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Matrix metalloproteinases activity in fibrosarcoma Wehi-164 cells

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Fibrosarcoma is a malignant disorder of fibroblasts and extremely metastatic cancer. Angiogenesis plays an important role in metastasis. Several factors including matrix metalloproteinases (MMPs) involve in angiogenesis. MMPs are a large group of endopeptidases which degraded the extracellular matrix. The present study was conducted to evaluate the patterns of MMP-2/MMP-9 activity in Wehi-164 cells *in vitro*. Wehi-164 cells were cultured in complete RPMI-1640 medium. Then the cells were seeded at a density of 4×10^5 cells/ml and were incubated with phorbol myristate acetate (PMA) (25 ng/ml) or phytohaemagglutinin (PHA) (10 μ g/ml) for 24, 48 and 72 h. The MMP-2/MMP-9 activity in cell-conditioned media was then evaluated by gelatin zymography. PHA/PMA significantly increased MMP-2 activity in Wehi-164 cells in a time-dependent manner. MMP-9 activity was shown in unstimulated Wehi-164 time-dependently. PHA did not show any significant effect on MMP-9 activity whereas PMA induced MMP-9 activity in Wehi-164, which increased time dependently. According to the result of the present study, Wehi 164 fibrosarcoma cells could potentially exhibit MMP-2/MMP-9 activity. However, the patterns of MMP-2/MMP-9 activity, varies according to the presence or absence of stimulator as well as duration of incubation time. Thus, Wehi 164 cell line could be a useful screening tool for of MMPs modulators.

Key words: Fibrosarcoma, cell lines, matrix metalloproteinase-2 (MMP-2), matrix metalloproteinase-9 (MMP-9).

INTRODUCTION

Fibrosarcoma is a malignant disorder of fibroblasts originated from mesenchymal cells (Steinstraesser et al., 2011). Tumor invasion and metastasis are suggested to be the main cause of death in cancers and fibrosarcoma has been recognized as an extremely metastatic cancer (Zhao et al., 2011; Castoria et al., 2011; Dong et al., 2010). Many investigators (Xian et al., 2011; Pratheeshkumar and Kuttan, 2011; Rykala et al., 2011) have reported the important role of angiogenesis in tumor progression and metastasis. Angiogenesis, the process of neof ormation of blood vessels from pre-existing vessels, is regulated by several factors including angiogenic cytokines, matrix metalloproteinases (MMPs) and MMP- inhibitors (Martins et al., 2011; Viothini et al., 2011; Wang et al., 2011). MMPs are zinc-dependent endopeptidases have an important role in reconfiguration

of the extracellular matrix through proteolysis of extracellular proteins (Brauer et al., 2011; Yang and Rosenberg, 2011). MMP-2/MMP-9 are important factors in tumor cell invasion and their expression is increased in a number of cancers and has been correlated with poor prognosis (Ranogajec et al., 2011; Shou et al., 2010; Bagnoli et al., 2010). The production of MMP-2/MMP-9 in fibrosarcoma cells has been reported (Hamedani et al., 2005; Lee and Kim, 2011; Choo et al., 2011). Moreover, a positive correlation between MMPs expression and fibrosarcoma metastasis has been revealed (Hwang et al., 2011; Hwang and Jeong, 2010).

Current treatment of fibrosarcoma, is usually associated with poor prognosis. Furthermore, the anti-invasive effect of some MMP-suppressors in fibrosarcoma cells has been shown (Hwang et al., 2010; Yang et al., 2011). Regarding the important role of angiogenesis in fibrosarcoma invasion and metastasis (Lee et al., 2010) and also the key role of MMPs in angiogenesis (Martins et al., 2011; Viothini et al., 2011; Wang et al., 2011), pharmacological modulation of MMPs

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production may be useful for fibrosarcoma therapy. On the other hand, the exact patterns of MMPs expression in fibrosarcoma have not been well elucidated. Thus determining the expression profiles of MMPs in fibrosarcoma cells, may be useful in screening of potent inhibitors/enhancers of MMPs expression in the related malignancies.

In the present study, the expression patterns of MMP-2 and MMP-9 in fibrosarcoma Wehi-164 cells has been evaluated *in vitro*. Phytohaemagglutinin (PHA) and phorbol myristate acetate (PMA) were used as MMP-2/9 inducers. PHA and PMA are potent stimulators of MMPs activities (Martinesi et al., 2010; Tahanian et al., 2011).

MATERIALS AND METHODS

Reagents

RPMI-1640 medium, penicillin, streptomycin, phytohaemagglutinin (PHA), phorbol myristate acetate (PMA) and trypan blue (TB) were from Sigma (USA). Fetal calf serum (FCS) was from Gibco (USA). Microtiter plates, flasks and tubes were from Nunc (Falcon, USA).

Cell line

Mouse fibrosarcoma Wehi-164 cells (NCBI C200), were obtained from NCBI (National Cell Bank of Iran, Pasteur Institute of Iran, Tehran). The cells were maintained in RPMI-1640 medium supplemented with 10% FCS and incubated in 5% CO₂ at 37°C.

Cell culture and treatment

The method has been described in detail elsewhere (Hajighasemi and Mirshafiey, 2010). Briefly, the human leukemic cells were cultured in RPMI-1640 medium supplemented with 10% FCS, penicillin (100 IU/ml) and streptomycin (100 µg/ml) at 37°C in 5% CO₂. The cells were then seeded at a density of 4×10⁵ cells/ml and prior to experiments, cells were treated in fresh medium. Then the cells were incubated with PMA (25 ng/ml) or PHA (10 µg/ml) according to our previous studies (Hajighasemi and Hajighasemi, 2009, 2010, 2011; Hajighasemi, 2011) for 24, 48 and 72 h. The supernatants of cell cultures were collected, centrifuged and stored at -20°C for next experiments. All experiments were done in triplicate.

Evaluation of MMP-2/ MMP-9 activity by gelatin zymography.

MMP-2/ MMP-9 activities in cell-conditioned media were evaluated by gelatin zymography technique according to the modified Kleiner and Stetler-Stevenson method as described elsewhere (Hajighasemi and Hajighasemi, 2009). Briefly, cell culture supernatants were subjected to SDS-PAGE on 10% polyacrylamide gel copolymerized with 2 mg/ml gelatin in the presence of 0.1% SDS under non-reducing conditions at a constant voltage of 80 V for three hours. After electrophoresis, the gels were washed in 2.5% Triton X-100 for one hour to remove SDS and then incubated in a buffer containing 0.1 M Tris-HCl, pH 7.4 and 10 mM CaCl₂ at 37°C overnight. Afterwards, the gels were stained with 0.5% Coomassie brilliant blue and then destained. Proteolytic activity of

enzyme was detected as clear bands of gelatin lysis against a blue background. The relative intensity of lysed bands to the control band was measured by using UVI Pro gel documentation system (Vilber Lourmat, Marne-la-Vallee Cedex 1, France) and expressed as relative gelatinolytic activity. Conditioned media from HT1080 fibrosarcoma cells was used as a molecular weight marker of MMP-2 and MMP-9 as explained somewhere else (Wan et al., 2008; Dagnell et al., 2007). The activity of MMP-2 and mmp-9 appears as white bands correspond to 72 and 92 kDa, respectively.

Statistical analysis

MMP-2/ MMP-9 activity quantification in cell-conditioned media was performed in three independent experiments and the results were expressed as mean ± SEM. Statistical comparisons between groups were made by analysis of variance (ANOVA). P<0.05 was considered significant. Test of multiple comparison of Tukey was applied (5%) for statistically significant differences. The software SPSS 11.5 and Excel 2003 were used for statistical analysis and graph making, respectively.

RESULTS

Patterns of MMP-2 activity in Wehi-164 cells

Wehi-164 cells cultured alone (with no inducer), showed definite bands related to MMP-2 activity. The intensity of bands increased with time in this order: 72 > 48 > 24 h as represented in Figure 1A and B. PHA significantly increased MMP-2 activity in Wehi-164 cells after 24 h incubation time compared with untreated control cells. The PHA- increased MMP-2 activity in Wehi-164 cells was time-dependent and increased with time in this order: 72 > 48 > 24 h, as illustrated in Figure 1B. Moreover, PMA increased MMP-2 activity in Wehi-164 cells after 24 h incubation time onwards compared with untreated control cells. The enhancement of MMP-2 activity in Wehi-164 cells by PMA was time dependent and increased with time in this order: 72 > 48 > 24 h, as depicted in Figure 1B.

Patterns of MMP-9 activity in Wehi-164 cells

Wehi-164 cells cultured alone (with no inducer), did not show any bands related to MMP-9 activity after 24 and 48 h incubation time. However, faint bands related to MMP-9 activity were detected in un-stimulated Wehi-164 cells after 72 h incubation time as represented in Figure 2A and B. PHA did not show any significant effect on MMP-9 activity in Wehi-164 cells at all time intervals (24, 48 and 72 h incubation time) compared with untreated control cells, as shown in Figure 2B. Moreover, PMA induced MMP-9 activity in Wehi-164 cells after 24 h incubation time compared with untreated control cells. The induction of MMP-9 activity in Wehi-164 cells was time dependent and increased with time in this order: 72 > 48 > 24 h, as illustrated in Figure 2B.

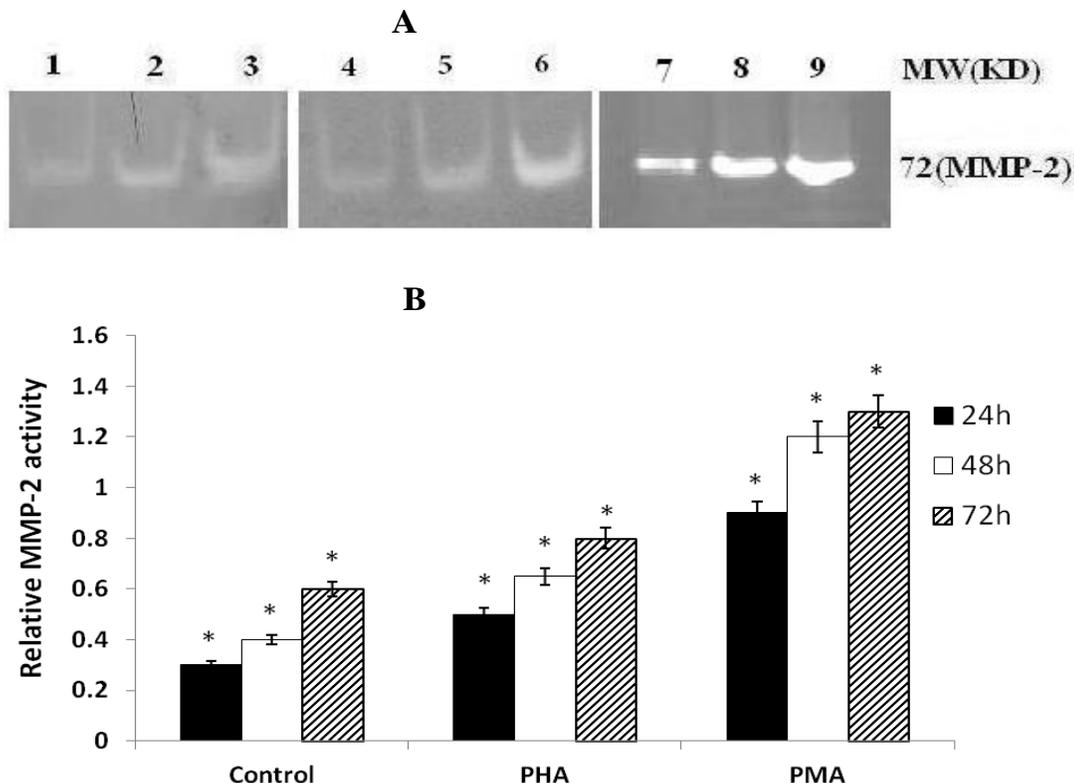


Figure 1. Patterns of MMP-2 activity in Wehi-164 cells. The Wehi-164 cells (4×10^5 cells/ml), were cultured in serum free RPMI-1640 medium and then incubated with PHA (10 μ g/ml) or PMA (25 ng/ml) for 24, 48 and 72 h. At the end of treatment, MMP-2 activity in conditioned medium was measured by gelatin zymography. (A) Zymogram of MMP-2 activity in Wehi-164 cells. Lanes 1 to 3 represent untreated Wehi-164 cells after 24, 48 and 72 h incubation respectively. Lanes 4 to 6 represent PHA-treated Wehi-164 cells after 24, 48 and 72 h incubation, respectively. Lanes 7 to 9 represent PMA-treated Wehi-164 cells after 24, 48 and 72 h incubation respectively. (B) MMP-2 activity in Wehi-164 cells was measured by scanning the zymograms and densitometric analysis of MMP-2 bands. Data are mean \pm SEM of three independent experiments. $P < 0.05$ was considered significant.

DISCUSSION

According to the results of the present study, we found out that Wehi 164 fibrosarcoma cells could potentially exhibit MMP-2/MMP-9 activity. However, the patterns of MMP-2/MMP-9 activity in these cells, varies according to the presence or absence of stimulator as well as duration of incubation time. Patterns of MMPs expression differ in different cancers and particular MMPs increase in particular carcinomas (Roomi et al., 2009; Roomi et al., 2009a; Takeuchi et al., 2011). Numerous studies have demonstrated a relation between the increase of particular MMPs and tumor expansion (Yang et al., 2009; Morrison et al., 2011; Srivastava et al., 2011). Determination of the expression patterns of particular biomarkers in specific cancers could be useful in developing anti-cancer agents for distinct neoplasm's (Szarvas et al., 2010). In the MMP family, MMP-2/MMP-9 are the important ones, which their expression is increased in several cancers and has been correlated with poor prognosis (Ranogajec et al., 2011; Shou et al.,

2010; Bagnoli et al., 2010). A positive correlation between MMP-2/MMP-9 production and cancer metastasis has been described (Ahmed and Mohammed, 2011; Tang et al., 2011). Several current clinical researches have focused on the developing of anti-metastatic agents for particular tumors (Lee et al., 2011; Hamsa and Kuttan, 2011). According to the results of this study, MMP-2 and MMP-9 were differently expressed by Wehi-164 cells. Wehi-164 cells showed MMP-2 activity without any stimulation after 24 h incubation time onwards. Furthermore, PHA and PMA both increased MMP-2 activity in Wehi-164 cells. Production of MMP-2 by Wehi-164 cells has been reported by other studies (Hamedani et al., 2005; Sharifabrizi et al., 2006).

Nevertheless, we did not find any report about PHA/PMA effect on MMP-2 activity in Wehi-164 cells. In Roomi et al. (2009a), production of MMP-2 by HT1080 fibrosarcoma cells (similar to us) and decrease of MMP-2 activity in PMA stimulated HT1080 cells (contrast to us) has been reported. In our study, PMA increased MMP-2 activity in Wehi-164 cells. The discrepancy between our

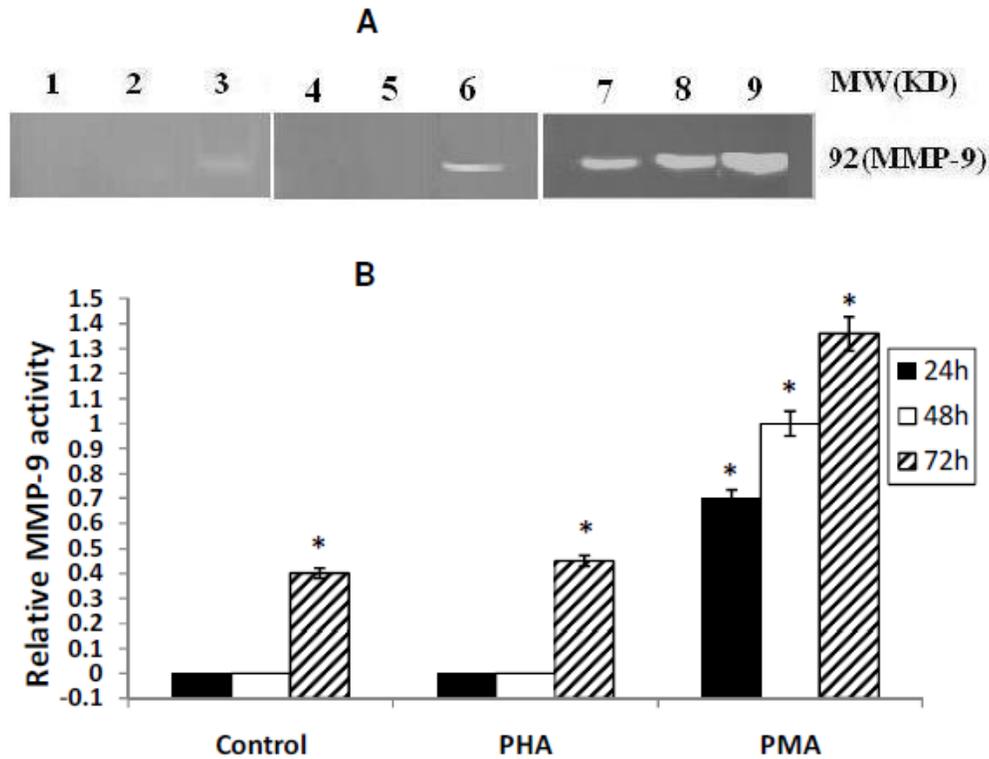


Figure 2. Patterns of MMP-9 activity in Wehi-164 cells. The Wehi-164 cells (4×10^5 cells/ml, were cultured in serum free RPMI-1640 medium and then incubated with PHA (10 $\mu\text{g/ml}$) or PMA (25 ng/ml) for 24, 48 and 72 h. At the end of treatment, MMP-9 activity in conditioned medium was measured by gelatin zymography. (A) Zymogram of MMP-9 activity in Wehi-164 cells. Lanes 1 to 3 represents untreated Wehi-164 cells after 24, 48 and 72 h incubation respectively. Lanes 4 to 6 represent PHA-treated Wehi-164 cells after 24, 48 and 72 h incubation respectively. Lanes 7 to 9 represent PMA-treated Wehi-164 cells after 24, 48 and 72 h incubation, respectively. (B) MMP-9 activity in Wehi-164 cells was measured by scanning the zymograms and densitometric analysis of MMP-9 bands. Data are mean \pm SEM of three independent experiments. $P < 0.05$ was considered significant.

results and Roomi et al. (2009a) study, may be due to two reasons: Type of used cells and concentration of PMA. Roomi et al. (2009a) used HT1080 fibrosarcoma cells whereas we used Wehi-164 cells. Different cells have different sensitivity to PMA (Roomi et al., 2009a; Hajighasemi and Hajighasemi, 2010, 2011). Also in Roomi et al. (2009a), PMA was used at 100 ng/ml concentration but we used PMA at 25 ng/ml concentration. However Lee et al. (2008) have reported enhancement of MMP-2 and MMP-9 activation by PMA, in HT1080 fibrosarcoma cells. Up regulation of MMP-2, activity by PHA has been reported in several leukemic and human peripheral blood mononuclear cells (Hajighasemi and Hajighasemi, 2011; Hajighasemi, 2011). In addition, increase of MMPs activity by lectins such as Concanavalin A (Con A) or Artocarpus lakoocha agglutinin (ALA), in human peripheral blood mononuclear cells has been shown (Saja et al., 2007a, b). Furthermore, there are several reports about induction or enhancement of MMP-2 activity in PMA-stimulated

cancer cells including fibrosarcoma (Hwang et al., 2011; Roomi et al., 2009b; Lee et al., 2008; Hajighasemi, 2011). It has been demonstrated that enhancement of MMP activity by PMA is associated to NF-Kappa B activation (Hwang et al., 2010).

Also in our study, Wehi-164 cells (in unstimulated condition) did not show any MMP-9 activity after 24/48 h incubation time and showed MMP-9 activity only after 72 h incubation time. Furthermore, PHA did not show any effect on MMP-9 activity in Wehi-164 cells.

The induction of MMP-9 activity in Molt-4 and Jurkat leukemia cells and enhancement of MMP-9 activity in U937 monocytic cells and human peripheral blood mononuclear cells by PHA has been reported (Hajighasemi and Hajighasemi, 2010, 2011). The discrepancy between Hajighasemi and Hajighasemi (2010, 2011) and present study may be somewhat due to two facts: Type and number of used cells. Different cells were used in Hajighasemi and Hajighasemi (2010, 2011) and the present study. Different cells have different

sensitivity to PHA. Moreover, Hajighasemi and Hajighasemi (2010, 2011) used 10^6 cells/ml in the case of leukemic cells and 10^5 cells/ml in the case of peripheral blood mononuclear cells while in the present study, 4×10^5 cells/ml were used.

Furthermore, in the present study, PMA induced MMP-9 activity in Wehi-164 cells after 24 and 48 h incubation time and increased MMP-9 activity after 72 h incubation time. Induction or increase of MMP-9 activity by PMA in cancer cells has been reported by various studies (Roomi et al., 2009a; Hajighasemi and Hajighasemi, 2010; Lee et al., 2008). According to the results of present study, MMP-2 precedes MMP-9 expression in unstimulated Wehi-164 cells. Because Wehi-164 cells showed MMP-2 activity after 24 h incubation time whereas MMP-9 activity was seen only after 72 h incubation time. Another fibrosarcoma cell line (HT1080) also produces both MMP-2 and MMP-9 (Lee and Kim, 2011). However, HT1080 cells have shown MMP activity, only after stimulation and MMP activity in HT1080 cells have not been evaluated and compared in different time intervals (Lee and Kim, 2011).

Taken together the results of this study showed that Wehi-164 fibrosarcoma cells could potentially display MMP-2/MMP-9 activity. Nevertheless, the patterns of MMP-2 activity were different from that of MMP-9 in this cancerous cell line.

Determination the patterns of MMPs expression in particular cancers could be of benefit in tumor therapy (Szarvas et al., 2010). Accordingly, regulation of MMPs is potentially helpful for cancer prevention. Targeting of these enzymes may have promising implication in control of malignancies.

Wehi-164 cells with potential MMP-2/9 activity could be a useful tool for screening of MMP modulators and so planning the novel therapeutics for fibrosarcoma. Moreover, Wehi-164 cell line may offer an appropriate system to study the mechanisms regulating MMPs expression in fibrosarcoma patients.

Conclusions

MMP-2 and MMP-9 activities were differently regulated in Wehi-164 cells. Wehi-164 cells with potential MMP-2/9 activity could be useful tool for screening of MMP modulators and may suggest an appropriate system to study the mechanisms regulating MMPs expression in fibrosarcoma patients.

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