

Full Length Research Paper

Zymographic analysis of gelatinases patterns in peripheral blood mononuclear cells

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Accepted 30 May, 2011

Gelatinases belongs to a large family of proteolytic enzymes named matrix metalloproteinases (MMPs) have a key role in inflammation and autoimmunity. In this study patterns of Gelatinase-A (MMP-2) and Gelatinase-B (MMP-9) activity in human peripheral blood mononuclear cells (hPBMCs) have been evaluated in vitro. hPBMCs were cultured in complete RPMI-1640 medium. Then the cells were seeded at a density of 10^5 cells/ml and were incubated with PMA (25ng/ml) or PHA (10 µg/ml) for 24 hours. Afterward the MMP-2 and MMP-9 activities in cell-conditioned media were evaluated by gelatin zymography. Statistical comparisons between groups were made by student T-test. PHA significantly increased MMP-9 activity in hPBMCs after 24 hour incubation time compared with untreated control cells. On the contrary, PMA extensively decreased MMP-9 activity in hPBMCs after 24 hour incubation time compared with untreated control cells. Besides hPBMCs did not show any MMP-2 activity and PHA/PMA had no effect on MMP-2 activity in hPBMCs compared with untreated control cells. According to the results of this study hPBMCs could exhibit MMP-9 activity. PHA and PMA had opposite effect on MMP-9 activity. PHA up-regulated while PMA down-regulated MMP-9 activity in hPBMCs. Thus, PHA/PMA stimulated hPBMCs, may provide valuable screening tools for MMP-9 enhancers/inhibitors and study of the regulatory mechanisms of MMP-9 activity.

Keywords: Gelatinases, peripheral blood, mononuclear cells

INTRODUCTION

Peripheral blood mononuclear cells (PBMCs) have a central role in inflammation (Cuttica et al., 2010). PBMCs exert their inflammatory effects through production of a number factors including cytokines, reactive oxygen intermediates and proteinases (Townsend et al., 2010; Ofiteru et al., 2003; Opdenakker and Van Damme, 2002). Proteases, mainly matrix - metalloproteinases (MMPs), have a fundamental role in degradation of extracellular matrix, tissue remodeling and inflammation (Fang et al., 2010; Hald et al., 2010; Löffek et al., 2010). A relation between MMP-9 secretion in PBMCs of systemic lupus erythmatosus (SLE) and disease activity has been reported (Hou and Zhang, 2008). MMP-2 (Gelatinase A) and MMP-9(Gelatinase B) with 72 KDa and 92 KDa molecular weight respectively, are the major MMPs produced by macrophages/monocytes (Strek et al., 2007; Ou et al., 2010). Also Production of MMP-9 by

T and B- lymphoblastoid cell lines and also T-lymphocytes have been reported (Yakubenko et al., 2000; Bauvois et al., 2002). Furthermore up-regulation of MMP-9 by Phorbol ester in T-lymphocytes has been revealed (Ou et al., 2010). Also production of MMP-2 by T-lymphocytes under restricted situations has been detected (Yakubenko et al., 2000). Moreover induction of MMP-9 expression in PBMCs by mycobacterium Leprae at the mRNA and protein level and its relation with inflammatory states of Leprosy has been reported (Teles et al., 2010). At the other hand the therapeutic effect of some anti- inflammatory drugs or agents has in part attributed to their suppressive effect on gelatinases secretion (Wu et al., 2009; Ma et al., 2010; Zhang et al., 2009). Furthermore, the preventive effect of a number MMP-suppressors in autoimmunity has been shown (Zhang et al., 2009). Due to important role of PBMCs and also gelatinases in a number of physiologic and pathologic conditions such as inflammatory and autoimmunity states (Cuttica et al., 2010; Löffek et al.,

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2010) and also potential of PBMCs in gelatinases production, determination of expression profile of gelatinases in PBMCs could be valuable in screening of gelatinases enhancers/inhibitors, selection the ideal drugs with gelatinase modulatory effects and designing of novel drugs to modulate gelatinases action in related disorders. In this study, the patterns of Gelatinase A and B expression by human PBMCs were assessed in vitro using phytohemagglutinin (PHA) and phorbol myristate acetate (PMA) as gelatinase inducers.

MATERIALS AND METHODS

Reagents

RPMI-1640 medium, penicillin, streptomycin, PHA, 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).

PBMCs isolation

PBMCs from the venous blood of healthy adult volunteers were isolated by ficoll-hypaque-gradient centrifugation. Subsequently, the cells were washed three times in phosphate buffer saline (PBS). Then the cells resuspended in RPMI-1640 medium supplemented with 10% FCS and were incubated in 5% CO₂ at 37°C.

Cell culture and treatment

The method has been described in detail elsewhere (Hajighasemi and Mirshafiey., 2010). Briefly, human PBMCs 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 seeded at a density of 10⁵ cell/well and then incubated with PHA (10 µg/ml) or PMA (25 ng/ml) for 24 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 and MMP-9 activity by gelatin zymography

MMP-2 and MMP-9 activity in cell-conditioned media were evaluated by gelatin zymography technique according to the modified Kleiner and Stetler-Stevenson method (1994) as described before (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 supernates from serum-free cultured HT1080 cells obtained from NCBI (National Cell Bank of Iran, Pasteur Institute of Iran, Tehran) was used as molecular weight marker of MMP-9 as described else where (Yodkeeree et al., 2008).

The relative intensity of lysed bands to the control was measured by using UVI Pro gel documentation system (Vilber Lourmat, Marne-la-Vallee Cedex 1, France) and expressed as relative gelatinolytic activity.

Statistical analysis

MMP-2 and MMP-9 activity 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 student T-test. P<0.05 was considered significant. The software SPSS 11.5 and Excel 2003 were used for statistical analysis and graph making respectively.

RESULTS

1. Zymographic analysis of PMA-induced gelatinases activity in hPBMCs

PMA effect on gelatinase-A(MMP-2) activity in hPBMCs

According to the results of this study hPBMCs did not show any MMP-2 activity and PMA had no effect on MMP-2 activity in hPBMCs (Table 1).

PMA effect on gelatinase-B(MMP-9) activity in hPBMCs

hPBMCs cultured alone without any inducer showed clear bands relating to MMP-9 activity. PMA significantly decreased MMP-9 activity in hPBMCs after 24 hour incubation time compared with untreated control cells as illustrated in table 1 and figure 1(A and B).

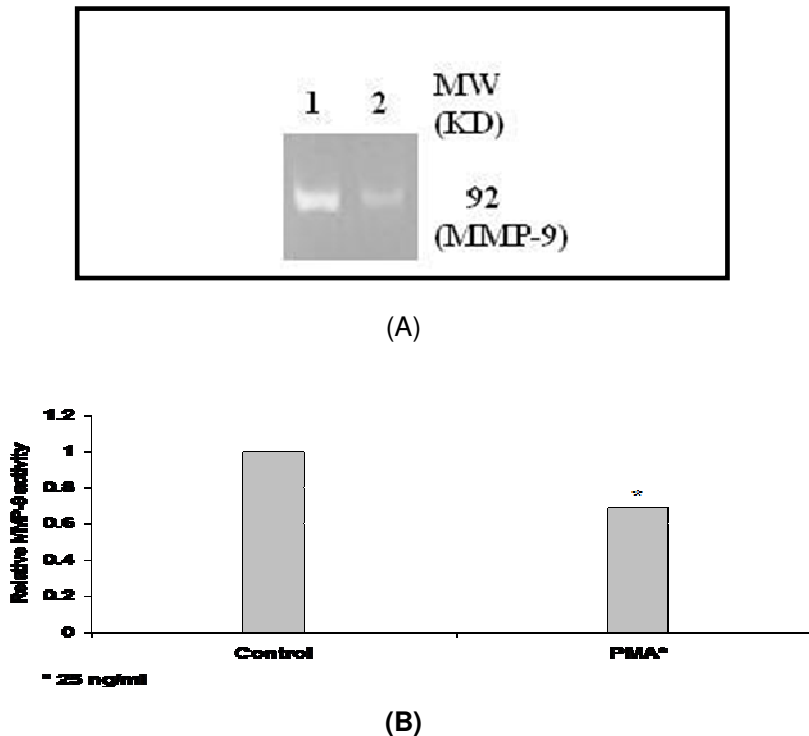
2. Zymographic analysis of PHA-induced gelatinases activity in hPBMCs

PHA effect on gelatinase-A(MMP-2) activity in hPBMCs

hPBMCs cultured alone, showed no bands demonstrating MMP-2 activity and PHA had no effect on MMP-2 activity in hPBMCs after 24 hour incubation time compared with untreated control cells(Table 1).

Table 1. Patterns of MMP-2 and MMP-9 secretion in hPBMCs

hPBMCs	MMP-2	MMP-9
Control	0%	100%
PHA	0%	200%
PMA	0%	20%

**Figure 1:** Effect of PMA on gelatinase-B (MMP-9) activity in human peripheral blood mononuclear cells.

Human peripheral blood mononuclear cells (1×10^6 cells/ml) were cultured in serum free RPMI-1640 medium and then treated with PMA (25ng/ml) for 24 hours. At the end of treatment, MMP-9 activity in conditioned medium was measured by gelatin zymography. (A) Zymogram of MMP-9 activity in hPBMCs. Lane 1 and 2 represent untreated and PMA-treated hPBMCs respectively. (B) MMP9 activity in hPBMCs 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.

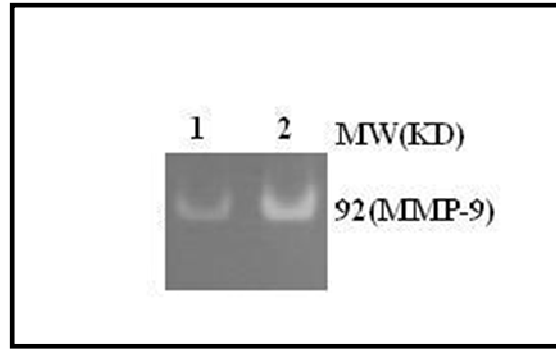
PHA effect on gelatinase-B(MMP-9) activity in hPBMCs

hPBMCs cultured alone without any inducer showed clear bands relating to MMP-9 activity. PHA significantly increased MMP-9 activity in hPBMCs after 24 hour incubation time compared with untreated control cells as illustrated in table 1 and figure 2(A&B).

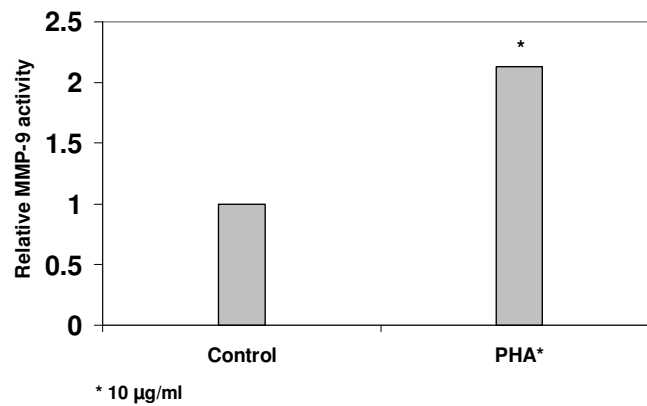
DISCUSSION

In the present study we found out that MMP-9 is expressed by normal human PBMCs. We also found that PHA increases whereas PMA decreases MMP-9 activity

in PBMCs. At the other hand, normal human PBMCs did not show any MMP-2 activity and PHA/PMA had no effect on MMP-2 activity of PBMCs. Different patterns of MMPs are expressed in different normal and cancer cells (Hajighasemi and Hajighasemi., 2009; Roomi et al., 2009). In Roomi et al. study (2009), all normal human cell lines tested expressed MMP-2 activity. The discrepancy between Roomi et al and us may be in part due to that the origin of cell lines we used was different from that of the Roomi et al. The cell lines used by Roomi et al. (2009) were originated from epithelial, connective and muscle tissues and the normal hPBMCs were not included in their study. Besides, in Roomi et al. study (2009), depending on the origin of cells, PMA decreased



(A)



(B)

Figure 2: Effect of PHA on gelatinase-B (MMP-9) activity in human peripheral blood mononuclear cells.

Human peripheral blood mononuclear cells (1×10^6 cells/ml) were cultured in serum free RPMI-1640 medium and then treated with PHA (10 μ g/ml) for 24 hours. At the end of treatment, MMP-9 activity in conditioned medium was measured by gelatin zymography. (A) Zymogram of MMP-9 activity in hPBMCs. Lane 1 and 2 represent untreated and PHA-treated hPBMCs respectively. (B) MMP9 activity in hPBMCs 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.

or had not any effect on MMP-2 activity. As in our study, normal hPBMCs did not show any MMP-2 activity, assessment the exact effect of PMA on MMP-2 activity in normal hPBMCs was impossible for us.

By the way there are contradictory data about the MMP-2 production by human PBMCs. Fang et al. (2010), reported that PBMCs do not express MMP-2 (similar to us) while in another study, Streck et al. (2007), showed MMP-2 activity in PBMCs. This inconsistency between Streck et al. (2007) and our results may be in part due to the number of cells used. Streck et al. used 10^6 cells/ml but we used 10^5 cells/ml. Hence in Streck et al. study, the cell numbers was ten fold of us and probably the

concentration of MMP-2 in cell conditioned medium in Streck et al. study (2007), was enough to be detected.

Besides Streck et al. (2007), measured MMP-2 activity after 48 h incubation time while we assessed MMP-2 activity after 24 h incubation time. Therefore it seems that in order to study the effect of PMA on MMP-2 activity in PBMCs we firstly need make the cells to produce detectable amounts of MMP-2. This may be done by increasing the number of cells in culture and also the incubation time or stimulating the cells by an MMP-2 inducer.

Several studies reported various patterns of MMP-9 expression in different normal cells (Roomi et al., 2009;

Weeks et al., 1993). In Roomi et al. study (2009), the normal cells did not express MMP-9 activity and had different sensitivity to PMA induced MMP-9 expression. Accordingly PMA-stimulated endothelia and stromal connective tissue did not express MMP-9 whereas muscle, supportive connective tissue and glandular epithelia produced MMP-9 after stimulation with PMA. However Roomi et al. (2009), did not use PBMCs in their study. In the present study, PBMCs expressed MMP-9 activity without any stimulation and PHA/PMA differently regulated MMP-9 activity in these cells. Accordingly PHA

(a lectin) increased but PMA decreased MMP-9 activity in PBMCs.

Production of MMP-9 by a number of immunocompetent cells such as T- lymphocytes (Weeks et al., 1993; Leppert et al., 1995), PBMCs (Strek et al., 2007) and macrophages (Strek et al., 2007; Ou et al., 2010; Opendakker et al., 2002), has been reported by several studies. Consistent to our results, enhancing effect of some lectins such as concanavalin A (Con A) or Artocarpus Lakoocha agglutinin (ALA), on MMP production and MMP activity of monocytes or human PBMCs has been reported (Saja et al., 2007a, b). The inhibitory effect of PMA on MMP-2 activity in PBMCs, in our study, may be explained by the different sensitivity of different normal cells to PMA-stimulated MMP-9 expression as described by Roomi et al. (2009). In contrast to our results, Nelissen et al. (2002), reported that PMA slightly increased gelatinase B (MMP-9) production in PBMCs. This inconsistency between our data and Nelissen et al. (2002), may be partly due to the concentration of PMA used. We used PMA at 25 ng/ml while in Nelissen et al study, PMA was utilized at 10 ng/ml concentration. Thus it seems that PMA effect on MMP activity in PBMCs is dose-dependent.

Several studies demonstrated an elevated production of MMP-9 by PBMCs in inflammatory conditions and autoimmunity (Fang et al., 2010; Weeks et al., 1993; Sternberg et al., 2009). Also there is accumulating data suggesting that inhibition of MMP-9 is useful for preventing of inflammation and autoimmunity (Hishikari K, 2010; Lesiak A, 2010).

Altogether, PHA/PMA stimulated PBMCs could be useful tools in screening of gelatinases modulators and thus helpful for development of new and successful therapeutic methods in correlated disorders such as inflammation and autoimmune diseases. Study of gelatinases inducers /inhibitors using the stimulated PBMCs could also be valuable in understanding the contributory mechanisms of related abnormalities.

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