumin-treated co-cultured PDLs (98.95±5.7% in curcumin-treated co-culture compared to normal co-culture by densitometry of western blots normalized to beta-actin) (Fig. 5A). Cytoplasmatic levels of NFκBα have not changed in control, co-cultured and Curcumin-treated co-cultured PDLs (Fig. 5A, lowest panel). In the cell nuclear fraction NFκBα levels increased in co-cultured PDLs related to cytoplasmatic levels (1.49±0.15 by densitometry), and this increase was lower in Curcumin-treated co-cultured PDLs (1.23±0.65 by densitometry) (Fig. 5B).

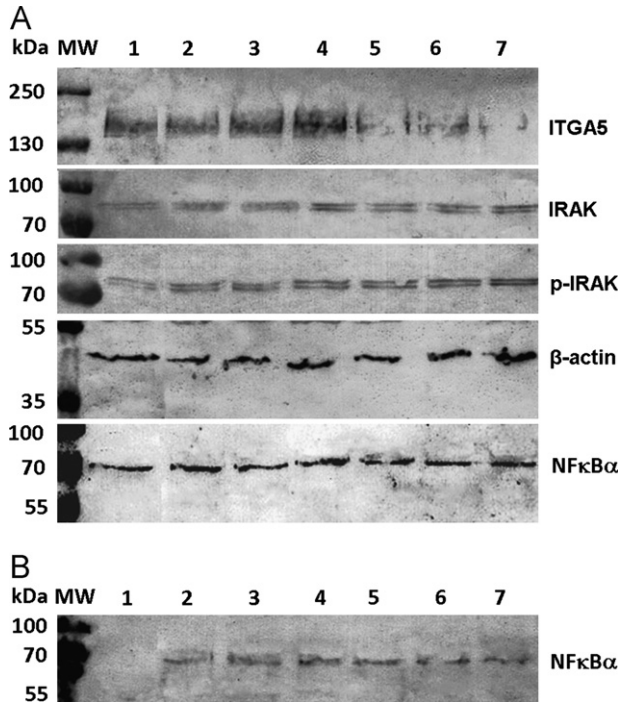

Fig. 5 – Effects of Curcumin on the signal transduction of PDLs in co-culture. After 4 days of co-culture (PDLs with SCC-25 cells) and in some experiments treatment of the co-culture with 2 μM Curcumin: non-nuclear (A) and cell nuclear (B) fractions of PDLs were analyzed by western blot using β-actin as loading control. In non-nuclear protein fraction (A) integrin αv (ITGA5), IRAK, p-IRAK and NFκBα, in cell nuclear fraction: NFκBα have been analyzed. Samples loaded on polyacrylamide gels: MW: molecular weight marker, control (1), co-cultured (2–4), co-cultured and 2 μM Curcumin-treated co-cultured (5–7) PDL cells.

Discussion

In the current study we hypothesized that Curcumin targets the dynamic mutual interaction of CAFs and tumor cells in head and neck squamous cell carcinoma at such low concentration levels that are achievable *in vivo*. We have clearly evidenced that Curcumin treatment of co-culture between SCC-25 cells and PDL oral fibroblasts resulted in decrease of tumor cell migration and invasivity, reversal of EMT in tumor cells, and decrease of the EMT mediators' gene expression and synthesis in fibroblasts. The modification of the interaction between carcinoma associated fibroblasts and tumor cells is achieved at low Curcumin concentrations, which do not have growth suppression effects. This study proves that Curcumin signal modulatory effects can be studied independently from growth suppression effects.

The most important and clinically relevant result is the reduction of tumor cell invasivity, based partially on the reduction of MMP-9 enzyme activity in tumor cells. The observation that Curcumin might decrease MMP-9 gene expression has been published before [26]. In that study of Mohan et al. this issue has been related to inhibitory effect on activator protein-1 (AP-1). Nevertheless, MMP-9 expression and activation might be also related with the transcription factor NFκBα [26], which is one of the main targets of Curcumin [7]. Chun et al. have reported that Curcumin dissolved in acetone, applied from 1 μM to dorsal skin of mice induced a dramatic reduction of – tetradecanoylphorbol-13-acetate (TPA)-induced NFκBα – activation, and a slight reduction of ERK1/2 activity [7]. These cell nuclear targets have also been confirmed in our study in co-cultured SCC-25 cells. The interaction of SCC-25 tumor cells with fibroblasts resulted in increased ERK1/2 and NFκBα—levels, both of which decreased if the co-culture was treated with Curcumin (Fig. 3B). Nevertheless, the relative function of both ERK1/2 and NFκBα was not influenced. Interestingly, Curcumin caused slightly decreased NFκBα cell nuclear translocalization in co-cultured PDLs (Fig. 5B).

As we have previously published, the ITGA5 is upregulated both in SCC-25 cells and in PDL fibroblasts in co-culture, and might also contribute to regulation of MMP-9 [5]. Here, the upregulation of ITGA5 is confirmed at protein level both in co-cultured SCC-25 cells and PDLs (Figs. 3 and 5). Curcumin treatment of co-culture caused a decrease of the ITGA5 levels both in fibroblasts and tumor cells. Interestingly, this effect was more enhanced in fibroblasts. In fact, reduction effects of Curcumin on gene expression of ITGA5 have been evidenced previously in metastatic melanoma, which was accompanied by increased expression of E-cadherin [27]. Increased expression of E-cadherin has been also observed in Curcumin-treated co-culture of SCC-25 cells (Fig. 2F), which is a sign of reversal of EMT, and was accompanied with decreased expression of vimentin (Fig. 2E).

Previous studies of our laboratory [2] and of others [28] confirmed an important participation of BDNF-TrkB system in regulation of EMT. BDNF gene expression, as a mediator of EMT, is upregulated in carcinoma-associated fibroblasts (CAFs), which are functionally comparable with co-cultured PDLs [2]. TrkB, the receptor of BDNF is expressed in SCC-25 cells, and its phosphorylated form is increased in co-cultured SCC-25 cells. Curcumin treatment of co-culture decreases both the gene expression of the EMT-mediator BDNF and the phospho-TrkB levels in SCC-25 tumor cells, providing a plausible mechanistic background for the reversal of EMT. Similarly to BDNF, also SDF gene expression has been down regulated in PDLs of curcumin-treated co-culture. Kupferman et al. suggested that TrkB is functioning through AKT signaling [28]. AKT (another kinase of transcription, also known as protein kinase B) is a protein kinase involved in signal transduction from oncogenes and growth factors [6]. The data on Curcumin's effect on the AKT pathway is varying; while Curcumin has been shown to act independently of AKT in HNSCC as well as melanoma, whereas Curcumin suppresses the AKT pathway in other tumors such as malignant gliomas and pancreatic cancer [6]. Confirming this knowledge, Curcumin has also not effected AKT in co-cultured SCC-25 cells (Fig. 3A).

The interaction of CAFs with tumor cells in supporting tumor cell-EMT and invasivity is a complex one, where the involvement of inflammatory cytokines produced by the tumor cells has been evidenced [3]. These cytokines, exemplified by IL-1β, might have receptors on fibroblasts and can regulate the expression of EMT-mediators as SDF-1 and BDNF in them [3]. This regulation in case of IL-1β, is mediated by IRAK. IRAK phosphorylation is increased in co-culture in fibroblasts and Curcumin-treatment of co-culture has not affected it.

Taken together, the most visible changes in Curcumin-treated co-culture in tumor cells were the decrease of p-TrkB, NFκBα and ERK-levels, and in fibroblasts: reduction of ITGA5 expression and of NFκBα cell nuclear translocalization. These (and presumably other) signal modulatory changes caused decreased release of EMT-mediators in CAFs and reversal of EMT in tumor cells. Reversal of EMT was associated with decreased invasion. These data confirm the palliative potential of Curcumin in clinical application and underline the need of improved formulation for *in vivo* delivery.

Conflict of Interest Statement

Authors declare no interest conflicts.

Role of the Funding Source

The funding source has no influence on the direction and the outcome of the study, it is an independent granting.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.yexcr.2012.12.001.

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