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Journal of Cellular Biochemistry 114:650–657 (2013)

Journal of Cellular Biochemistry

uPA and MMP-2 were Involved in Self-Assembled Network Formation in a Two Dimensional Co-Culture Model of Bone Marrow Stromal Cells and Endothelial Cells

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ABSTRACT
Two dimensional (2D) co-cultures of human bone marrow stromal cells (HBMSCs) and human umbilical vein endothelial cells (HUVECs) stimulate osteoblastic differentiation of HBMSCs, induce the formation of self-assembled network and cell interactions between the two cell types involving many vascular molecules. Because of their strong activities on angiogenesis and tissue remodeling, urokinase plasminogen activator (uPA), plasminogen activator inhibitor-1 (PAI-1), matrix metalloproteinase-2 (MMP-2) as well tissue inhibitors of matrix metalloproteinase-2 (TIMP-2) were investigated in this 2D co-culture model. We found that the expression of uPA, MMP-2 in the co-cultured cells was significantly higher than those in mono-cultured cells. In opposite, PAI-1, expressed only by HUVECs is not regulated in the co-culture. Inhibition assays confirm that uPA played a critical role in the formation of self-assembled network as neutralization of uPA disturbed this network. In the same context, inhibition of MMP-2 prevented the formation of self-assembled network, while the inhibition of uPA abolished the over expression and the activity of MMP-2. This upregulation could initiate the uPA expression and proteolysis processes through the MMP-2 activity, and may contribute to endothelial cell migration and the formation of this self-assembled network observed in these 2D co-cultured cells. J. Cell. Biochem. 114: 650–657, 2013. © 2012 Wiley Periodicals, Inc.

KEY WORDS: CO-CULTURE; ENDOTHELIAL CELLS; MMP-2; uPA; ANGIOGENESIS

Angiogenesis is very important for bone tissue engineering as engineered bone with critical defect size needs blood vessels to supply nutrition and remove waste products, as well as establish biological communications [Santos et al., 2009; Yu et al., 2009]. Many methods have been applied to stimulate angiogenesis for bone tissue engineering [Dennis and Rifkin, 1990; Montesano et al., 1993; Bischoff, 1995; Cockerill et al., 1995; Bishop et al., 1999], among which the co-culture of endothelial cells with other cell types has attracted great interest with the anticipation of stimulating angiogenesis [Nehls and Drenckhahn, 1995; Sorrell et al., 2007]. Many studies have shown that two dimensional (2D) co-cultures of human mesenchymal stem cells and human primary endothelial cells not only induced the osteoblastic differentiation of mesenchymal stem cells but also stimulated the formation of self-assembled network, in which the latter is often considered as a prevascularization [Finkenzeller et al., 2006; Fuchs et al., 2007; Grellier et al., 2009; Li et al., 2010, 2011]. As angiogenesis is a physiologic process that involves finely cell–cell and cell–matrix interactions, to elucidate the molecular events that initiate and play important roles in angiogenesis is very important for pharmacologically controlling angiogenesis [Haas and Madri, 1999]. It is undoubted that, in our 2D co-culture model where human bone marrow stromal cells (HBMSCs) and human umbilical vein endothelial cells (HUVECs) are in direct contact, the interactions between HBMSCs and HUVECs are in great complexity. We have previously shown the role of a gap junction molecule (i.e., Connexin 43) [Villars et al., 2002], adherens junction molecules (vascular endothelial-cadherin and neural-cadherin), and specific secreted diffusible factors, such as vascular endothelial growth factor (VEGF), in the interactions between HBMSCs and HUVECs [Grellier et al., 2009; Li et al., 2010, 2011].

In addition to cell–cell interactions, the cell–matrix interaction for angiogenesis is also important for angiogenesis. A large

Grant sponsor: « *Agence Nationale de la Recherche*; Grant numbers: ANR 06-PNANO-022-06, ANR 07 TECSAN 011-01.
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Manuscript Received: 25 July 2012; Manuscript Accepted: 21 September 2012
Accepted manuscript online in Wiley Online Library (wileyonlinelibrary.com): 11 October 2012
DOI 10.1002/jcb.24407 • © 2012 Wiley Periodicals, Inc.

literature shows that the migration of endothelial cells is a critical step in angiogenesis process, which needs the proteolysis of the underlying basement membrane matrix [Arnold and West, 1991; Cockerill et al., 1995; Folkman, 1995; Lamalice et al., 2007]. Therefore, cells have to express proteolytic enzymes allowing cleavage of matrix proteins for favoring cell migration. Many studies demonstrated in vitro in 3D culture systems that plasminogen activators and plasmin (serine protease), as well matrix metalloproteinases (MMPs) are the two main families of proteases and work intimately to promote angiogenesis [Mignatti and Rifkin, 1996; Carmeliet and Collen, 1998; Collen et al., 2003]. Two physiological plasminogen activators (PA) have been identified: tissue-type PA (tPA) and urokinase-type PA (uPA). The tPA is primarily involved in fibrin homeostasis while the uPA is mainly involved in phenomena such as cell migration and tissue remodelling [Garcia-Touchard et al., 2005]. The plasminogen activators convert plasminogen to plasmin, which works more efficiently in proteolysis of the fibrin, whereas the MMPs family is the main proteases for fibronectin, laminin, elastin, and collagens, which are the extracellular matrix proteins commonly found in endothelial cell basement membranes. In addition, plasminogen activators activate several MMPs [Liotta et al., 1981; Bini et al., 1999]. The Plasminogen Activator Inhibitor-1 (PAI-1), is a serine protease inhibitor that functions as the principal inhibitor of tissue plasminogen activator (tPA) and uPA. This well known signaling could also contribute to the MMPs activities.

Among the MMPs, it has been reported that endothelial cells mainly produce MMP-1 or MMP-13 (interstitial collagenases), MMP-3 (stromelysin-1), MMP-2, MMP9 (gelatinase A and B), and MMP-14 [Cornelius et al., 1995; Lewalle et al., 1995]. More recently, MMP-2 has received recognition that they play an important role in the process of angiogenesis [Sato et al., 1997; Koike et al., 2002; Finkenzeller et al., 2006]. In addition, there is always balance between concentrations of specific MMPs and tissue inhibitors of metalloproteinases (TIMPs) that defines the extent of proteolysis via MMPs [Corcoran et al., 1996]. Among them, TIMP-2 is not only an inhibitor of MMP-14 but also stimulates cleavage of MMP-2 to the active form [Cao et al., 1995; Pei and Weiss, 1996]. It is undoubtedly critical for the angiogenesis process involving at least MMP-2.

In our previous work describing 2D co-cultures of human endothelial cells and mesenchymal stem cells, we found that the expression of uPA was highly upregulated in co-cultured cells as compared to the mono-cultured cells, which indicated that the cell–matrix interactions might be active during the formation of self-assembled network. In this study, we further investigated the cell–matrix interactions that stimulated the formation of self-assembled network observed in this 2D co-culture model of HBMSCs and HUVECs. We further investigated the activities and production of MMP-2, and TIMP-2 under the control of uPA. Besides, since uPA is mainly involved in cell migration, we inhibited the activity of uPA to investigate its effect on production of MMP-2, and TIMP-2 as well the possible regulation of PAI-1 in this co-culture system. Then, we have identified new functions of these molecules on the formation of self-assembled network that contributes to cell-to-cell communication and osteogenesis in this co-culture system.

MATERIALS AND METHODS

CELL CULTURE

HUVECs were isolated and cultured according to methods described previously and with respect of the French Legislation [Grellier et al., 2009; Li et al., 2010]. For human bone marrow stromal cells (HBMSCs), bone marrow was aspirated from the femoral diaphysis or iliac bone after obtaining consents from patients (age 30–80 years) undergoing hip prosthesis surgery after trauma. The human bone marrow was then sequentially filtered with syringes fitted with 16-, 18-, and 21-gauges needles. These human cells were either mono-cultured or co-cultured in six-well plates with the initial seeding density of 20,000 HBMSCs/cm^2 and 40,000 HUVECs/cm^2. Culture medium is Iscove's Modified Dulbecco's Medium (IMDM, Gibco) supplemented with 1% (v/v) fetal bovine serum (FBS, Gibco) for all cells. In some experiments, the medium was supplemented with MMP-2 inhibitor I (20 μM) from Calbiochem or with vehicle (0.2% DMSO) [Berton et al., 2001]. At 14 and 24 h, culture medium from all cells were transferred into centrifuge tubes and centrifuged at 2,000*g* for 10 min, after which the supernatant was collected and conserved at -80°C for further analysis. The cultured cells were removed from the culture plates by scraping on ice with a lysate buffer containing 50 mM Tris HCl, pH8, 150 mM NaCl, 0.1% (v/v) Nonidet P-40, 10 μg/ml Aprotinine, 10 μg/ml Leupeptin, and 1 mM (4-(2-Aminoethyl)-benzenesulfonyl fluoride hydrochloride (AEBSF) (Fluka). Then, the collected mixture of cell pellet and lysate buffer were agitated at 4°C for 30 min, then centrifuged at 20,000*g* and 4°C for 20 min to extract total protein from cells. Self-assembled network formation was monitored by phase contrast microscopy (Zeiss Axiovert 25, Seli, France).

QUANTITATIVE REAL TIME POLYMERASE CHAIN REACTION (Q-PCR)

Total RNA was prepared from cells using Total RNA Isolation kit (NucleoSpin ® RNA II, MACHEREY-NAGEL) according to the manufacture's guidelines. Complementary DNA (cDNA) was synthesized according to the protocols described by Grellier et al. [2009] Primer of uPA, PAI-1 (Eurogentec) were used as the final concentration of 250 nM and P0 was used as a housekeep gene (P0 primer's sequence is BC015690, whose forward sequence is 5′ATG CCC AGG GAA GAC AGG GC 3′ and reverse sequence is 5′ CCA TCA GCA CCA CAG CCT TC 3′). uPA primer's sequence is NM 001145031.1, whose forward sequence is 5′ CAC GCA AGG GGA GAT GAA 3′ and reverse sequence is 5′ ACA GCA TTT TGG TGG TGA CTT 3′. PAI-1 primer's sequence is BC010860, whose forward sequence is 5′ TGT TTG TCT GCG GCG ATG TTA 3′ and reverse sequence is 5′ GTA TGC GGC TTG TCA CCT CCT 3′. Data were analyzed with the iCycler IQTM software and compared by the $\Delta\Delta$Ct method and each Q-PCR was performed in triplicate for PCR yield validation. Data were normalized to P0 mRNA expression of each condition. uPA and PAI-1 relative gene expression were standardized to expression in HBMSCs or HUVECs after 14 h of monoculture.

ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

uPA from cell lysates was quantified using a specific uPA ELISA kit (American Diagnostica). Briefly, cell lysates from 14 and 24 h mono-cultured or co-cultured cells were submitted to the immuno-

enzymatic detection, according to the protocol described by the supplier. The quantification of total proteins in cells was performed using BCA (bicinchoninic acid) protein assay kit (Pierce, Perbio Science, Bezons, France). Results are expressed in pg of uPA per μg of total protein.

WESTERN BLOT

Western blot analysis was carried out as previously described [Li et al., 2010]. Briefly, electrophoresis and mini trans-blot were used to separate and blot the protein to a polyvinylidene difluoride membrane (PVDF) (Immobilon-P; Millipore). Then, membranes were incubated with primary antibodies anti-MMP-2 (mouse monoclonal antibody, Calbiochem), and TIMP-2 (mouse monoclonal antibodies, R&D Systems) diluted in Tris-Buffered-Saline-Tween blocking buffer (TBS-T: 20 mM Tris HCl, pH 7.4, 150 mM NaCl, 0.05% (v/v) Tween 20) containing 5% (w/v) non fat milk). Loading control was performed by incubating membrane with a mouse monoclonal antibody against α-tubulin (Sigma) diluted at 1/30,000 in blocking buffer. Immunoreactive bands were visualized using horseradish-peroxidase-conjugated secondary antibodies (HRP, goat anti-mouse) (Chemicon, Euromedex, France) diluted at 1/15,000 in blocking buffer. Membrane was immersed in enhanced chemiluminescence detect reagent (Amersham ECL-plus, GE Healthcare) and exposed to hyperfilm ECL (GE Healthcare). The intensities of the bands were quantified by a Bio Imaging System (Gene Genius, Syngene) with GeneTools software.

GELATIN ZYMOGRAPHY

Gelatinolytic activities of secreted MMP-2 were analyzed by zymography on gelatin-containing polyacrylamide gels. The previously collected and conserved culture media were used for these assays. Briefly, samples were applied to 10% (w/v) polyacrylamide gels copolymerized with 1 mg/ml gelatin. After electrophoresis, the gels were washed four times for 15 min in 2.5% (v/v) Triton X-100 to remove the SDS, followed by one wash of 10 min in 5 mM Tris-HCl, pH 7.4, containing 5 mM $CaCl_2$, 0.2 M NaCl, 0.1% (v/v) Triton X-100 and incubated in the same solution for 18 h. The gels were stained with Coomassie brilliant blue for 90 min at room temperature, followed by discoloration in a water solution containing 50% (v/v) methanol and 10% (v/v) acetic acid.

FUNCTIONAL STUDIES

For uPA blocking experiments, HBMSCs and HUVECs were separately pre-treated with 10 μg/ml monoclonal mouse antibody (IgG isotype) against human uPA (American Diagnostica Inc.) overnight before they were cultured in six-well plates with 15 μg/ml of the same antibody. As controls, non-pre-treated cells were cultured without the neutralizing antibody and isotype mouse antibody (IgG) has been used also as control antibody. The LIVE/DEAD viability/cytotoxicity assay kit (Invitrogen) was applied to the cells cultured with or without neutralizing antibody to confirm their cell viability using ethidium homodimer that enter in dead cell and produces a red fluorescence (ex/em 495/635 nm) and calcein-AM reagents a non-permeant calcein AM converted by the esterase activity of live cells in calcein producing a green fluorescence (ex/em 495/515 nm).

STATISTICAL ANALYSIS

All data were expressed as means ± standard deviation (SD) for n = 3 (three independent experiments) and were analyzed using standard analysis of Student's *t*-test. Differences were considered significant when $P \leq 0.05$ (*) or $P \leq 0.01$ (**).

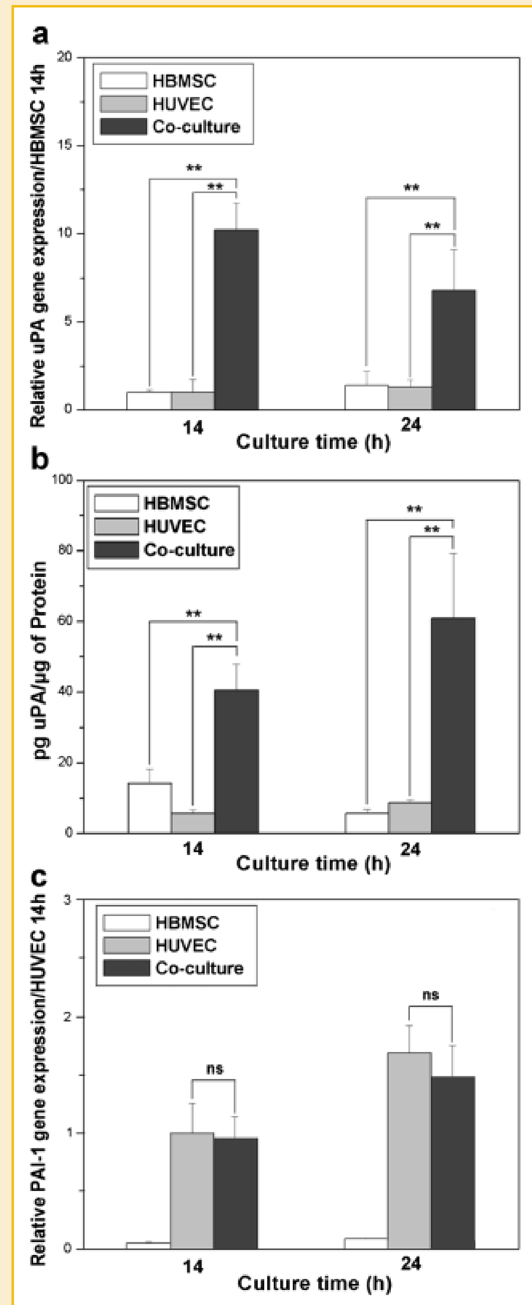

Fig. 1. Expression of uPA, PAI in HBMSCs, HUVECs, and HBMSCs-HUVECs co-culture. The uPA and PAI-1 expressions were detected by Q-PCR for mRNA level (a, c) and by ELISA for uPA protein level (b). Data of gene expression were quantified relative to uPA or PAI-1 gene expressions of HBMSCs after 14 h of culture. Data of protein expression were quantified relative to the total proteins in cell lysates measured using BCA protein assay. (n = 3; $^{*}P \leq 0.05$; $^{**}P \leq 0.01$; ns: not significant).

RESULTS

EXPRESSION OF uPA AND PAI-1 DURING SELF-ASSEMBLED NETWORK FORMATION IN CO-CULTURE

In our previous studies, it has been reported that co-cultures of HBMSCs with HUVECs induced self-assembled network structure after 24 h and that uPA gene expression was upregulated [Li et al., 2011]. To assess whether the uPA upregulation was at mRNA and protein level, we checked the expression of uPA in the cells by Q-PCR and ELISA and the results are shown in Figure 1. Obviously, the expression of uPA in co-cultured cells is significantly higher than those in mono-cultured cells, as evidenced by the gene expression (Fig. 1a) and protein expression (Fig. 1b). In the same time of co-culture, from 14 to 24 h, the expression of PAI-1, only quantified in HUVECs, was unchanged by the co-culture and the direct contact with HBMSCs (Fig. 1c) whatever the time of co-culture.

SELF-ASSEMBLED NETWORK FORMATION IN CO-CULTURE IS DEPENDENT OF uPA

Interestingly, the formation of self-assembled network was partly disturbed after addition of specific uPA neutralizing antibody. In Figure 2, the left column shows the phase contrast images of co-cultured cells and the right column shows the live-dead assay of co-cultured cells with or without uPA neutralizing antibody. Neutralization of uPA successfully suppressed the self-assembled network formation (Fig. 2) without impairing the cell viability (Fig. 2). Addition of isotype control mouse antibody had no effect on the formation of self-assembled networks.

EXPRESSION AND ROLE OF MMP-2 IN SELF-ASSEMBLED NETWORK FORMATION

The production of MMP-2 in all cells after being cultured for 14 and 24 h were detected by western blot analysis (Fig. 3a) in cell lysates and by gelatin zymography in media (Fig. 3b). Regarding the protein production (Fig. 3a), the co-cultured cells expressed significantly more MMP-2 than mono-cultured cells at 14 and 24 h. Inhibition of uPA decreased the MMP-2 protein level in co-cultured cells at 14 h but showed no effect on mono-cultured cells. Interestingly, the neutralization of uPA increased the MMP-2 production in HBMSCs. The significant increase of MMP2 protein level in HBMSCs treated with uPA neutralizing antibody compared to untreated cells after 14 and 24 h of culture, is not confirmed at protease activity level of MMP-2 (Fig. 3b).

Obviously, MMP-2 in co-cultured cells showed a stronger activity than MMP-2 in mono-cultured cells (Fig. 3b). Neutralization of uPA

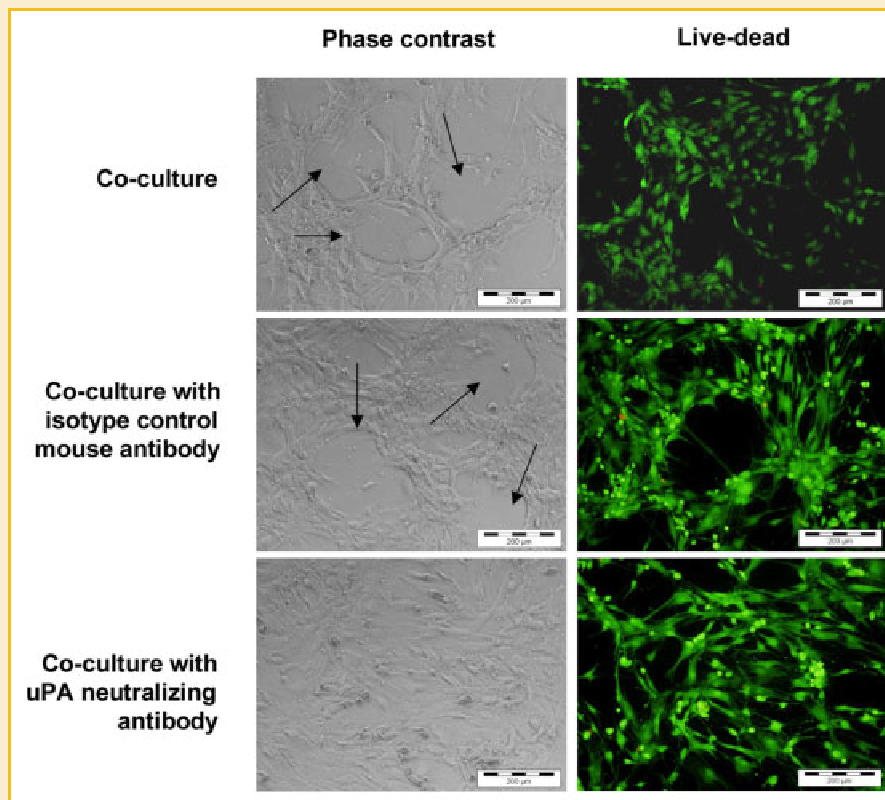

Fig. 2. Functional effects of uPA in co-culture. HBMSCs and HUVECs were co-cultured with neutralizing antibodies against uPA used at 15 μg/ml for 24 h or with the same concentration of the isotype control mouse antibody. Left panel: Phase contrast images of the co-cultures. Right panel: Live dead assays performed on each co-culture conditions. Briefly, after 24 h of treatment with the antibodies, cells were incubated with a LIVE/DEAD® Viability/Cytotoxicity Kit (calcein AM/ethidium homodimer-1; Molecular Probes, Invitrogen) for 30 min at room temperature. Arrows show the self-assembled network formation. Scare bars represent 200 μm analysis.

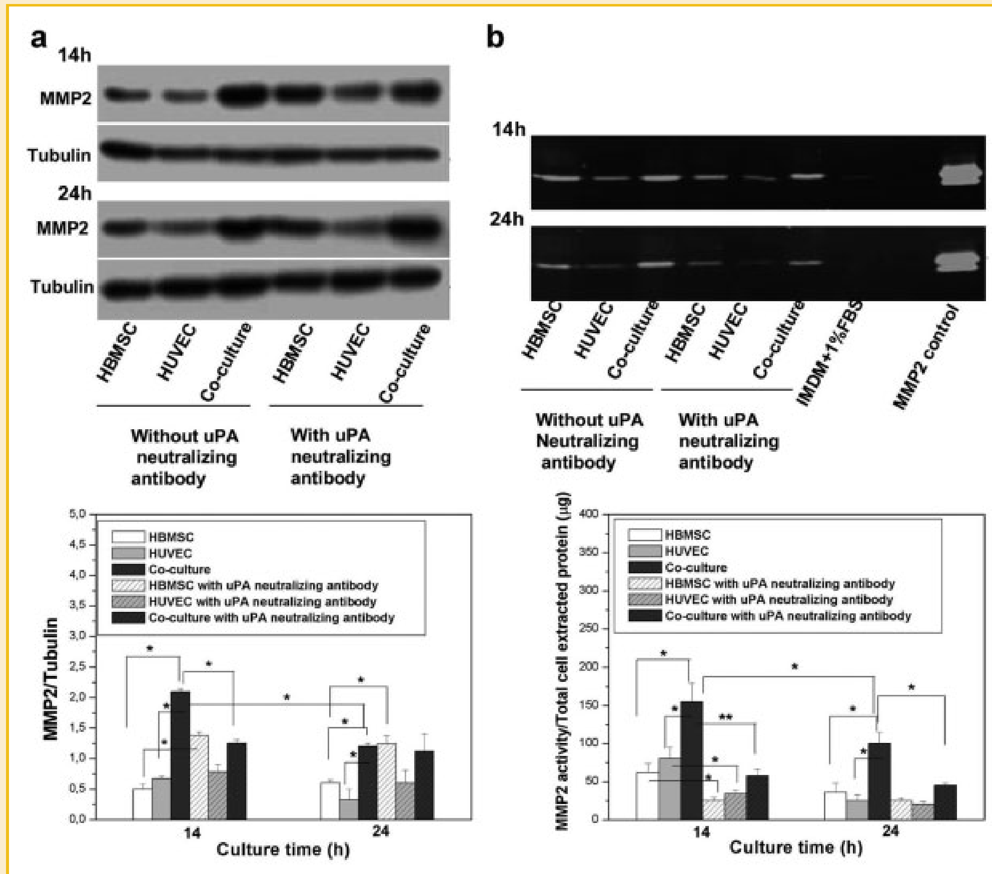

Fig. 3. Expression of MMP-2 in HBMSCs, HUVECs and HBMSCs-HUVECs co-culture. a: Expression of MMP-2 was analyzed and quantified by Western blot in cell lysates in mono- and co-cultured cells at 14 and 24 h as described in Materials and Methods section after treatment with the uPA neutralizing antibody or the corresponding control used at 15 µg/ml. HBMSCs and HUVECs were separately pre-treated with 10 µg/ml of the antibodies before co-culture assays. α-Tubulin was analyzed as loading control in western blot analysis. b: MMP-2 was detected and quantified by gelatin zymography in media in the different culture conditions (mono- and co-culture) in presence or not with antibodies. IMDM containing 1% (v/v) of FCS was used as negative control for checking a residual activity of MMP-2. (n = 3; $^{*}P \leq 0.05$; $^{**}P \leq 0.01$).

significantly weakened the activities of MMP-2 in all cells at 14 h but downregulated the activity of MMP-2 only in co-cultured cells at 24 h, although at this same time of culture the application of uPA neutralization antibody had no effect on the MMP-2 protein production in co-cultured cells and in HUVECs (Fig. 3a).

To determine the role of MMP-2 in self-assembled network formation, study has been performed by adding in medium 20 µM of MMP-2 inhibitor I, a potent inhibitor of MMP-2 [Berton et al., 2001]. The cytotoxicity of this inhibitor was previously performed on both cell types from 5 to 40 µM. No cytotoxicity was observed for both cell types (data not shown). As shown in Figure 4, the MMP-2 inhibitor I suppressed the self-assembled network formation in co-culture of HBMSCs and HUVECs but not the DMSO, used here as vehicle.

EXPRESSION AND REGULATION BY uPA OF TIMP2, A REGULATOR OF MMP-2

The expression of TIMP-2 was found by western blot in all cells at 14 and 24 h of culture, as shown in Figure 5, meanwhile HUVECs expressed highest amount of TIMP-2 compared to HBMSCs. The expression of TIMP-2 increases with time of culture in HBMSCs and in HUVECs but is not regulated in the co-cultured cells. Addition of uPA blocking antibody did not affect the expression of TIMP-2 in mono-cultured cells and co-cultured cells whatever the time of culture (14 and 24 h).

DISCUSSION

In this study we have shown that the uPA and MMP-2 were involved in the 2D self-assembled network observed when HUVECs and HBMSCs were co-cultured. Our previous studies, focused on the HUVECs-HBMSCs cell–cell interactions, have demonstrated that several molecules participated in the formation of self-assembled network, such as the VEGF, vascular endothelial cadherin, and neural-cadherin [Li et al., 2010, 2011]. However, the formation of self-assembled networks not only involves cell–cell interactions but also needs a dynamic cell–matrix interaction since the cells have to migrate to self-assemble.

Many literatures have studied the role of different types of MMPs in endothelial cell migration and capillary tube formation, but mainly performed in three dimensional (3D) culture systems, on various matrices, such as fibrin, collagen, or fibrin/collagen mixture

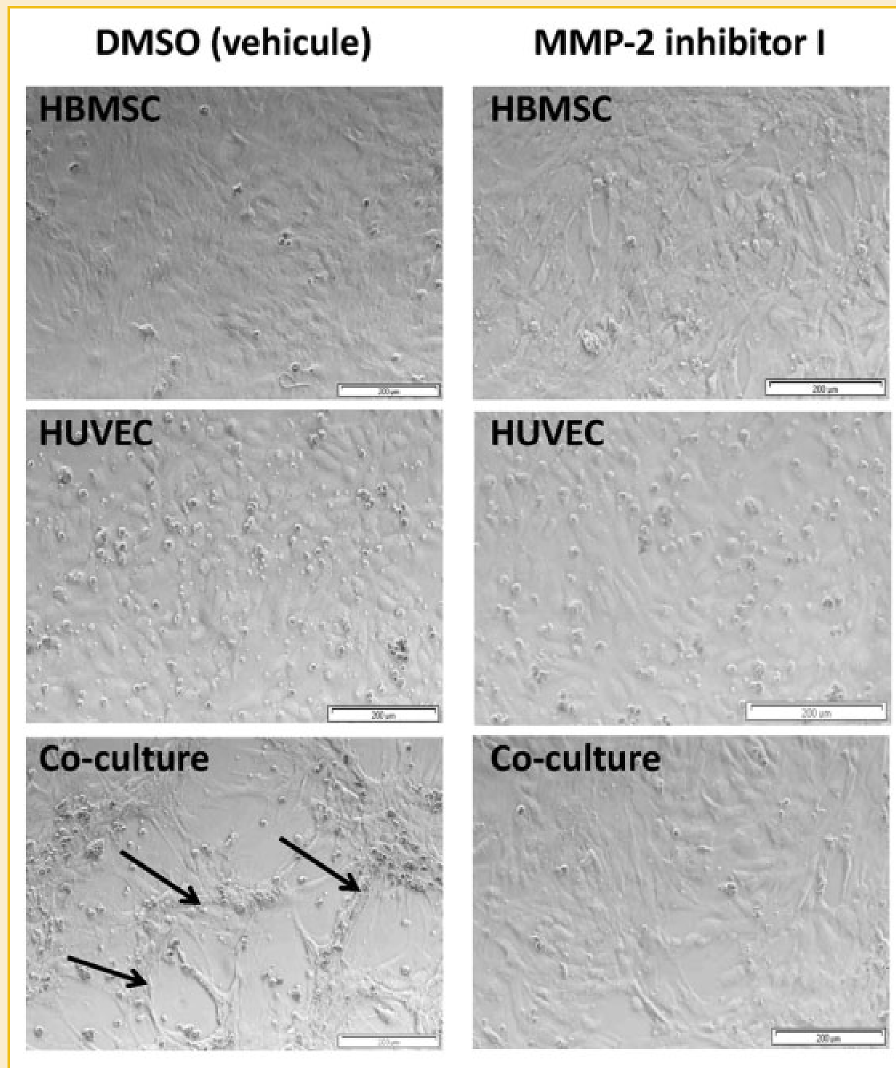

Fig. 4. Effect of MMP-2 inhibitor in the self-assembled network of HBMSCs-HUVECs co-culture. Phase contrast images of HBMSCs and HUVECs cultured alone or co-cultured in the absence (vehicle: DMSO) or presence of 20 μM of MMP2 inhibitor I after 14 h.

[Haas and Madri, 1999; Koike et al., 2002; Collen et al., 2003]. Among these studies, the MMP-2 and MMP-14 are the most studied for their role in angiogenesis and the significant upregulation of MMP-2 and MMP-14 during the migration and tube formation process of endothelial cells on 3D matrix. In addition, there is a complex relationship between the uPA/plasmin and MMPs system suggested by the facts that the uPA/plasmin system located at the cell membrane directly or indirectly activates a number of pro-MMPs and MMPs can also control plasminogen activation by uPA/uPA receptor/PAI-1 systems [Ugwu et al., 1998]. In this study performed using a 2D co-culture system, we found that the expressions of uPA, and MMP-2 were upregulated in the co-cultured cells, which indicated that these molecules may be required for the formation of self-assembled network.

Many studies on endothelial cells migration and angiogenesis of 3D matrix demonstrated the capability of MMP-14 of binding and activating latent MMP-2 [Sato et al., 1994; Cao et al., 1995; Pei and Weiss, 1996; Zucker et al., 1998; Haas and Madri, 1999]. However, in our study, active MMP-2 could not be detected in all cells with or without the uPA neutralizing antibody either by zymography or by western blot. The discordance between our results and the literature may result from the different culture model. Here, we used a specific 2D co-culture model while the studies in literature normally used a 3D mono-culture model. However, it has been reported that endothelial cells could constitutively express low levels latent MMP-2 on 2D collagen coated culture plates while the culture in a 3D collagen matrix increased the total amount of MMP-2 and expressed much higher active MMP-2 than that culture on 2D [Haas and Madri, 1999]. In the same context, our preliminary data obtained in 3D with our co-cultured cells seeded within a polymer scaffold, revealed the presence and an upregulation of the active form of MMP2 (data not shown).

A major mechanism for pro-MMP-2 activation involves its association with MMP-14 and TIMP-2 and the level of active MMP-

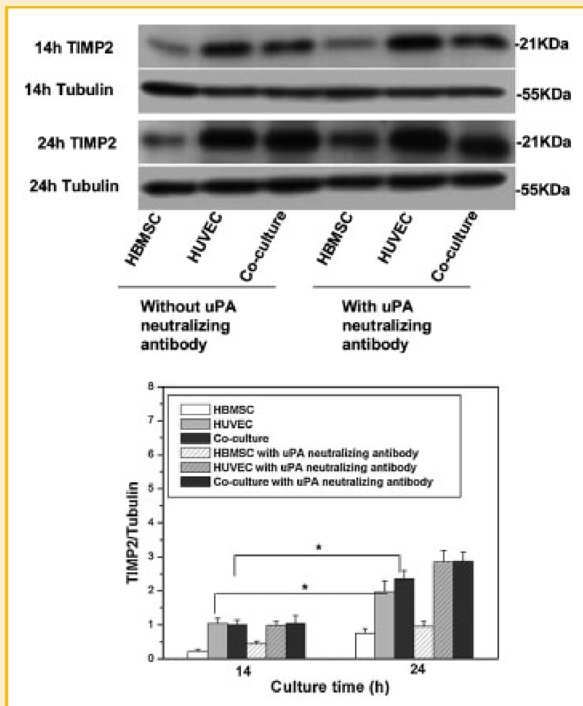

Fig. 5. Expression of TIMP-2 in HBMSCs, HUVECs and HBMSCs-HUVECs co-culture. Expression of TIMP-2 was detected by Western blot in cell lysates (top) and quantified (bottom) in mono- and co-cultured cells at 14 and 24 h as described in Materials and Methods section after treatment with the uPA neutralizing antibody or the corresponding control used at 15 μg/ml. HBMSCs and HUVECs were separately pre-treated with 10 μg/ml of the antibodies before co-culture assays. α-Tubulin was analyzed as loading control in western blot analysis (n = 3, $^*P \leq 0.05$).

2 depends on the concentrations of each component [Haas and Madri, 1999]. There is evidence that low concentrations of TIMP-2 stimulate the activation of MMP-2 while high levels of TIMP-2 inhibit MMP-14 mediated generation of active MMP-2 [Haas and Madri, 1999; Koike et al., 2002]. As we could easily detect TIMP-2 in the cell lysates by western blot, abundant TIMP-2 may be another factor for preventing the activation of MMP-2, though TIMP-2 was not regulated in our co-culture model. Since the absence of active soluble MMP-2 did not affect the self-assembled network formation, we may conclude that the active soluble MMP-2 was not critical for the formation of self-assembled network in this 2D co-culture model of HBMSCs and HUVECs. However, we cannot exclude the possibility that MMP-2 bound to the cell surface with MMP-14 may influence the cell migration and self-assembled network formation as the MMP-2 inhibitor I prevented the self assembled network. Because the MMP-2 inhibitor I is known to block the active form of MMP-2, and inhibit here the self assembled network formation, we could hypothesize that in our experimental conditions, the level of active form is too low to be detected by zymography, despite this method is more sensitive than western blot and similar to ELISA technique [Snoek-van Beurden and Von den Hoff, 2005].

TIMP-2, a regulator of MMP2, maintained a low expression in all cells, at 14 h. When the networks formed at 24 h, the expression of TIMP-2 increased, in co-cultured cells as well as in mono-culture of HUVECs. However, the neutralization of uPA did not affect the expression of TIMP-2 all the time. This suggests that TIMP-2 is not involved in the self-assembled network. A same result was obtained with MMP14 that is also upregulated in co-cultured cells but unaffected by uPA blocking antibody (data not shown).

The role of uPA/uPAR/plasmin in migration and self-assembled network formation have been extensively studied in mono-culture and have been commonly considered as proteolytic enzymes for degradation of matrix to initiate cell migration and angiogenesis [Carmeliet, 2000; Pepper, 2001]. In the present study, the role of PAI-1 in the regulation of uPA/uPAR was not evidenced by a regulation of its expression in co-culture. It has been widely recognized that uPA/uPAR/plasmin proteolytic process mostly depends on VEGF. Prager et al. [2004] demonstrated that VEGF initiated proteolysis by activation of pro-uPA via the VEGF-receptor 2 and uPAR redistributed to focal adhesions at the leading edge of endothelial cells in response to $VEGF_{165}$.

Thus, the proteolysis process could be initiated and the migration of cells could be stimulated as it has been shown previously, by using neutralizing antibodies against uPAR or VEGF that both affect cell migration in co-culture. However, these results also indicated that the partly disturbance of neutralization of uPA on the self-assembled network formation have no relationship with TIMP-2. In addition, the disturbance of network by uPA may involve MMP-2 since the neutralization of uPA downregulated the expression of MMP-2 although MMP-2 in its active form is not found.

In summary, we investigated the role of uPA, PAI-1, MMP-2, and TIMP-2 in the formation of self-assembled network in the 2D co-culture of HBMSCs and HUVECs. Our data evidenced that uPA played an important role in the network formation, while the process appears independent on TIMP-2 but involved MMP-2. The upregulation of uPA and uPAR of VEGF may increase their both expression. Taken together these new data improve our knowledge on cell to cell communication between these two cell types and on matrix remodeling that could explain the cell migration and formation of a tubular-like network observed in this 2D co-culture system.

ACKNOWLEDGMENTS

This work was supported by Institut National de la Sante et de la Recherche Medicale (INSERM), University Bordeaux Segalen, l'Agence Nationale de la Recherche (ANR 06-PNANO-022-06), and (ANR 07 TECSAN 011-01).

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