Abstract. In the present study, a polysaccharide extract was obtained from *Ocimum basilicum* (basil polysaccharide, BPS) and the effects of curcumin and BPS on the invasion activity of the SKOV3 ovarian cancer cells and human monocyte-derived dendritic cells (DCs) were investigated. SKOV3 cells and immature or mature DCs were treated with 50 \( \mu \)M curcumin or 100 \( \mu \)g/ml BPS. A transwell invasion assay demonstrated that curcumin and BPS differentially regulate the invasion of SKOV3 cells and DCs. Curcumin significantly decreased the invasion of SKOV3 cells and immature and mature DCs, while BPS only decreased SKOV3 cell invasion. Osteopontin (OPN) mRNA and protein expression were significantly reduced in curcumin and BPS -treated SKOV3 cells and curcumin‑treated DCs. Furthermore, flow cytometry showed that curcumin significantly inhibited the surface expression of CD44 in SKOV3 cells and DCs, while BPS had a minimal effect on CD44 expression. Matrix metallopeptidase -9 (MMP-9) mRNA and protein expression were also reduced in all curcumin-treated cells and BPS-treated SKOV3 cells. The results indicated that curcumin and BPS regulated invasion of SKOV3 cells and DCs by distinctly downregulating OPN, CD44 and MMP-9 expression. Therefore, Curcumin and BPS may be suitable candidates for DC-based vaccines for ovarian cancer immunotherapy.

Introduction

Ovarian cancer is one of the most common types of malignancy in females worldwide, with an estimated 225,500 novel cases and 140,200 cancer-related mortalities occurring annually (1). Although chemotherapy for ovarian cancer has been well established throughout the last three decades, the 5-year survival rate remains <50%, the lowest of all gynecological malignancies. The cause for this is that ovarian cancer tends to have distantly metastasized by the time the majority of cases are diagnosed (1). Local invasion is an initiating step in cell migration and commonly occurs prior to distant metastasis; therefore, decreasing invasion activity of cancer cells may improve the outcome of ovarian cancer patients.

In healthy individuals, malignant cells are under the surveillance of immune cells to resist invasion and metastasis, which is an essential aspect of anticancer immunity. Dendritic cells (DCs) are known to be the most specialized antigen-presenting cells and are important in the development of anticancer immune responses as they control the initiation and polarization of adaptive immunity (2). A previous clinical trial showed promising results achieved by DC vaccination in advanced ovarian cancer patients, with 90% overall survival during 36 months of observation (3). Fulfilling this function, however, is dependent upon the migration of DCs to T-cell-rich lymph organs following the uptake of the vaccination and the presentation of tumor antigens. Inhibition of DC invasion activity facilitates tumor escape from host immune surveillance. A previous study indicated that intratumoral injection of DCs generated in vitro, fails to initiate systemic antitumor immunity as the DCs migrated into regional lymph nodes less efficiently (4). Thus, a complementary therapy, which decreases the motility of cancer cells without markedly affecting DC invasion, may be beneficial in preventing ovarian cancer metastasis.

Cell invasion results from cross-talk between cells and the extracellular matrix (ECM). Osteopontin (OPN), a phosphorylated glycoprotein with pleiotropic properties, is expressed on the ECM and on the surface of a number of cells, including malignant cells, lymphocytes and vascular smooth muscle cells. OPN promotes cell adhesion and invasion in various
physiological or pathological conditions (5-7). These functions are primarily achieved by its interaction with receptors on the cell surface, including the \(\alpha 5\beta 3\) integrin and CD44 (8), and the initiation of downstream signaling events, for example, matrix metalloproteinase-9 (MMP-9) expression (9-11). A previous study suggested that OPN decisively enhanced the migration of DCs into draining lymph nodes (12). In ovarian cancer, OPN serves as one of the biomarkers for early detection and predictors of prognosis (13,14), indicating its involvement in cancer development and progression. However, the specific mechanisms of OPN in ovarian cancer invasion remain unknown.

Among chemotherapeutic agents against cancer, natural bioactive molecules possess a unique advantage of milder side-effects. Curcumin, a polyphenolic pigment extracted from turmeric (Curcuma longa), is a prime example, due to its low toxicity and high anticancer potency. The application of curcumin as a complementary therapy for ovarian cancer appears promising as it induces apoptosis (15) and sensitivity to cisplatin (16) in ovarian carcinoma, without decreasing quality of life (17). Moreover, the enhancement of adaptive immunity was involved in curcumin-mediated tumor growth retardation (18,19). However, to the best of our knowledge, no systematic analysis of curcumin on the invasion of ovarian cancer cells and DCs has been reported. Sweet basil (Ocimum basilicum) is commonly used in Chinese traditional medicine for detoxemesis, anti-inflammation and promoting circulation, and previous studies have shown its cytotoxic effects in cancer cells (20). In the current study, a polysaccharide extraction was obtained from Ocimum basilicum (basil polysaccharide, BPS) and was compared with curcumin for its ability to effect the regulation of invasion of SKOV3 ovarian cancer cells and DCs. The underlying mechanisms were investigated. The results indicated that curcumin and BPS significantly inhibit the invasion of SKOV3 cells, while curcumin prevented the invasion of DCs to a greater extent compared with BPS. This diversity was achieved, at least partly, by distinctly regulating OPN, CD44 and matrix metalloproteinase-9 (MMP-9) expression.

Materials and methods

Materials. For flow cytometry, the following monoclonal antibodies: anti-CD14, anti-CD1a, anti-CD80, anti-CD86, anti-CD83, anti-human leukocyte antigen (HLA)-DR, anti-CD209, anti-CD44 and their isotype antibodies were purchased from BD-Pharmaning (Heidelberg, Germany). Recombinant human interleukin-4 (IL-4) and recombinant human granulocyte-macrophage colony-stimulating factor (GM-CSF; 1,000 U/ml) and curcumin (50 µM) or BPS (100 µg/ml) were added to the medium 24 h prior to experiments.

Invasion assay. In preparation for the assay, a 24-well Transwell chamber with 8.0 µm pore size (CoStar, Cambridge, MA, USA) was pre-coated with 30 µg Matrigel (Becton Dickinson) diluted in phosphate-buffered saline. Cell suspensions (1x10² cells/well in serum-free growth media + 0.1% bovine serum albumin) were treated and added to the upper compartment of the insert. Media containing a chemoattractant (10% FBS) was added to the bottom chamber of the Transwell plates. Following incubation at 37°C for 12 h, non-invaded cells (which remained on the upper surface of the filter) were removed and invaded cells (on the lower surface of the filter) were stained with Eosin Y and counted by light microscopy (Olympus CKX31, Tokyo, Japan). The number of migrating cells was adjusted by the cell viability assay to correct for possible toxic effects of curcumin or BPS treatment using the following equation: corrected migrating
cell number = counted migrating cell number/percentage of viable cells.

Quantitative polymerase chain reaction (qPCR). Total mRNA was extracted from cells by an RNeasy mini kit and purified by RNeasy mini spin columns (Qiagen Inc., Valencia, CA, USA), according to the manufacturer's instructions. cDNA synthesis was performed with oligo dT16 primers and Moloney murine leukemia virus reverse transcriptase according to the manufacturer's instructions (Invitrogen Life Technologies, Carlsbad, CA, USA). qPCR was performed on the LightCycler 2.0 instrument (Roche Diagnostics GmbH, Penzberg, Germany). Primer sequences were listed as follows: Forward: 5'-GGACAGGGAGACTTTG-3' and reverse: 5'-TGTGGGGACAACTGGAATGAA-3' for OPN; forward: 5'-CAGAGATGCGTGAGAGTGC-3' and reverse: 5'-CAAAGGCGTCGTCAATCACC-3' for MMP-9, and, forward: 5'-AGCGACGAATCCCCAAGTTT-3' and reverse: 5'-GGGCACGAAGGCTCATCATT-3' for β-actin. Gene-specific amplifications were demonstrated by melting-curve data.

Western blot analysis. A total of 20 µg protein (cell lysates) was subjected to electrophoresis in 10% sodium dodecyl sulfate-polyacrylamide gels and transferred onto polyvinylidene difluoride membranes (Immobilon™; Millipore, Bedford, MA, USA). Following transfer, gels were blocked with 5% skimmed milk in Tris-buffered saline with 0.05% Tween 20 (TBST) for 1 h, followed by overnight blotting with primary antibodies at 4˚C. Primary antibodies included a rabbit anti-human OPN polyclonal antibody (1:1,000) and a mouse anti-human β-actin mAb (1:1,000). Membranes were washed with TBST prior to incubation with secondary antibodies conjugated with horseradish peroxidase (1:2,000). An enhanced chemiluminescence system was used to detect horseradish peroxidase enzyme activity. Briefly, membranes were washed three times with buffer after the incubation with secondary antibodies and treated with enhanced chemiluminescent substrates according to the manufacturer's instructions (Pierce Biotechnology, Inc., Rockford, IL, USA). The immunobands were visualized using the Kodak Digital Image Station IS2000 (Kodak, Rochester, NY, USA) and subsequently analyzed using densitometry with AlphaEaseFC software (version 4.0.0, Alpha Innotech Corp., Santa Clara, CA, USA).

Enzyme-linked immunosorbent assay (ELISA). OPN and MMP-9 in the cell supernatant were measured using ELISA kits (R&D Systems, Weisbaden, Germany), according to the manufacturer's instructions. Marker concentration was calculated from the standard curve. A subset of samples was assayed five times in every ELISA plate for quality control.

Flow cytometry. Surface receptor expression on SKOV-3 and DCs in the respective groups was detected using a FACS CALibur flow cytometer (Becton Dickinson). Two- or three-color immunofluorescence was performed using the following panel of fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)- or PE-carbocyanin (Cy) 5 labeled monoclonal antibodies against CD14, CD1a, CD83, CD80, CD86, HLA-DR, CD209 and CD44. Isotype controls were run in parallel. Cell debris was eliminated from the analysis by forward and side scatter gating. For viability assays, cells were stained with Annexin V and propidium iodide, according to the manufacturer's instructions (Bender MedSystems, Vienna, Austria). The mean fluorescence intensities were determined by CellQuest software (Becton Dickinson).

Statistical analysis. The SPSS software package (version 13.0; SPSS Inc., Chicago, IL, USA) was used for all statistical analysis. Data is expressed as the mean ± SD from at least three independent experiments. Statistical analysis was performed using a t-test or analysis of variance. P<0.05 was considered to indicate a statistically significant difference.

Results

Distinct regulation of curcumin and BPS on invasion of SKOV3 cells and DCs. The regulation of BPS on the invasion

![Figure 1. Effects of curcumin and BPS on invasion of SKOV3 cells and DCs.](image-url)
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of SKOV3 cells and DCs was investigated and compared with that of curcumin, as a number of previous studies have indicated that curcumin inhibited the motility of ovarian cancer cells and DCs (23-25). Results of the invasion assay showed that curcumin and BPS inhibited the invasion of SKOV3 cells and no difference was identified between the inhibitory efficiency of these two substances (Fig. 1). Curcumin significantly inhibited the invasion of immature and mature DCs, whereas the inhibitory effect was not exerted by BPS on immature or mature DCs (Fig. 1). These results indicated that BPS possesses the same inhibitory efficiency on the invasion of ovarian cancer cells as curcumin, but exerts minimal inhibition on DCs.

Curcumin and BPS alter the expression of OPN in SKOV3 cells and DCs. Previous studies have suggested that OPN promotes the motility of ovarian cancer cells and DCs (12,14,26). To investigate the underlying mechanisms of curcumin- and BPS-regulated cell invasion, mRNA and protein levels of OPN were measured. qPCR analysis showed that curcumin inhibited OPN mRNA expression in SKOV3 cells and immature and mature DCs, whereas BPS decreased OPN mRNA levels in SKOV3 cells, but not in immature or mature DCs (Fig. 2A). Consistent with its mRNA level, the protein expression and secretion of OPN regulated by curcumin in SKOV3 cells and DCs were significantly decreased; however, this decrease was only observed in BPS-treated SKOV3 cells and not in DCs (Fig. 2B and C). These results indicated that OPN expression was closely correlated with the curcumin- and BPS-regulated motility of ovarian cancer cells and DCs.

CD44 is downregulated in curcumin- but not BPS-treated SKOV3 cells and DCs. The CD44 surface expression on SKOV3 and DCs was analyzed following curcumin or BPS treatment by flow cytometry, as CD44 is an important receptor of OPN and it facilitates the invasion of cancer cells (8). Results of the current study showed that CD44 surface expression was significantly decreased in curcumin-treated SKOV3 cells and DCs, but its expression was not affected by BPS (Fig. 3). As well as decreasing OPN expression, curcumin was hypothesized to dampen its signaling activity by downregulating the functional receptor, CD44, in ovarian
cancer cells and DCs. However, the effect of BPS on CD44 expression was not observed to be significant compared with that of curcumin.

**MMP-9 is involved in curcumin- and BPS-modulated invasion of SKOV3 cells and DCs.** OPN has been demonstrated to upregulate MMP-9 expression in the metastasis of a number of types of cancer (9-11). In addition, MMP-9 overexpression is markedly correlated with a higher risk of metastasis in ovarian cancer patients (27). Therefore, the effect of curcumin and BPS on MMP-9 expression of SKOV3 cells and DCs was investigated. qPCR results indicated that curcumin decreased MMP-9 mRNA expression in SKOV3 cells and DCs (Fig. 4A). By contrast, BPS decreased MMP-9 expression in SKOV3 cells, but not in immature or mature DCs (Fig. 4A). Consistent with its mRNA level, ELISA results indicated that the concentration of MMP-9 in the supernatant of curcumin-treated SKOV3 cells and DCs was significantly lower, compared with the control group. BPS decreased MMP-9 concentration in the supernatant of SKOV3 cells, but the MMP-9 level in the supernatant of DCs was not affected (Fig. 4B). This correlation between MMP-9 expression and invasion activity suggested that the alteration of MMP-9 expression was involved in curcumin and BPS modulated invasion of SKOV3 cells and DCs.

**Discussion**

In the current study, the regulation of two natural products, curcumin and BPS, on the invasion of SKOV3 ovarian cancer cells and DCs, and the underlying mechanisms were...
investigated. The observations indicated that curcumin impeded the invasion of SKOV3 cells and immature or mature DCs. Compared with curcumin, BPS showed similar inhibitory efficiency on SKOV3 cell invasion, but its effect on DCs was minimal. Further investigation showed that curcumin decreased the levels of OPN, CD44 and MMP-9 expression on the surface of ovarian cancer cells and DCs, while BPS decreased OPN and MMP-9 expression on ovarian cancer cells and showed no inhibitory effect on CD44 expression. Therefore, these results indicated that distinct regulation of OPN, CD44 and MMP-9 expression was at least partly responsible for the motility change of two types of cells mediated by curcumin or BPS.

The therapeutic effect of curcumin on ovarian cancer has been well established by in vitro and in vivo studies (16,28-30). Consistent with previous studies, which have demonstrated that curcumin decreased ovarian cancer cell migration (23,24), the current study confirmed that it significantly inhibited the invasion of SKOV3 cells, which provided more evidence for its anticancer mechanisms. However, the effect of curcumin on the immune system appears to be controversial, primarily dependent on different types of immunocytes. Although curcumin was shown to directly enhance T cell-mediated antitumor immunity, it inhibited the antigen-presenting properties of DCs by blocking maturation marker expression and inducing differentiation towards a tolerogenic phenotypes (25,31,32). In the current study, curcumin was observed to inhibit the invasion of immature and mature DCs. Compared with curcumin, BPS appears more beneficial as a complementary therapy in ovarian cancer treatment, as the results showed that it inhibited SKOV3 cell invasion, almost as efficiently as curcumin, whereas no inhibition was observed on the invasion of immature or mature DCs. These results indicated that BPS may achieve more robust antitumor immunity in ovarian cancer patients than curcumin as the invasion of activated DCs to secondary lymph organs is vital for the subsequent excitation of T cells. However, in addition to motility, the immune-initiating potency of DCs is also dependent upon its phagocytotic activity, surface molecule expression and cytokine secretion. Therefore, this hypothesis may not be confirmed until further studies regarding the modulation of BPS on DCs biological properties are conducted.

OPN enhances cell invasion by interacting with integrins and CD44 and initiating subsequent reactions (12,14,26), for example, enhancing MMP secretion (9,11). In the present study, curcumin was observed to inhibit OPN expression in SKOV3 cells and DCs, while BPS decreased OPN expression in SKOV3 cells, but not in DCs. The correlation between the levels of OPN and cell motility indicated that curcumin and BPS modulated SKOV3 cells and DC invasion by altering the expression of OPN. The results indicated that the protein level and secretion of OPN was significantly affected following treatment with curcumin or BPS and a significant decrease of OPN mRNA may be responsible for this change in protein levels. However, more modifications may have occurred at the translational or post-translational level after the mRNA was formed, such as methylation, phosphorylation and acetylation. All these modulations would affect the protein synthesis of OPN, but their effects were not investigated in the present study. In addition, the distinct effect of BPS on OPN expression in cancer cells and DCs is noteworthy. This may be explained by various activation pathways of OPN expression in cancer cells and DCs; however, further studies are required to investigate the underlying mechanisms.

Cellular adhesion molecules (CAMs) mediate contact among or between cells and the ECM, and dysregulation of their expression is involved in tumor progression. CD44 has been shown to be involved in cancer metastasis (33). In addition, the close correlation between CD44 and OPN-mediated cell invasion has been well described as OPN was observed to upregulate CD44 surface expression (34) and CD44 is known to promote cell invasion by binding with hyaluronan in ECM. However, OPN directly collaborates with CD44 to activate downstream signaling pathways in an autocrine manner and subsequently promotes cell invasion and chemotaxis (35,36). The current study demonstrated that curcumin significantly downregulated the expression of CD44 on the surface of SKOV3 cells and DCs, while BPS had no marked effect on CD44 expression. These results indicated that curcumin not only decreased OPN expression, but also dampened its activity by inhibiting the expression of its functional receptor.

OPN induced MMP expression, was involved in the metastasis of cancer cells (9,11) and curcumin was shown to inhibit the expression of MMP-2 by inactivating the OPN-mediated nuclear factor-κB (NF-κB) pathway (37). Curcumin and BPS were observed to modulate MMP-9 expression in a manner consistent with the OPN level. This was achieved by decreasing the OPN level or directly interfering with OPN-MMP-9 pathway activity or a combination of the two. Moreover, activation of CD44 promotes the binding of MMP-9 to the cell surface and its maturation (38), and CD44-MMP-9 aggregates, enhanced cancer metastasis in a murine mammary carcinoma model (39). Although curcumin and BPS decreased MMP-9 secretion in SKOV3 cells to a similar efficacy, the maturation of MMP-9 may differ, given that curcumin significantly downregulated CD44 expression when compared with BPS. The current results showed that curcumin and BPS modulated MMP-9 secretion in a manner that was consistent with cell invasion and OPN expression, thereby it may be a potential mechanism for the modulation of cell motility.

In conclusion, to the best of our knowledge, this was the first study to demonstrate that BPS inhibited the invasion of SKOV3 cells while curcumin affected SKOV3 cells and DCs. This diversity was achieved, in part, by the distinct expression of OPN, CD44 and MMP-9 in the two types of cells. The present study indicated that curcumin and BPS may serve as efficient complementary therapies for ovarian cancer. Notably however, BPS may be a superior candidate for maintaining anticancer immunity activity in ovarian cancer patients, considering its low inhibitory effect on DC invasion. However, further investigation is required to investigate whether these compounds are equally effective in vivo.

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