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AT1 Receptors Activation Enhances the Expression of MMP-2, MMP-13 and VEGF but not MMP-9 in B16F10 Melanoma Cells

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Abstract: Melanoma is one of the most aggressive cancers of all solid tumors. The effect of angiotensin II on expression of three Matrix Metalloproteinases (MMPs) and Vascular Endothelial Growth Factor (VEGF) in B16F10 melanoma cells was evaluated. Also the blocking effect of losartan on angiotensin II induced effects was assessed. B16F10 murine melanoma cells were cultured in RPMI-1640 medium supplemented with 10% Fetal Bovine Serum (FBS) and 24 h prior to experiment the serum free medium was used. Angiotensin II (0 M, 10^{-10} M, 10^{-9} M or 10^{-8} M) alone or in combination with Losartan (10^{-6}M) in RPMI-1640 replaced the medium for experiments. After the incubation time (0, 1, 2, 6 and 12 h) cells were harvested using 0.05% (w/v) Trypsin and then recovered by centrifugation. The expression of MMP-2, MMP-13, MMP-9 and VEGF in B16F10 cell lysate was assessed by immunoblotting. Angiotensin II significantly enhanced the expression of MMP-2, MMP-13 and VEGF by concentrations as low as 0.1 nM. But angiotensin II could not stimulate any significant increase in MMP-9 expression by angiotensin II in B16F10 cells. Losartan abolished the enhancing effect of every concentration of angiotensin II on MMP-2, MMP-13 and VEGF expression completely and in all incubation times. As a result, angiotensin II through activation of AT1 receptors can stimulate the expression of MMP-2, MMP-13 and VEGF in B16F10 melanoma cells. This is an important conclusion because of the importance of these factors in melanoma invasiveness and the possible important role that angiotensin receptor blockers may play as cancer medications.

Key words: Losartan, angiotensin II, melanoma, B16F10, tumor

INTRODUCTION

Although angiotensin II (AngII) as the main effector protein of the Renin Angiotensin System (RAS) is playing an important role in homeostasis, the place that it takes in tumor progression needs to be more clarified (Egami *et al*., 2003; Mahabeleshwar and Byzova, 2007; Walter *et al*., 2003; Jain and Chaturvedi, 2008). AngII increases the expression of many factors in favor of neo-angiogenesis in tumor tissues (Otake *et al*., 2010; Herr *et al*., 2008). The dependence of tumor cells to angiogenesis for their growth is not a new finding. As a result, inhibiting the formation of new blood vessels which interfere with tumor blood supply has become an approach in cancer therapy (Kerbel and Folkman, 2002; Eskens , 2004; Mocellin, 2006). On the other hand, investigating downstream events and factors to AngII receptor activation which may be involved in different steps of tumor progression, is critical. Matrix Metaloproteinases (MMPs) are among such factors which belong to a family of at least 21 members. These endopeptidases are involved in degradation of the Extracellular Matrix (ECM) components in both physiological and pathological situations (Sahib *et al*., 2010; Ao *et al*., 2008). Members of this family of enzymes may classify into different subgroups including collagenases, stromelysins, matrilysins or gelatinase and also membrane-type MMPs (Pasco *et al*., 2004). MMP-2, MMP-9 and MMP-13 are among the important members of this family regarding their role in cancer progression especially melanoma progression (Pasco *et al*., 2004; Zigrino *et al*., 2009; El-Meghawry *et al*., 2006). However, considering melanoma as the most aggressive and the first cause of mortality among cutaneous cancers (Hofmann *et al*., 2000), there is still no report regarding the possible role of AngII in expression of these MMPs in melanoma cells.

On the other hand, the dependence of tumor cells to angiogenesis for their growth has been highlighted previously (Mahabeleshwar and Byzova, 2007). Several cell types and also mediators are required for the formation of new capillaries from pre-existing blood vessels which is called angiogenesis (Guruvayoorappan and Kuttan, 2007). Among the main angiogenic regulators is Vascular Endothelial Growth Factor (VEGF) which is

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over-expressed in most tumors (Leung *et al*., 1989; Shibuya, 1995). VEGF is a cytokine with key regulating effects on processes such as vasculogenesis, angiogenesis and increase in vascular permeability which are important for growth and metastasis of malignant tumors (Huang *et al*., 1998; Lee *et al*., 2006; Rmali *et al*., 2006). It has been reported that AngII increases VEGF production in vascular smooth muscle cells, mouse podocytes (Kang *et al*., 2006) and mesenchymal stem cells (Shi *et al*., 2009). On the other hand recently it has been revealed that AngII blocking agents may play an important role as therapeutic agents in gastric cancer (Huang *et al*., 2008), renal cancer (Miyajima *et al*., 2002) and melanoma (Otake *et al*., 2010).

Malignant melanoma is a highly aggressive tumor which is resistant to many available therapeutic methods (Ramadan *et al*., 2007; Timar *et al*., 2006). Therefore, it is of importance to find new therapeutic processes or mechanisms to treat or suppress this kind of malignancy. Furthermore renin-angiotensin system components and AngII receptors have been found in many tissues including skin (Steckelings *et al*., 2004) which may provide locally produced ligand for AngII receptors in skin or skin tumors to be activated. Considering the reports mentioned above, this study reports the direct effect of AT1 receptors activation by AngII on expression of MMP-2, MMP-13, MMP-9 and VEGF production in cultured B16F10 melanoma cells and the effect of losartan as an AT1 receptor antagonist on this process.

MATERIALS AND METHODS

Using western blot analysis the changes in expression levels of several factors caused by AngII was studied in a melanoma cell line. The inhibitory effect of losartan was also studied separately. The 25 cm^2 cell culture flasks were used for cell culture of B16F10 cells which took about 4 days to reach the confluence when the tests were started from 1×10^6 cells for each flask. After the process of incubation with the agonist and antagonist the cells were stored in -80°C until used during several western blot tests using antibodies against the target proteins. Highly metastatic murine melanoma cell line B16F10 (NCBI C-540) was obtained from national cell bank of Iran (Tehran, Iran). Angiotensin II, aprotinin, leupeptin and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma (St. Louis, MO, USA), Losartan was obtained from Santa Cruz and dissolved in Dimethyl Sulfoxide (DMSO) which was from Merck. RPMI-1640, Fetal Bovine Serum (FBS), trypsin, penicillin and streptomycin were purchased from GIBCO. All primary antibodies against MMP-2, MMP-9, MMP-13, VEGF and actin and also secondary HRP conjugated antibodies were purchased from Abcam. Micro BCA protein assay kit, CL-XPosure X-ray film and ECL western blotting substrate were purchased from Pierce (Rockford, IL, USA). Polyvinylidenedifluoride (PVDF) membranes were from Millipore (Bedford, USA). All other materials and reagents used were from Merck.

Cell culture: B16F10 murine melanoma cells were cultured in RPMI-1640 medium supplemented with 10% Fetal Bovine Serum (FBS) in an incubator maintained at 5% CO_2 and 37°C. For experiments, cells (1×10^6 cells for each flask) were plated in 25 cm^2 flasks with 8 ml medium and allowed to growth to confluence. 24 h before the experiments the cell culture medium was changed to serum-free RPMI-1640. Experimental cells were incubated with serum-free RPMI-1640 (vehicle) or AngII (10^{-10} M, 10^{-9} M or 10^{-8}M) alone or in combination with Losartan (10^{-6} M) in serum-free RPMI-1640. Whenever needed to use losartan, it was added to flasks 1 h before the agonist. Different incubation times (0, 1, 2, 6 and 12 h) were used to determine the effect of AngII and its AT1 receptor antagonist on MMP-2, MMP-9, MMP-13 and VEGF expression in B16F10 cells. After the incubation time, cells were harvested using 0.05% (w/v) trypsin in 0.02% (w/v) EDTA for 3 min at room temperature. Then trypsin was diluted using 3 times volume of RPMI-1640 and cells were recovered by centrifugation at 2000 g for 10 min at 4°C. The supernatant was dumped and 1 mL RPMI-1640 was added to each tube and centrifuged further at 12000 g for 15 min at 4°C. The supernatants were dumped again and the precipitated cells were stored at -80°C for later use.

Total protein measurement: B16F10 cells extract were prepared in lysis buffer (137 mMNaCl, 20 mMTris-HCl pH 8.0, 1% NP-40, 10% glycerol, 1 mMPMSF, 10 μg mL^{-1} aprotinin and 1 μg mL^{-1} leupeptin and 0.5 mM sodium vanadate). Cell lysate was centrifuged to remove insoluble components (12500 g for 20 min at 4°C). Then total protein concentration was determined using Micro BCA protein assay kit according to the kit procedure. Cell extracts were stored at -80°C until used.

Western blotting: The expression of MMP-2, MMP-9, MMP-13 and VEGF in B16F10 cell lysate was assessed by immunoblotting. For SDS-PAGE, equal amounts (90 μg) of protein from each sample was loaded on 10% polyacrylamide gels and separated by electrophoresis. Proteins were electrotransferred to PVDF membranes which were then blocked with 5% skim milk and 0.1% Tween-20 in Tris-buffered saline at room temperature for 1 h. Membranes were incubated with primary antibodies against target proteins for 1 h at room temperature which followed by incubation with appropriate secondary antibodies for 1 h at room temperature. Actin was used as internal standard for western blot analysis and the result of densitometric film scanning for quantification of target proteins was normalized for actin levels. For MMP-2 quantification anti-MMP-2 primary antibody (ab7032),

(1:500) and anti-mouse IgG HRP conjugate (ab6728) (1:2000), for MMP-9 quantification anti-MMP-9 antibody (ab38898) (1:4000) and anti-rabbit IgG HRP conjugate (ab6721) (1:2000), for MMP-13 quantification anti-MMP-13 antibody (ab58836) (1:200) and anti-mouse IgG HRP conjugate (ab6728) (1:2000), for VEGF quantification anti-VEGF antibody (ab1316) (1:1000) and anti-mouse IgG HRP conjugate (ab6728) (1:2000) and finally for actin quantification anti-actin antibody (ab8227) (1:2500) and anti-rabbit IgG HRP conjugate (ab6721) (1:2000) were used. Chemiluminescence detection of the bands was performed using the ECL kit according to the manufacturer recommendation and the result was then quantified using Gel-Pro analyzer imaging software.

Statistical analysis: Values represent the Mean±Standard error of the mean (SEM). A one-way Analysis of Variance (ANOVA) was conducted to compare the data between two different groups using SPSS-16 software. Statistical differences were considered significant when $p<0.05$. The results are representative of those from three independent experiments.

RESULTS

The amount of total protein concentrations was quantified by micro BCA method and the level of MMP-2, MMP-9, MMP-13 and VEGF expression in B16F10 cells was measured by western blotting analysis. The expression of MMP-2, MMP13 and VEGF were significantly induced by Ang II in concentrations as low as 0.1 nM but in different exposure times. At the same time, the expression of MMP-9 did not significantly increased by AngII in any concentration and at any exposure time. Also to confirm the involvement of AT1 receptors in observed effect of AngII on MMP-2 MMP-13 and VEGF expression we performed an independent series of experiments with the same AngII concentrations and incubation times with melanoma cells exposed to losartan (10^{-6} M) 1 h before AngII.

As it is shown in Fig. 1a, AngII (0.1, 1 and 10 nM) significantly induced MMP-2 protein expression in B16F10 melanoma cells. This induction was time-dependent and started with 0.1 nM Ang II concentration in 2 h incubation (123.5±1.99, 117.5±3.3 and 120.4±4.6% of

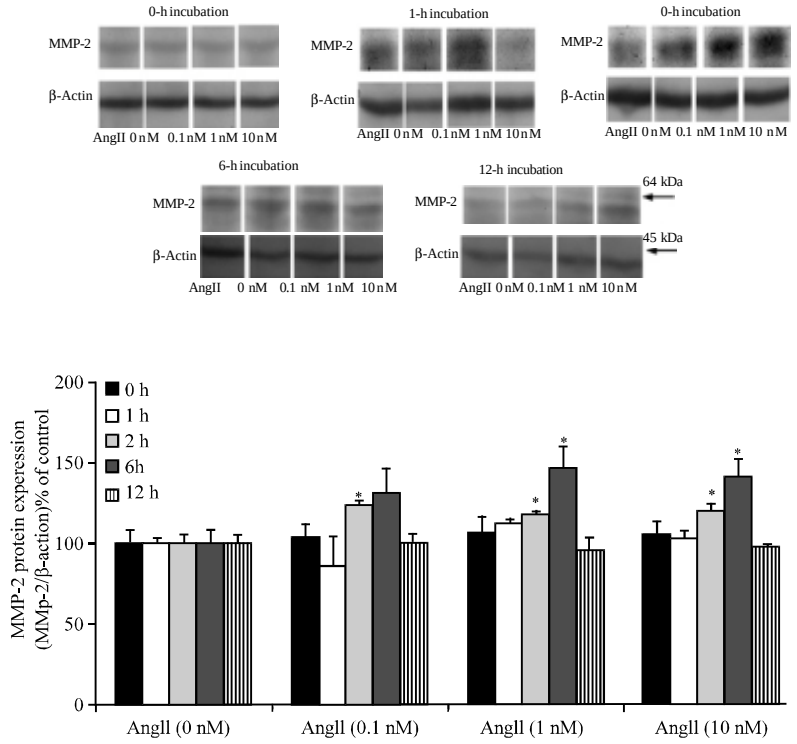

Fig. 1a: B16F10 cells were harvested after being exposed to AngII (0.1-10 nM) for different exposure times (0, 1, 2, 6 and 12 h). Western blot analysis revealed that AngII increased the expression of MMP-2 protein in melanoma cell line. AngII could significantly increase the MMP-2 expression in concentrations as low as 0.1 nM after 2 h incubation. Results are displayed as percentage of control groups for each exposure time. Each value represents the Mean±SEM (N = 3) (ANOVA, *$p<0.05$, **$p<0.01$)

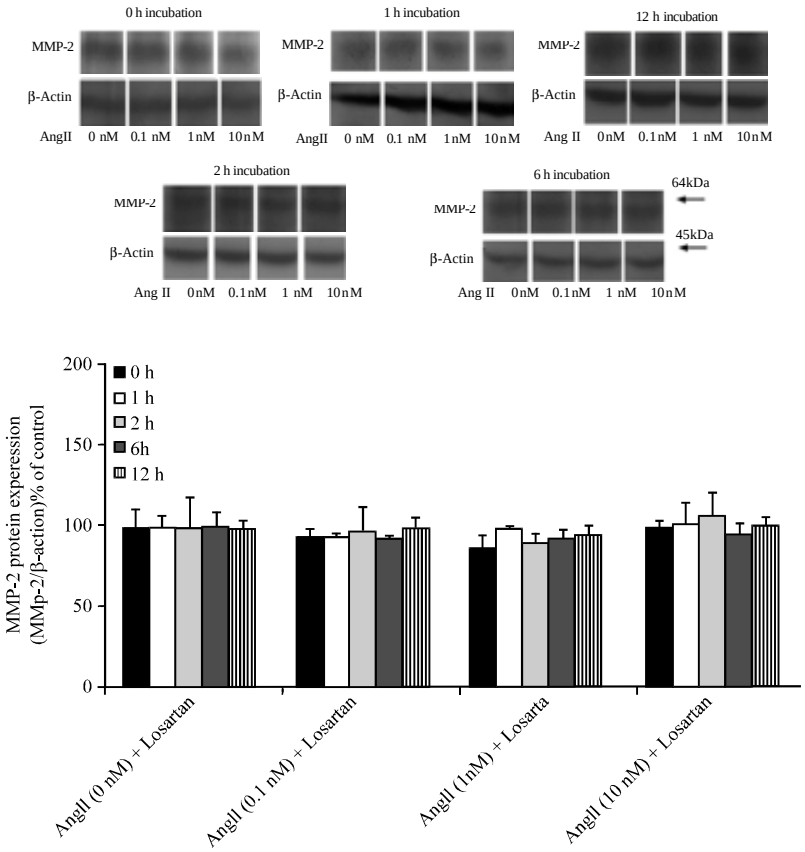

Fig. 1b: B16F10 cells were harvested after being exposed to AngII (0.1-10 nM) and Losartan (10^{-6} M, 1 h before AngII) for different exposure times (0, 1, 2, 6 and 12 h). Western blot analysis revealed that losartan completely blocked the observed effect of AngII on MMP-2 expression. Each value represents the Mean±SEM (N = 3)

control for 0.1, 1 and 10 nM Ang II, respectively during 2 h incubation) and peaked after 6 h of incubation with 1 nM Ang II concentration (146.8±13.6 and 141±11.7% of control for 1 and 10 nM Ang II, respectively during 6 h incubation). The increase in MMP-2 expression was declined in longer incubation times in a way that after 12 h incubation AngII could not increase the expression of MMP-2 significantly. Also, incubating for 1 h did not resulted in any increase in MMP-2 expression which shows the ineffectiveness of AngII in induction of MMP-2 from melanoma cells in this time point. Figure 1b represents the blocking effect of losartan on AngII effect on MMP-2 expression. Losartan could completely suppress the enhancing effect of AngII on MMP-2 expression. This suppression happened in every studied time point and Ang II concentration. Figure 2a and b show the stimulatory effect of AngII (0.1, 1 and 10 nM) and inhibitory effect of losartan (10^{-6} M) on MMP-13 expression respectively. AngII significantly increased the expression of MMP-13 in melanoma cells up to 166±12.6% of control in concentration as low as 0.1 nM after 2 h incubation. This effect was happened in other tested concentrations of AngII 1 nM and 10 nM (151.4±14.5 and 156.1±16.2% of control, respectively) but was measurable only after 2 h incubation and at longer incubation times AngII could not enhance the expression of MMP-13 significantly. Also losartan could abolish the enhancing effect of AngII on MMP-13 expression completely. According to this result AngII may increase the expression of MMP-13 significantly for a short period of time. Figure 3 show that AngII could not increase the expression of MMP-9 in B16F10 melanoma cells in any tested concentration or exposure time. Figure 4a and b represents the effect of AngII with and without pretreatment with losartan on VEGF expression in B16F10 melanoma cells respectively. As it is shown in these

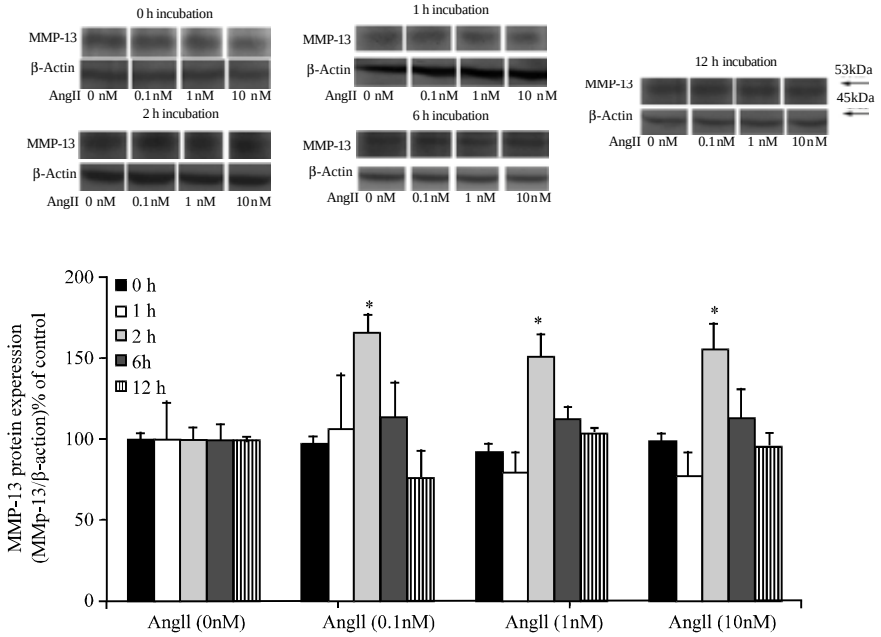

Fig. 2a: B16F10 cells were harvested after being exposed to AngII (0.1-10 nM) for different exposure times (0, 1, 2, 6 and 12 h). Western blot analysis revealed that AngII increased the expression of MMP-13 protein in melanoma cell line. AngII could significantly increase the MMP-13 expression in concentrations 0.1, 1 and 10 nM after 2 h incubation. Results are displayed as percentage of control groups for each exposure time. Each value represents the Mean±SEM (N = 3) (ANOVA *p<0.05)

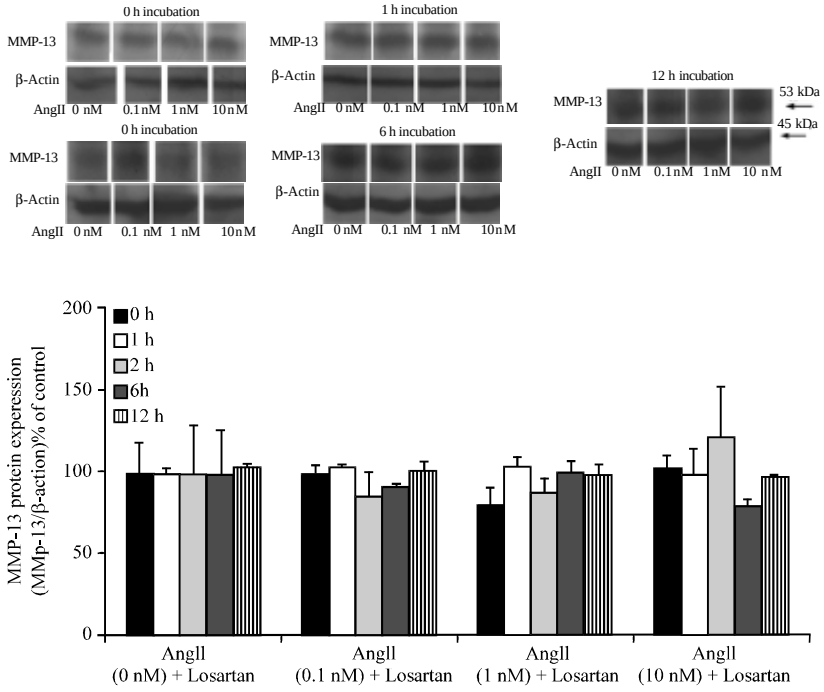

Fig. 2b: B16F10 cells were harvested after being exposed to AngII (0.1-10 nM) and Losartan (10^{-6} M, 1 h before AngII) for different exposure times (0, 1, 2, 6 and 12 h). Western blot analysis revealed that losartan completely blocked the observed effect of AngII on MMP-13 expression. Each value represents the Mean±SEM (N = 3)

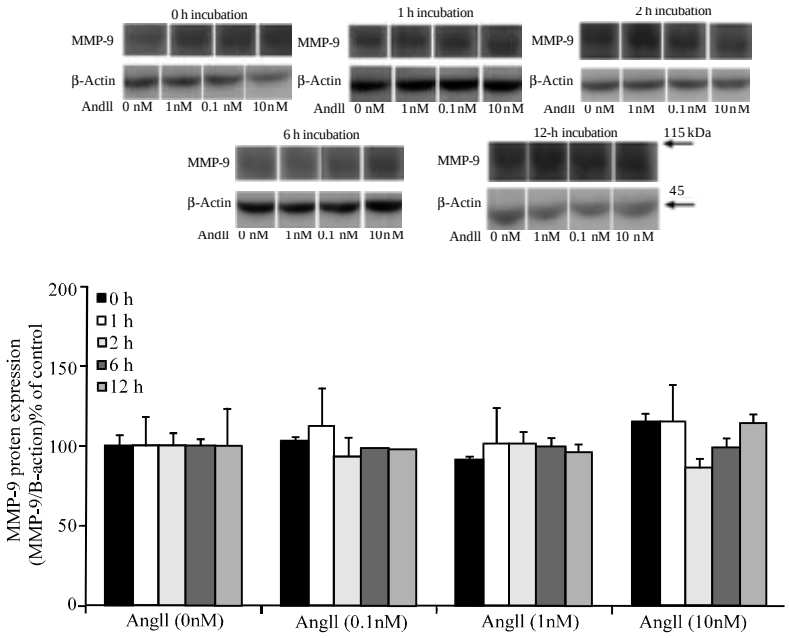

Fig. 3: B16F10 cells were harvested after being exposed to AngII (0.1-10 nM) for different exposure times (0, 1, 2, 6 and 12 h). Western blot analysis revealed that AngII could not significantly increase the expression of MMP-9 protein in melanoma cell line in any concentrations and after any incubation times. Results are displayed as percentage of control groups for each exposure time. Each value represents the Mean±SEM (N = 3)

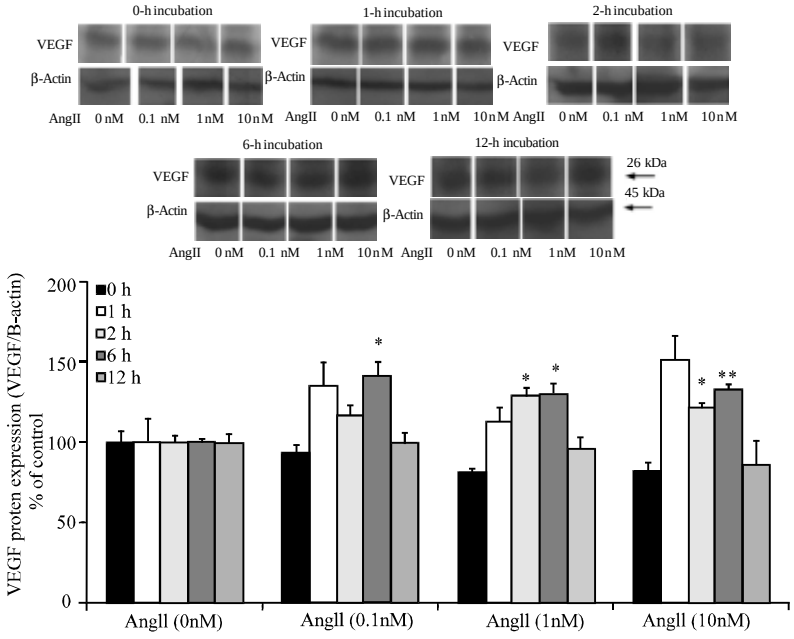

Fig. 4a: B16F10 cells were harvested after being exposed to AngII (0.1-10 nM) for different exposure times (0, 1, 2, 6 and 12 h). Western blot analysis revealed that AngII increased the expression of VEGF protein in melanoma cell line. AngII could significantly increase the VEGF expression in concentrations as low as 0.1 nM after 6 h incubation. Results are displayed as percentage of control groups for each exposure time. Each value represents the Mean±SEM (N = 3) (ANOVA, *p<0.05, **p<0.01)

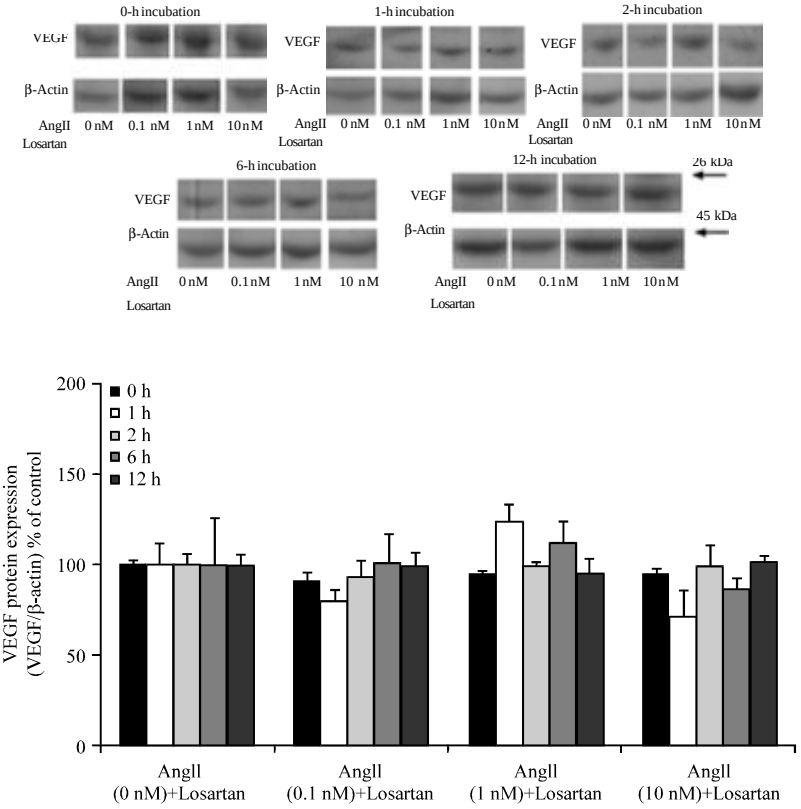

Fig. 4b: B16F10 cells were harvested after being exposed to AngII (0.1-10 nM) and Losartan (10^{-6} M, 1 h before AngII) for different exposure times (0, 1, 2, 6 and 12 h). Western blot analysis revealed that losartan completely blocked the observed effect of AngII on VEGF expression. Each value represents the Mean±SEM (N = 3)

figures AngII could significantly increase the VEGF expression by concentrations as low as 0.1 nM after 6 h incubation time (141.8±9.1%, 130.5±6.9%, 132.4±4.5% of control for 0.1, 1, 10 nM AngII, respectively). This effect was also time dependent and in grater concentrations (128.5±5.3% and 122±2.4% of control for 1 nM and 10 nM AngII, respectively) AngII increased the VEGF expression after 2h exposure. Losartan completely abolished this effect in any concentration and any incubation time. This shows that the mechanism of AngII induced effect on MMP-2, MMP-13 and VEGF expression in B16F10 melanoma cells is through activation of AT1 receptors located on these cells.

DISCUSSION

The broad presence of AT1 receptors and the evidences suggesting the inhibitory effect of AngII blocking agents in cancer growth and invasiveness (Otake *et al*., 2010; Ino *et al*., 2006; Fujita *et al*., 2002) shows the importance of RAS in cancer biology. Although the mechanisms of the therapeutic effect of AngII blocking agents still need clarification, however the role of AngII and its AT1 receptor in tumor angiogenesis has been highlighted (Egamı *et al*., 2003). Using immunohistochemical methods it has been shown that human melanoma express AT1 receptor and AngII protein (Otake *et al*., 2010) which is an evidence for the local production of this octapeptide and its availability to melanoma cells. It has been shown that in $AT1a^{-/-}$ mice not only the growth of engrafted B16F1 tumors, but also the tissue capillary density of the tumor was less than the wild type mice. The production of VEGF was also less in $AT1a^{-/-}$ mice which has been explained to be partly related to less infiltration of tumor-associated macrophages (Egamı *et al*., 2003). In one report losartan suppressed significantly gastric tumor development and lymphangiogenesis by inhibiting VEGF-C expression in a mice model (Wang *et al*., 2008). In another report, losartan limited murine melanoma growth by reduction of tumor

volume and micro vessel density which shows the importance of AT1 receptors in melanoma growth (Otake *et al*., 2010). However the effect of AngII on melanoma growth and invasion still needs to be clarified and it is of importance to study the anti-cancer effects of AT1 receptor blockers in different tumor models. The differences between a tumor tissue and cell culture models of cancer research to some extend is related to the presence of different kind of cells in a tumor mass. Although in a tumor tissue such as melanoma a significant part of the cells are melanoma cells (Smith *et al*., 2006), however the decrease of the percentage of cancer cells in a tumor tissue relative to the cell culture model may decrease the concentration of any protein which may be expressed or released from cancer cells and also does not allow the study to be specifically focused on the malignant cells. On the other hand using the cell cultured cancer cells actually eliminate any interplay between different cells and their released factors that are present in a real cancer tissue. Therefore it should be keep in mind that different results obtained in these studies does not necessarily contradicted. In the process of cancer invasion and metastasis, the breakdown of the ECM is a crucial step. MMP-2, MMP-9 and MMP-13 are among the most important matrix metalloproteinase family members which are expressed by melanoma cells (Kuphal *et al*., 2005). Although several reports suggest the importance of MMPs in progression of cancer tumors however the effect of AngII on MMPs expression in melanoma has not been studied (Kuphal *et al*., 2005; Pasco *et al*., 2004; Williams *et al*., 2005; Brinkerhoff and Matrisian, 2002). MMP-2 and MMP-9 are among the factors that facilitate the degradation of basement membrane type IV collagen and increase the bioavailability of matrix associated growth factors (Brinkerhoff and Matrisian, 2002). The elevated levels of MMP-2 have been reported in various human malignancies including melanoma tumors (Pasco *et al*., 2004) and this enzyme has been identified as critical in the process of angiogenesis (Zhang *et al*., 2004). It has been reported that blocking the activity of MMP-2 and MMP-9 in a nude mice model of human MGLVA-1 gastric adenocarcinoma xenografts reduced the tumor size by 40-50% (Williams *et al*., 2005). However there are controversial reports regarding the effect of AngII on MMP-2 or MMP-9 expression in different cells. For example, it has been reported that AngII increased the expression of MMP-2 and MMP-9 in MKN-28 cells (Huang *et al*., 2008). But it has been shown that AngII could not affect the MMP-2 and MMP-9 activity in subcutaneous tissues of C57Bl/6 mice (Tamarat *et al*., 2002). In B16F10 cultured cells, AngII could up-regulate MMP-2 (but not MMP-9) protein synthesis in B16F10 melanoma cells and blocking the AT1 receptors on these cells could abolish the effect of AngII completely. This result provides a mechanism which explains the effect of AT1 blocking agents in suppression of melanoma progression. On the other hand, It has been reported that in MMP-13$^{-/-}$ mouse engrafted with B16F1 cells, tumor growth was significantly impaired and also tumor metastasis to various organs was reduced (Zigrino *et al*., 2009) whish shows the significant importance of MMP-13 in melanoma progression. As AngII could increase the expression of MMP-13 in B16F10 cells this may explain one of the important mechanisms through which AngII may benefit the malignant tumor progression.

Also the possible effect of AngII on VEGF expression in melanoma cells needs to be clarified. As any other cancer tissues, melanoma needs angiogenesis which provide cancer tissue with blood supply to be able to growth (El-Habashy *et al*., 2006). However there is controversy in reports regarding the possible effect of Ang II on VEGF expression in various cancer models. It has been reported that AngII significantly increased the expression of VEGF in A549 human lung carcinoma cells (Feng *et al*., 2010) and some of the human prostate cancer cell lines (C4-2 and C4-2AT6 cells but not in LNCaP cells) (Kosaka *et al*., 2010). On the other hand it has reported recently that in mice engrafted with B16F10 melanoma cells, losartan could reduce the expression of VEGFR1 (Flt-1) and VEGFR2 (Flk-1) but not their ligand VEGF in cancer tissue (Otake *et al*., 2010). According to the results presented here, AngII significantly increased the VEGF expression in cultured B16F10 melanoma cells. This is of importance because it demonstrates the effect of AngII in expression of a powerful angiogenic cytokine in melanoma cells which depends significantly on angiogenesis (Egami *et al*., 2003).

CONCLUSION

The presence of AT1 receptors in different cells in tumor tissues including tumor associated macrophages, endothelial cells, vascular smooth muscle cells and melanoma cells (Egami *et al*., 2003) dictate the necessity of analyzing the effect of AngII on some important molecules such as MMP-2, MMP-9 and MMP-13 and VEGF expression in cancer cells and tissues. The exposure of B16F10 melanoma cells to AngII increased the expression of several factors with proved enhancing effects on tumor growth and metastasis. This result may explain at least a part of observed ability of AT1 receptor blocking agent losartan in suppressing the growth of melanoma tumors. And also provide a mechanism for

AngII potentiating effect on tumor growth and metastasis. As losartan is presently in market as a drug, it may be recommendable to consider its use for suppressing the progress of possible melanoma cases in human.

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