RESEARCH ARTICLE

Oleuropein Induces Anti-metastatic Effects in Breast Cancer

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Abstract

Breast cancer causes death due to distant metastases in which tumor cells produce matrix metalloproteinase (MMP) enzymes which facilitate invasion. Oleuropein, the main olive oil polyphenol, has anti-proliferative effects. This study aimed to investigate the effect of oleuropein on the metastatic and anti-metastatic gene expression in the MDA human breast cancer cell line. We evaluated the MMPs and TIMPs gene expression by semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR) in treated and untreated cells. This study demonstrated that OL may induce anti-metastatic effects on human breast cancer cells. We found that TIMP1,-3, and -4 were over-expressed after all periods of incubation in treated cancer cells compared to untreated cells, while MMP2 and MMP9 genes were down-regulated, at least initially. Treatment of breast cancer cells with oleuropein could help in prevention of cancer metastasis by increasing the TIMPs and suppressing the MMPs gene expressions.

Keywords: Breast cancer – oleuropein - metastasis - TIMPs expression - MMPs expression

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Introduction

Breast cancer causes death due to distant metastases in which tumor cells produce Matrix Metalloproteinase (MMP) enzymes permitting invasion (Kohrmann et al., 2009). MMPs upregulation in tumor cell are linked to metastasis and tumor progression is positively correlated with the expression of MMP family members (Overall and Lopez-Otin, 2002). The tissue inhibitors of metalloproteinases (TIMPS) family have apoptosis-inducing properties and are down-regulated in a variety of human cancer cell lines. Over-expression of TIMPs reduces the experimental metastasis of melanoma. The overproduction TIMP-1 slows carcinogenesis (Buck et al., 1999). TIMP-2 causes cancer progression and metastasis (Stetler-Stevenson and Seo, 2005) and is downregulated in prostate cells and tumor samples (Pulukuri et al., 2007). The over-expression of TIMP-3 results in apoptosis of lung cancer cell line. In breast cancer the expression of MMP-1,-2, -3, -9, and inhibition of TIMP-1, -2 were stronger in tumor than in inflammatory cells (Baker et al., 2002). Olive oil is identified as effective agent in initiation, promotion and progression of multistage carcinogenesis.

The olive tree, known as *Olea europaea*, provides a variety of commercial products. The pharmacological properties of olive is important in medicine owing to their phenolic constituents presents in all parts of the olive plant (Visioli et al., 2002). In *Olea europaea*, oleuropein, represents the predominant phenolic compound and has several pharmacological properties including anti-inflammatory (Visioli et al., 1998; Coni et al., 2000) and anti-cancer (Owen et al., 2000; Park et al., 2011). Oleuropein decreases breast cancer cell viability (Menendez et al., 2007) and induces strong tumoricidal effects in HER2-overexpressing breast carcinomas (Menendez et al., 2008). Oleuropein (200 μg/mL) reduces the viability of MCF-7 cells and decreases the number of MCF-7 cells by inhibiting the rate of cell proliferation and inducing cell apoptosis (Han et al., 2009). The crude extracts, with oleuropein as the dominant compound, inhibit cell proliferation of human breast adenocarcinoma (MCF-7), human urinary bladder carcinoma and bovine brain capillary endothelial (Goulas et al., 2009). With this premise and aiming at a better understanding the present study was designed to investigate the effect of Oleuropein on the MMPs and TIMPS genes, in search of promising molecular targets to inhibit breast cancer metastasis.

Materials and Methods

MDA-cell line was cultured in a mixture (1:1, v/v) of DMEM and Ham's F12 medium (Invitrogen) supplemented with 2 mmol/L L-glutamine (Invitrogen), 0.02 mmol/L nonessential amino acids (Mediatech), and 5% fetal bovine serum. Cells were treated with 200 μg/mL of Oleuropein (Sigma, Cat No. 08889) and were incubated

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Table 1. Primers sequendec.

Gene	Forward primer	Reverse primer
MMP-2	5'-TTTCCATTCCGCTTCCAGGGCAC-3'	5'-TCGCACACCACATCTTTCCGTCACT-3'
MMP-9	5'-CCTGCCAGTTTCCATTCATC-3'	5'-GCCATTCACGTCGTCCTTAT-3'
TIMP1	5'-ACAACCGCAGCGAGGAGT-3'	5'-AGGTGACGGGACTGGAAGC-3'
TIMP2	5'-TTGACCCAGAGTGGAACG-3'	5'-ACCAAAGACGGGAGACGA-3'
TIMP3	5'-GTTGTAGGGTTTCTGTTGT-3'	5'-GTGTTGTCTGCTGCTTTT-3'
TIMP4	5'-TACCAGGCTCAGCATTAT-3'	5'-CCACTTGGCACTTCTTATT-3'
GAPDH	5'-AAGGATAATGGCTTACAAC-3'	5'-TCACTTAGGGCTTCTCAC-3'

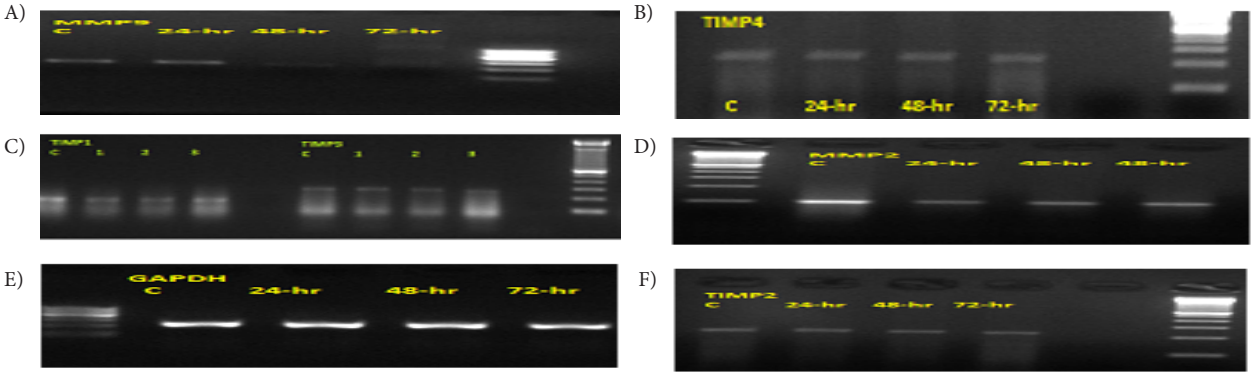

Figure 1. The Expression of TIMP1, TIMP3, TIMP4, MMP2, MMP9 and GAPDH Examined by RT-PCR.

for 24-hr, 48-hr and 72-hr and the untreated cell line was act as control.

Gene expression profile

Total RNA was extracted from the Oleuropein -treated and control cell line with TRIzol (Gibco BRL), in accordance with the manufacturer's instructions. Concentrations and purity of RNA were quantified spectro-photometrically by measuring A_{260} and A_{280}; the ratio A_{260}/A_{280} of pure RNA is approximately 1.8.

Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA isolated from treated and untreated cells was analyzed for selected genes by semi-quantitative reverse transcriptase-PCR. cDNA was prepared from total RNA as described in manufacturer's protocol (Invitrogen, USA). Reverse transcription using oligo-dT primers in a 20µl total volume reaction mixture using a superscript system (Invitrogen, USA) and PCR were performed as previously, with an endogenous control gene GAPDH as a control. Sequences of primers are listed in table 1. Experiments were performed in triplicate. Prior to amplification of each gene normalization was carried out with endogenous control gene GAPDH. Aliquots of the PCR reaction were subjected to electrophoresis on 2% agarose gels and PCR fragments were visualized by ethidium bromide staining and photographed on gel documentation system. mRNA gene expression of the housekeeping gene was used as a quality control for the samples showing equal cDNA in all samples.

Statistical Analysis

All experiments were performed in triplicate and analyzed by one way ANOVA (Excel; Microsoft) for significant differences. P values of <0.05 were considered statistically significant. Where appropriate, the data are presented as the mean±SD.

Results

To investigate the effect of Oleuropein on the MMPs and TIMPs genes, we treated MDA human breast cancer cell line with 200 μg/mL concentration of Oleuropein and incubated for 24-hr, 48-hr and 72-hr. After semi-quantitative PCR the band size was observed on 2% agarose gel electrophoresis for each gene Figure 1.

There was no significant difference observed in the TIMP1 gene expression at 24-hr incubation. After 48-hr incubation, the TIMP1 gene expression was significantly increased by 2.5 folds at the concentration of 200 μg/mL in treated than in untreated cells. TIMP1 was increased after 72 hours of treatment to 3.7 folds (Figure 1C).

There was no significant difference observed in the TIMP2 gene expression at 24hrs incubation and with slight increasing for the other two lengths of time in treated compared to the untreated cells (Figure 1F).

The expression of TIMP3 gene was increased significantly in response to treatment with Oleuropein after 48-hr and 72-hr incubations (Figure 1C).

There was no difference observed in the TIMP4 gene expression at 24-hr in treated compared to the untreated cells. After 48-hr, the TIMP4 gene expression was increased significantly in response to Oleuropein-treatment compared to untreated cells and no further up-regulation was observed after 72-hr incubation (Figure 1B).

MMP2 gene expression was reduced significantly to 0.5 and 0.3 folds in response to Oleuropein -treatment after 48-hr and 72-hr incubations respectively (Figure 1D). The expression of MMP9 gene was significantly down-regulated in response to Oleuropein -treatment to 0.4 folds after 48-hr and to 0.2 folds after 72-hr incubation (Figure 1A). These results suggested that Oleuropein

induces anti-metastatic effect in a time- dependent manner in MDA cells.

Discussion

There are number of studies on health beneficial effects of olive oil showing that olive oil is more favorable against cancer than other forms of added lipids due to its high content of monounsaturated fatty acids (Visioli and Galli, 2001). The phenolic compounds of olive oil and leaf are complex mixture of compounds. Oleuropein (OL) is one of various phenolic compounds in olive with a powerful antioxidant and anti-angiogenic effect by inhibiting the proliferation and migration of advanced-grade tumor cell lines in a dose-dependent manner (Sirianni et al., 2010; Santiago-Mora et al., 2011). Due to its little or no toxic side effects and good bioavailability OL targets multiple steps in cancer progression (Abe et al., 2011).

Some epidemiological studies showed correlation between olive products consumption and incidence of breast cancer. The anticancer properties of oleuropein are studied in-vitro with different cell lines but its metastatic effect on breast cancer has not been demonstrated. In this study we investigate the possible effect of oleuropein on breast cancer using human breast cancer cell line MDA.

MMPs are up regulated and often associated with a poor prognosis for patients as they function in the remodeling of the extracellular matrix that is integral for many normal and pathological processes (Forget et al., 1999; Curran et al., 2004; Ranogajec et al., 2012). In this study no difference was observed in the treated cells with OL at 24-hr in MMP2 and MMP9 genes expression levels. The expression of MMP2 was reduced significantly in response to OL-treatment to 0.5 folds after 48-hr incubation and to 0.3 folds after 72-hr incubation. The expression of MMP9 was significantly down-regulated in response to OL-treatment to 0.4 fold after 48-hr and to 0.2 fold respectively after 72-hr incubation. The observed efficient reduction of MMP-2 and -9 gene expression levels during the OL treatment of MDA breast cancer cells in time-dependent suggests that OL can suppress breast cancer metastasis in a time-dependent manner in MDA cells. MMP-2 and MMP-9 help in forming neovascularization and are therefore involved in tumor angiogenesis mainly through their matrix-degrading capacity (John and Tuszynski, 2001). Upregulated expression of MMP-2 and -9 in tumors leads to the degradation of basement membranes (Iwasaki et al., 2002; Kato et al., 2002). MMP-9 gene upregulation is associated with shortening the relapse-free survival in breast cancer patients (Vizoso et al., 2007). There was a correlation between high expression of MMP-2 and the reduction in the survival and between the increased levels of MMP-9 with the tumor grade in breast cancer patients (Li et al., 2004). There was a correlation between high expression levels of MMP-2 and -9 and a higher rate of distant metastases (Vizoso et al., 2007).The expression of MMP-2, -9 genes was identified in breast cancer tissue (Decock et al., 2007) and with high expression levels in comparison to normal breast tissue (Pacheco et al., 1998).

The TIMPs family, including TIMP-1, 2, 3, and 4, regulates the activity of multifunctional MMPs. The degradation of matrix proteins is under the control of MMPs, which in turn are regulated by their own tissue inhibitors (TIMPs). TIMPs inhibit MMPs activities and could modulate critical signaling pathways independent of metalloproteinase inhibition (Olafsdottir et al., 2010). TIMPs are involved in biological processes in cancer and are decreased in some human cancer cell line. Control and modulation of MMPs transcription and/or activation by several naturally occurring substances are novel options for the control of MMP and TIMP activity. In this study data demonstrated that after 24-hr incubation no difference observed in the TIMP1 gene expression. In the OL-treated cells at 48-hr incubation, the expression of TIMP1 was significantly increased by 2.5 folds and after 72-hr was significantly increased by 3.7-folds. Overexpression of TIMPs reduced experimental metastasis of melanoma (Khokha, 1994; Montgomery et al., 1994). TIMP-1 overproduction slowed chemical carcinogenesis in skin and liver carcinogenesis in transgenic mice.

TIMPs, beside inhibiting MMP (Valente et al., 1998), could also suppress receptor tyrosine kinase signaling independent of metalloproteinase inhibition (Stetler-Stevenson, 2008). Our study showed that the increase of the TIMP-1 mRNA level in breast cancer cell line during OL treatment was associated with the down-regulation of MMP-9. Similarly a study found TIMP-1 gene has inhibitory activity against MMP-9 (Figueira et al., 2009). This suggests that OL may inhibit MMP-9 production, rather than decrease of its synthesis, leading to inhibition the degradation of ECM. Thus, it appears that OL significantly reduces the functional ability of MMP-9 by both decreasing the rate of production as well as increasing its natural inhibitor, TIMP-1. Administration of OL can return the relationship between MMPs and TIMPs to their normal concentration. OL controls cancer progression by either blocking tumor growth or inhibiting its invasive and aggressive potential.

TIMP-2 is involved in cancer progression and metastasis and its high expression inhibits the proMMP-2 activation (Munshi et al., 2004) . Study using breast cancer samples demonstrates that the inhibition of TIMP-1 and TIMP-2 were stronger in tumor cells than in inflammatory cells within the tumor section. In this study, there was no difference observed in the TIMP2 gene expression level at the OL concentration at all exposure periods compared to the untreated cells. TIMP-2 is normally expressed in breast stromal tissue; however, increased expression has been found in ductal carcinoma in situ and in invasive breast carcinomas (Brummer et al., 1999; Kim et al., 2006; Kohrmann et al., 2009) TIMP-2 has been found to stimulate cell growth and inhibit apoptosis in breast cancer cells, as well as to inhibit endothelial cell growth and abrogate angiogenesis (Chirco et al., 2006). Increased expression of TIMP-2 in breast cancer tissue has also been associated with tumor recurrence and development of metastasis (Ree et al., 1997; Zhang et al., 2007)

TIMP-3 has been found to induce apoptosis in both normal and malignant cells (Mannello et al., 2005). In addition to inhibit endothelial cell motility, proliferation and tumor growth, TIMP-3 has also been found to be a potent inhibitor of angiogenesis (Qi et al., 2003).

Overexpression of TIMP-3 resulted in apoptosis of lung cancer cells. The delivery of TIMP-3 gene inhibited the growth of tumors in nude mice, and was associated with a greater therapeutic effect than either TIMP-1 or -2 gene (Finan et al., 2006). In this study, the expression of TIMP3 was found to be increased insignificantly in response to treatment with OL followed by increasing in its expression in the 48-hr and 72-hr incubation period. The TIMP-3, a cell-cycle-regulated gene normally found in breast epithelium; suppression in breast tumor and peri-tumoral tissues has been linked to cell cycle deregulation and tumor cell proliferation (Mylona et al., 2006). Reduced expression of TIMP-3 in breast cancer tissue has been associated with poor disease-free survival (Kotzsch et al., 2005). Down regulation of TIMP3 can cause increase in MMP2.

According to our results there was no difference observed in the TIMP4 gene expression at 24-hr compared to the untreated cells. OL increases the expression of TIMP4 after 72-hr incubation of treatment in association with decrease in the expression level of MMP9 and MMP2. To our knowledge, currently there is no data available for the effect of OL on the TIMPs regarding their expression in breast cancer cell in literature. Overall, this study on MMPs and TIMPS in cancer provides a new principle for developing an anti-metastatic drug that targets TIMP and MMP activities.

In conclusion, oleuropein plays an important role in regulating MDA cell metastasis by suppressing the expression of MMP-2 and MMP-9 genes and upregulating the expression of TIMP1 and TIMP4 genes in breast cancer cells therefore it can help in tailoring new anti-metastatic cancer therapy.

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