Interleukin-6 induces secretion of tissue inhibitors of etalloproteinases by breast carcinoma cells

Abeer Mahmoud Badr
Zoology Department, Faculty of Science, Cairo University, Giza, Egypt

Abstract: Interleukin (IL)-6 can induce matrix metalloproteinases (MMPs) expression, which may be critical factors involved in tumor metastasis. Tissue inhibitors of metalloproteinases (TIMPs) are important inhibitory enzymes of MMP. This study was designed to investigate the effect of recombinant IL-6 on the MMP/TIMP expression in MDA-MB-231 breast cancer cell line in a dose dependent manner (10, 25 and 50ng/ml) in comparison to non-treated breast cancer cells (control). The data demonstrated that low dose (10 ng/ml) of IL-6 failed to induce TIMP-1 and -2 production by breast cancer cells compared to control cells whereas moderate (25 ng/ml) and high (50ng/ml) exposure levels promoted a significant expression of TIMP-1 ($P<0.01$ and $P<0.0001$) respectively as compared to control cells. TIMP-2 was significantly released ($P<0.0001$) from breast cancer cells higher than in control cells at moderate and high exposure levels of IL-6. This up-regulation of TIMP-1 and -2 was accompanied with undetectable levels of MMP-1, -2, -3, -8, -9, -10, and -13. Furthermore, IL-6 potentially increased the invasion potency of cancer cells significantly ($P<0.05$ and $P<0.01$) at moderate and high exposure levels respectively. These findings suggest that IL-6 could promote the invasion potency of breast cancer cells by inducing secretion of TIMP-1 and -2, causing a disturbance in TIMP/MMP balance.

Keywords: Interleukin-6, matrix metalloproteinases, tissue inhibitors of metalloproteinases, breast carcinoma MDA-MB-231 cells, cancer invasion.

INTRODUCTION

The link between immune system and cancer is considered as an important approach. Persistent of inflammation has been suggested to be one of the main causes that lead to cancer. Many research studies demonstrated that inflammatory state of tumor microenvironment can increase the risk of cancer by creating signals associated with poor prognosis (Fisher *et al*., 2014). In the tumor microenvironment, the release of inflammatory mediators such as tumor necrosis factor-α, interleukin (IL)-1 and IL-6 molecules either from infiltrating cells or tumor cells themselves could help in providing a suitable niche to promote tumorigenesis by inducing proliferation as well as angiogenesis and metastasis (Mantovani *et al*., 2008).

IL-6 is a glycoprotein composed of 184 amino acids with a molecular weight of 26 kilodaltons (kDa). It has been suggested as a multifunctional cytokine characterized with potential effects on the activity of cancer cells (Zarogoulidis *et al*., 2015). IL-6 being a putative pro-inflammatory cytokine, plays a functional role in hindering apoptosis (Guo *et al*., 2012), by binding to its receptor (IL-6Rα) and co receptor gp130 (glycoprotein 130), thus activating JAK tyrosine kinases and the transcription factor STAT3 as observed in many types of cancer including breast cancer (Hodge *et al*., 2005). Detection of high levels of serum IL-6 have been reported in patients with systemic cancers as compared to healthy controls or patients with benign cancer. It is also known as an indicator of malignancy, with sensitivity and specificity of about 60-70% and 58-90%, respectively (Heikkilä *et al*., 2008). Stimulation of IL-6 signaling pathways can profoundly increase growth of tumor cells by provoking molecules that are essentially implicated in tumor invasiveness and metastasis.

Extra cellular matrix-modifying enzymes such as matrix metalloproteinases (MMPs) are examples of these mediators that promote carcinogenesis (Liu *et al*., 2006). Human MMPs are a family of more than 20 zinc-dependent endopeptidases that hydrolyze most consistents of extra cellular matrix (ECM). Secretion of MMPs initially involved latent forms and subsequently promoted to active forms by releasing of zinc atoms and activation of catalytic site. The major subgroups of MMPs, identified by their substrate preferences are; Collagenases regulate remodeling of ECM, stromelysins prefer proteoglycans and glycoproteins as substrates, and gelatinases are particularly potent in degradation of fibral collagen (Vihinen *et al*., 2005; Klein and Bischoff, 2011). These proteinases are known also to play a central role in many biological processes such as normal tissue remodeling, embryogenesis, wound healing and angiogenesis (Hamacher *et al*., 2004). During cancer development, ECM degradation have been observed in tumor progression and spread. Several studies also showed a relation between high expression of MMPs and inflammation as well as tumor cell growth (Kessenbrock *et al*., 2010).

**Corresponding author:* e-mail: abeerbadr@gmail.com

Tissue inhibitors of metalloproteinases (TIMPs) are a small family of homologous and low molecular weight proteins including four members, TIMP-1, -2, -3, and -4 acting as inhibitor enzymes controlling the activities of metalloproteinases (Sun 2010). As mentioned that transcription of MMP and TIMP could be regulated by inflammatory cytokines and growth factors (Kossakowska *et al*., 1999). Moreover, overexpression of TIMPs has been associated with inflammatory mononuclear cells in breast cancer metastasis (Vizoso *et al*., 2007). The imbalance between MMP and their inhibitors is a crucial requirement to preserve tumor invasion and angiogenesis as overexpression of these inhibitory enzymes displayed protection from apoptosis in human melanoma cells (Sun and Stelter-Stevenson, 2009).

Based on the relation between IL-6 secretion and the increase of tumor growth, we hypothesize that treatment of breast carcinoma MDA-MB-231 cell line with recombinant IL-6 in gradient concentrations could have stimulatory effects on MMPs and TIMPs expression. The aim of the present study was to assess the influence of IL-6 on the balance between MMPs and TIMPs. Additionally, invasiveness of treated MDA-MB-231 cells was investigated by invasion assay.

MATERIALS AND METHODS

Cell Line and reagents

MDA-MB-231 human breast carcinoma cell line was obtained as a gift from Dr. Mona Mostafa (Cairo University, Giza, Egypt). Recombinant human IL-6 was purchased from R&D systems (Minneapolis, USA). Human Matrix Metalloproteinase Antibody Array C1 kit was attained from Ray Biotech (RayBio^{R}, Inc, USA). BD Matrigel™ invasion chambers were obtained from BD Biosciences (Billerica, MA, USA). Cells were cultured in RPMI with 10% fetal bovine serum (FBS), 2mM L-glutamine, 1.5g/l sodium bicarbonate, 4.5g/l glucose, 10mM HEPES, and 1.0mM Na-pyruvate. Cell culture, subculture, passage and seeding densities were performed as recommended by the instruction guidelines of ATCC. Unless otherwise stated cell culture media and all reagents were purchased from Sigma (St. Louis, MO).

Preparation of different concentrations of MDA-MB-231 conditioned media

MDA-MB-231 cancer cells were seeded at cell density of 250×10^3/ml in RPMI with 10% FBS medium and grown until about 70% confluent. To prepare a conditioned media, normal culture medium was exposed to recombinant IL-6 whereas cells were treated for 24h with IL-6 at different exposure levels; 10 (low), 25 (moderate), and 50 (high) ng/ml. After 24h, confluent cultures were washed twice with phosphate-buffered saline and incubated overnight in serum free-RPMI media at 37 C in humidified CO2 incubator. Conditioned media of MDA-MB-231 cells was collected and centrifuged at 7000 rpm for 5 min to pellet cells. Supernatant was collected, centrifuged in cooling centrifuge at 2000 for 10 min to get rid of cell debris and stored at -80 C until use. Supernatant was used in assessment of MMPs and TIMPs secreted by MDA-MB-231 cells in response to IL-6 exposure at different concentrations.

MMPs profiling

Ten proteins including MMPs and their inhibitors were determined using matrix metalloproteinase antibody based-array. According to the manufacturer instructions, all incubation and washing steps were performed under rotation, the provided washing and detection buffers were prepared as described. The antibody array membrane was placed into the incubation tray that is provided by the kit. Subsequently, membrane was blocked with 2ml of blocking buffer for 30 min at room temperature, followed by washing. The membrane was incubated with 1ml of undiluted MDA-MB-231 conditioned media overnight at room temperature, ended with washing. After that, the array membrane was applied with biotinylated antibody cocktail for 2h at room temperature, followed with the last wash. HRP-Streptavidin incubation was conducted for 2h at rotation, ended with washing. Finally, membranes were incubated with detection buffers for chemiluminescence detection, membranes were exposed to X-ray film at room temperature for 1 min. Membrane signal intensities were determined by using image J software (National institutes of Health MD, USA). The signal intensities of each spot is proportional to the relative concentration of protein in the sample. Relative differences in expression of MMP were detected by background subtraction. Spot signal intensities were normalized to positive control spots. Negative control spots were buffer with no antibodies used to measure the baseline responses.

In vitro matrigel invasion assay

Cell invasion assay was applied by invasion 24-well insert culture plates with 8μm pore diameter, coated with BD Matrigel™ matrix (BD Biosciences, Billerica, MA, USA). In this assay, the ability of cancer cells to be transferred from upper compartment to the lower compartment through these small pores in the presence of a stimulant was examined. The cells has to digest the Matrigel coat before moving to the other side. Tissue culture medium that provided with 10% FBS was added in the lower compartment as chemoattract. The conditioned medium was established by addition of the recombinant IL-6 to the culture medium. The MDA-MB-231 cells seeded onto (5×10^4 cells/well) the upper surfaces of coated membrane filters without (0 pg/ml, control) or with recombinant IL-6 at various concentrations (10, 25, 50pg/ml) in each 24-well insert invasion chamber. Then, the membranes were incubated for 24h in a humidified tissue culture incubator at 37ºC and 5% CO2 atmosphere. After that, non-invading cells

	A	B	C	D	E	F	G	H
1	POS	POS	NEG	NEG	MMP-1	MMP-2	MMP-3	MMP-8
2	POS	POS	NEG	NEG	MMP-1	MMP-2	MMP-3	MMP-8
3	MMP-9	MMP-10	MMP-13	TIMP-1	TIMP-2	TIMP-4	NEG	POS
4	MMP-9	MMP-10	MMP-13	TIMP-1	TIMP-2	TIMP-4	NEG	POS

Fig. 1: Schematic presentation of matrix metalloproteinase (MMPs) array map design detected by RayBio® Human Matrix metalloproteinase Antibody Array C1 Map. All MMPs antibodies detect both pro and active forms. POS: Positive Control Spot, NEG: Negative Control Spot, TIMP: Tissue inhibitor of metalloproteinase.

were removed from the upper surface of the membrane with cotton swabs, while invading cells on the lower surface of the membrane filter were fixed, stained with Diff-Quik. The invaded cells were counted by light microscope at 40X magnification in several fields.

STATISTICAL ANALYSIS

The data were analyzed by IBM Statistical Package for the Social Sciences version 20 (copyright by IBM SPSS software, US). One way-ANOVA was applied to analyze the effect of IL-6 exposure levels on TIMP-1 and -2; Post hoc (Least Significant difference, LSD) was utilized to compare between control and various levels of exposure. Data were expressed as mean ± standard deviation (SD). $P<0.05$ is significant at $\alpha=0.05$. Regression analysis and correlation coefficient (r) were used to fit the relation between levels TIMP-1 and -2 (dependent variable) and exposure level of IL-6 (independent variable).

RESULTS

The balance between MMPs and TIMPs

To investigate the influence of various exposure levels of recombinant IL-6 on one of the vigorous breast cancer cell line, we assessed MMPs and TIMPs in the conditioned media of control and treated MDA-MB-231 cells according to map-antibody array design (fig. 1). The appearance of the membrane antibody array demonstrated undetectable signal of MMP-1, -2, -3, -8, -9, and -10, and -13 (fig. 2). One way analysis revealed that the expression of TIMP-1 ($F_{3,11}$=632.97, $P<0.05$) and TIMP-2 ($F_{3,11}$=98.63, $P<0.0001$) were significantly increased as a direct exposure to the IL-6. By analysis of the membrane antibody array, conditioned media with low dose (10 ng/ml) of IL-6 had no significant effect on the TIMP-1 and -2 expression in comparison to control media (0 ng/ml) (figs 3A and B). On the other hand, moderate (25 ng/ml) and high (50ng/ml) exposure levels of IL-6 revealed that TIMP-1 (991.61±61.54 and 1966.99±13.53) was significantly increased ($P<0.01$ and $P<0.0001$) respectively compared to control (784.62±40.04) (fig 3A). As shown in fig. 3B, treated conditioned media with moderate and high exposure levels of IL-6 enhanced MDA-MB-231 cells to release a significant expression of ($P<0.0001$) of TIMP-2 (1584.56±67.40 and 3097.37±393.69) respectively versus control (1267.73 ±19.23). Conversely, MMPs antibody array showed undetectable signal intensity of TIMP-4 (fig. 2).

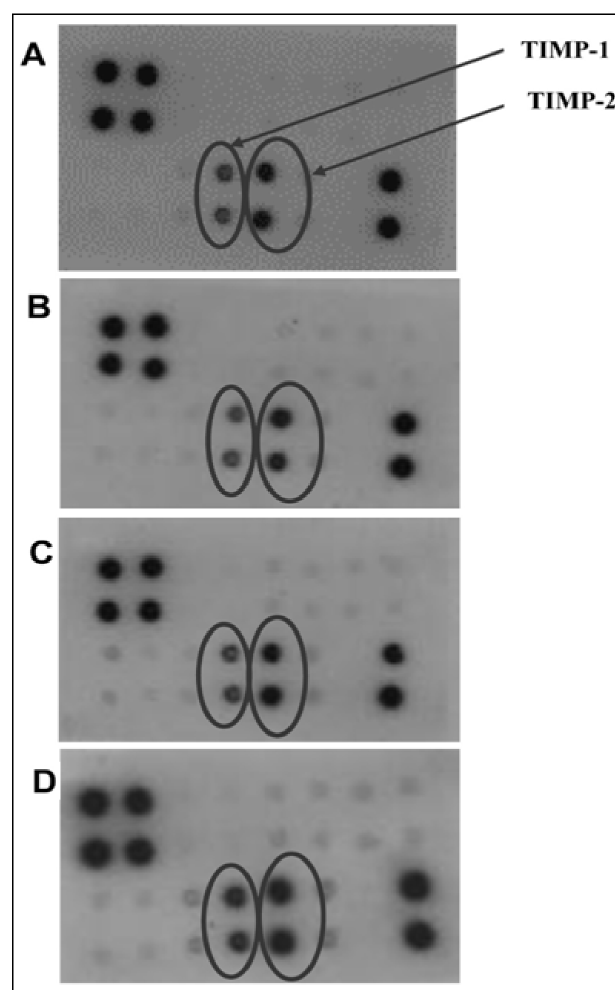

Fig. 2: Stimulation of TIMP-1 and -2 secretion from human breast MDA-MB-231 cell line treated with human recombinant IL-6. (A) Representative MMPs antibody array conditioned with untreated MDA-MB-231 cell line as control (0ng/ml of IL-6). Representative MMPs antibody array of media incubated with human

recombinant IL-6 at concentration (B) 10ng/ml, (C) 25 ng/ml and (D) 50ng/ml. The position on the array is circled in black, was upregulated. TIMP: Tissue inhibitor of metalloproteinase.

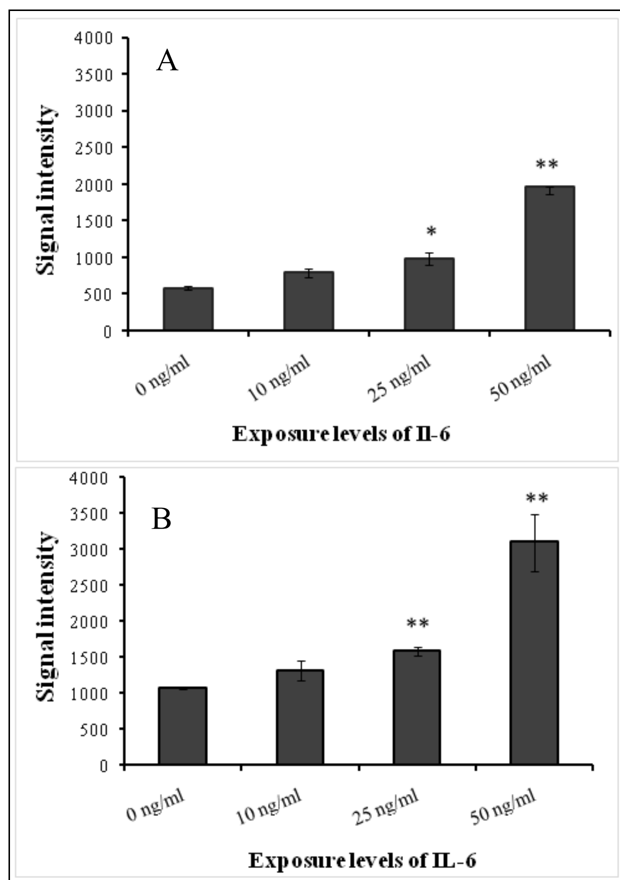

Fig. 3: Increased secretion of TIMPs by MDA-MB-231 cell line. MDA-MB-231 cells were grown for 24h in absence (0ng/ml, control) or presence2 of various concentrations of recombinant human IL-6. Release of (A) TIMP-1 and (B) TIMP-2 and after incubation with 10, 25, and 50 ng/ml of IL-6. Data represent mean ± standard deviation (SD) at least three independent experiments. Asterisks indicated to a significant difference compared to the control (*P<0.01, **P<0.0001). TIMP: Tissue inhibitor of metalloproteinase.

Here, the relationship between signal intensities of TIMP-1 and -2 secreted from MDA-MB-231 cells and exposure levels of IL-6 was determined (fig. 4). According to Pearson correlation, the various exposure levels of IL-6 showed a direct positive correlation with TIMP-1 (r= 0.95) and TIMP-2 (r= 0.94) respectively.

Recombinant IL-6 triggers the invasion potency of breast cancer cell line

The effect of recombinant IL-6 on the cancer cell properties that involved in the development of metastases was examined. The invasion potency of control (0ng/ml) and treated MDA-MB-231 cells with recombinant IL-6 (10, 25, and 50ng/ml) was determined as shown in fig. 5. The mean number of invaded cells (137.5±14.5) treated with low dose of IL-6 were not statistically different from the control cells (121.7±7.21). The moderate and high exposure levels of IL-6 enhanced a significant increase (P<0.05 and P<0.01) in the mean number of invaded cells (173.6±18.952 and 229.63±32.05) respectively to the lower compartment of the transwell membrane in comparison to control (121.7±7.21).

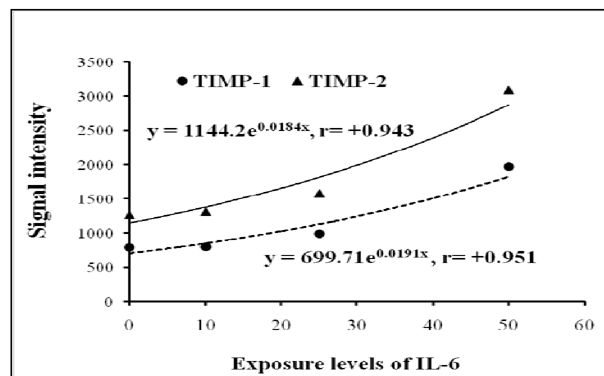

Fig. 4: Correlation between signal density of TIMPs and different exposure levels of recombinant IL-6. MDA-MB-231 human breast cell line was cultured with human recombinant IL-6 at concentrations of 10, 25 and 50 µg/ml. Each value was a mean of three samples± standard deviation (SD). r: correlation coefficient. TIMP: Tissue inhibitor of metalloproteinase.

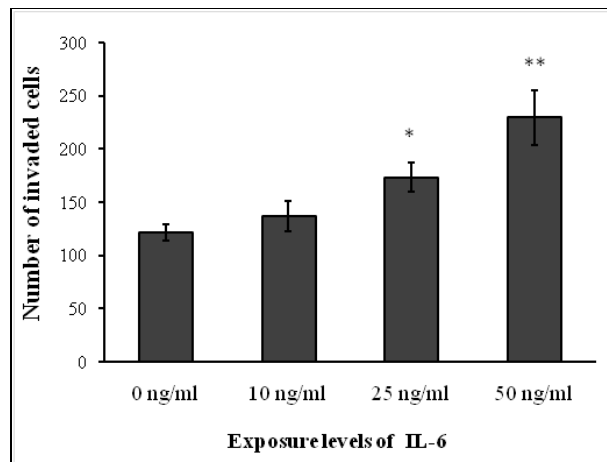

Fig. 5: Effect of IL-6 on the invasion power of MDA-MB-231 breast cancer cells. Normal growth medium of MDA-MB-231 cells without IL-6 was used as control (0 ng/ml). MDA-MB-231 cells were incubated for 24h on Matrigel coated filters in the presence of conditioned media at different exposure levels of IL-6 (10, 25, and 50ng/ml). The invaded cells were counted within three microscopic fields and expressed as average value. Data were expressed as mean ± standard deviation (SD) of three independent experiments. Asterisks indicated to a significant difference compared to the control (*P<0.05, **P<0.01).

DISSCUSION

Activation of a transcriptional factor NF-kB as a key regulator of inflammatory responses leads to secretion of inflammatory cytokines such as IL-6 and recruitment of tumor associated inflammatory cells. Thereby, persistent of this inflammatory state by these inflammatory cytokines can provide a suitable microenvironment for tumor growth and metastasis (Mantovani *et al*., 2008). In the present study, the MDA-MB-231 cells were treated with recombinant human IL-6 in a dose dependent manner (10, 25, and 50ng/ml) for 24h. The results indicated that there is no effect of various concentrations of recombinant human IL-6 on MMPs secretion. These proteases (MMP-1, -2, -3, -8, -9, -10 and -13) here were not recorded by antibody array in the conditioned media of control and treated MDA-MB-231 cells. In spite of the observed link between MMP-2 expression and breast cancer metastasis, the expression may be indirectly blocked by co-expression with other co-receptors (Jezierska and Motyl, 2009). In addition, MMP-2 expression showed a dependency on extra cellular MMP-inducer and suppression of its activity on β_3 integrin overexpression after stimulation of MDA-MB-231 cells with collagen type I (Liang *et al*., 2005; Borriukwanit *et al*., 2007). In the current study, MMPs array didn't detect MMP-8 either in control or treated MDA-MB-231 culture medium. However, Thirkettlee and coworkers observed an enhancement of MMP-8 mRNA expression by MDA-MB-231 cells to reach nearly 20% after incubation with human recombinant IL-6 at concentration of 100 ng/ml for 24 h. Moreover, positive control of MDA-MB-231 cells failed to reveal MMP-8 by western blot but a very small amount was detected at mRNA level (Thirkettlee *et al*., 2013). Thus, the increase of MMP-8 expression by IL-6 may be attributed to the relatively high dose of recombinant IL-6 compared to low doses that used in the current design.

TIMP-1 is 28.5 KDa glycoprotein and have been expressed on several cells such as breast carcinoma cells. TIMP-1, as a member of TIMP family, non-covalently associated with MMPs in 1:1 stoichiometric way inhibited the proteolytic actions of MMPs. Although the inhibitory action of TIMP-1 on MMPs, TIMP-1 has participated in enhancement of cell proliferation (Chesler *et al*., 1995). Thereby, we investigated the effect of IL-6 on TIMP level. The present results showed that treatment of MDA-MB-231 cells with recombinant human IL-6 in moderate and high exposure levels revealed a significant up-regulation of TIMP-1 and -2 versus control cells while low exposure had no effect of TIMP-1 and -2 in comparison to control. TIMP-1 and -2 also employed a highly positive ($r= +0.95$ and $r= +0.94$) correlation respectively with different exposure levels of IL-6. In contrary, TIMP-4 not affected by IL-6 that indicating to the selectivity action of IL-6 through different signaling pathways.

The process of ECM degradation is dependent on the balance of interaction between MMPs and their inhibitors (Figueira *et al*., 2007). The obtained data demonstrated a picture of up-regulation of TIMP-1 and inhibition of MMPs. Thus, these results inspired the effect of IL-6 on MMP/TIMP balance. MMPs inhibition is one function of multifunctional TIMP-1 protein, which is independent on stimulation of cell proliferation (Würtz *et al*., 2005). The mechanism of MMP inhibition by TIMP-1 have been attributed to its N-terminal moiety forming complex with zinc ion in MMPs. Furthermore, proliferative effect of TIMP-1 has been revealed in two breast carcinoma cell lines in a dose dependent manner (Luparello *et al*., 1999). High levels of TIMP-2 could inhibit pro MMP-2 activation and tyrosine kinase pathway without dependency on MMP inhibition (Stetler-Stevenon, 2008). These data may suggest the proposed idea that TIMP-1 can reinforce loop of IL-6 expression which could amplify the inflammation environment, promoted cell invasiveness. Several publications have revealed that there is an increase of TIMP-1 expression in tumor breast tissue compared to benign or normal breast tissue. The protein level of TIMP-1 was amplified in the tumor tissue in comparison to non-neoplastic tissue (Brummer *et al*., 1999). It has been reported that levels of TIMP-1 in plasma as well as tumor tissue extract have been associated with a poor prognosis in breast cancer patients (Schrohl *et al*., 2004; Wurtz *et al*., 2008). Cancer breast cells that expressing TIMP-1, have been associated with resistance to drug treatment (Hekmat *et al*., 2013).

In the current work, the invasion assay was conducted to assess the influence of IL-6 on the invasion potency of cancer breast cell line. The invasion potency of treated cells at moderate and high exposure levels exhibited a significant elevation regard to control cells. Furthermore, a study by Arihiro *et al.* observed a remarkable transition of the invasive MDA-MB-231 human breast carcinoma cells that induced by IL-6, but failed in MCF-7 (Arihiro *et al*., 2000). IL-6 protein level is forty-fold higher in MDA-MB-231 than MCF-7 breast cancer cell line by using ELISA (D'Anello *et al*., 2010). Additionally, it has been reported that secretion of IL-6 in the media of MDA-MB-231 cells provoked the invasion capacity (Chia *et al*., 2014). Epithelial or mesenchymal transition cells can produce IL-6, participating in creation of a tumor microenvironment favorable for reinforcement of invasiveness (Sullivan *et al*., 2010). *In vitro* studies also showed a full growth of myeloma cells in the presence of IL-6 (Van Camp and Van Riet, 1998). Thereby, these findings suggested that IL-6 may up-regulate the expression of TIMP-1 and -2 promoting the migration and invasiveness of breast cancer cells.

CONCLUSION

Thus, results denote that any change in MMP/TIMP profile may engage into an increase of human breast cells

invasiveness depending on IL-6 concentration. TIMP-1 and -1 up-regulation may be one of the mediators that enhance breast cancer cell invasion. These data also confirmed that TIMP-1 and -2 act as regulator of cancer invasiveness, independent on its inhibitory effect on MMPs. Thus, the invasion potency of breast carcinoma cell line might be enhanced by activation of TIMP-1 and -2. The mechanism of action has to be explored in further studies.

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