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Homocysteine Induces Metalloproteinase and Shedding of β-1 Integrin in Microvessel Endothelial Cells

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Abstract Although studies have suggested microvessel endothelial cells (MVEC) activation and induction of matrix metalloproteinases (MMPs) by homocysteine (Hcy), the transduction mechanism leading to endothelial activation was unclear. We hypothesized that Hcy induced metalloproteinase and altered the levels of integrin in MVEC. MVEC from mouse brain were isolated and characterized by CD-31 (PECAM-1) FITC labeling. The MVEC were activated with different doses (6–40 μM) of Hcy. The cultured-conditioned-medium was analyzed for MMP activity by gelatin gel-zymography. TIMP-1, -4, β-1 integrin, and a disintegrin and metalloproteinase-12 (ADAM-12) were quantified by Western blot analysis. We used MVEC in cell culture to study the effect of increasing concentrations of Hcy upon the secretion of various proteins into the culture medium. MMP-9, β-1 integrin, ADAM-12, and TIMP-1 were found in increased concentrations in the culture medium of Hcy-treated cells whereas TIMP-4 was decreased. We have shown that purified TIMP-4 blocked the increase of β-1 integrin shedding in Hcy-treated cells. Interestingly, our results suggest that TIMP-1 and TIMP-4 function antagonistically in Hcy-induced signaling pathways. J. Cell. Biochem. 93: 207–213, 2004. © 2004 Wiley-Liss, Inc.

Key words: MMP; TIMP; ADAM; homocysteine

Homocysteine (Hcy) is an important risk factor for the development of cardiovascular diseases and stroke [Hashimoto et al., 2003; Li et al., 2003]. Epidemiological studies have indicated that hyperhomocysteinemia (HHcy) is an essential contributory factor for the development of atherosclerotic lesions and arterial hypertension [Sutton-Tyrrell et al., 1997]. Numerous studies have demonstrated the role of Hcy in large conduct vessel endothelial cells in vivo and in vitro [Upchurch et al., 1997a,b; Lentz et al., 2000]. However, little is know about the role of Hcy in microvessel endothelial cells (MVEC). In particular, nothing is known about the role of Hcy in MVEC remodeling. Hcy induces endocardial endothelial dysfunction [Miller et al., 2000, 2002], and impairs microvascular endothelial function in vivo [Ungvari et al., 1999]. Hcy activates endothelial cells and increases matrix metalloproteinases (MMPs) activity [Hunt and Tyagi, 2002]. Tissue inhibitors of metalloproteinases (TIMPs) modulate MMP activity [Visse and Nagase, 2003]. The expression of TIMP is altered in the presence of elevated levels of Hcy and resulted in an abnormal MMP activity [Mujumdar et al., 2002]. In addition, Hcy induces the levels of TIMP-1 in mesangial cells [Yang and Zou, 2003]. The cell–cell and cell–matrix interactions are mediated by integrin receptors [Lampugnani et al., 1991]. A disintegrin and metalloproteinases (ADAMs) play significant role in cardiovascular development [Schwartzbauer and Robbins, 2002]. ADAM instigates disintegrin and shedding the cell surface receptor [Tyagi and Hoit, 2002]. Although Hcy induces cell adhesive molecules in leukocytes and platelets [Dardik et al., 2000], it is unclear whether Hcy induces integrins in MVEC. Furthermore, although inhibition of disintegrin and metalloproteinase ameliorates the Hcy-mediated constrictive vascular remodeling by endothelial cells [Mujumdar et al., 2002], it is unclear whether Hcy induces ADAM in MVEC. The hypothesis is that Hcy trans-

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duces MVEC by increasing metalloproteinase and shedding the integrin.

MATERIALS AND METHODS

Cell Isolation and Culture

Because brain MVEC can be isolated unambiguously to homogeneity, mouse brain endothelial cells were isolated from the grey matter of the cerebral cortex as described by Audus and Borchardt [1987]. In brief, the cerebral grey matter was repeatedly extracted from mouse brain and minced into pieces in minimal essential medium (MEM), pH 7.4. The tissue suspension was incubated with dispase (0.3% w/v) for 3 h at 37°C. The suspension was centrifuged and resuspended in 13% (w/v) dextran (average MW 70,000) and centrifuged at 5,800 × g for 10 min. The pellet was suspended in MEM, pH 7.4 and incubated with collagenase/dispase (1 mg/ml) for 2 h at 37°C. The suspension was then centrifuged 1,000 × g for 10 min and pellets were layered on 50% percoll gradient. The mixture was centrifuged 1,400 × g for 10 min. Centrifugation was repeated for 10 min, and resulted in cell suspension separated as two bands. The second band which was a thick red band below the white fatty band was recovered for MVEC. The recovered thick red band was washed by centrifugation for 10 min at 1,000 × g and pellet was resuspended in MEM pH 7.4. The cells were directly plated in the culture medium containing (50% v/v) MEM, 50% (w/v) F-12 nutrient mixture (Ham), 11% (v/v) plasma derived equine serum, 50 mg/ml heparin, 100 × g streptomycin, and 100 × g penicillin-G. Cells were then incubated at 37°C with 5% (v/v) CO_2. After formation of confluent monolayers (10–14 days), the experiments were performed.

Characterization of Endothelial Cell Primary Cultures

To verify purity of the isolated MVEC, the cells were stained with endothelial cell specific marker, CD-31, platelet endothelial cell adhesive molecule-1 (PECAM-1) as described by Strelow et al. [1998]. In brief, the cell monolayers were fixed at room temperature for 10 min in 95% (v/v) ethanol, 5% (v/v) glacial acetic acid. The cell monolayers were then permeabilized for 5 min at room temperature with 0.3% (v/v) Triton X-100 in PBS and blocked for 20 min at 37°C with 20% (v/v) horse serum in PBS. Cells were then incubated with 1:100 dilution of mouse anti-endothelial cell-fluorescein conjugated monoclonal antibody (Chemicon, Temecula, CA) for 3 h. The cells incubated without mouse anti-endothelial cell CD-31-fluorescein conjugated monoclonal antibody (Chemicon) were used as a negative control. The cells were viewed and images were captured with Leica inverted fluorescent microscope attached to a digital camera.

Treatment of MVEC With Hcy and Analysis of MMP Activity by Zymography

The confluent MVEC cells were washed with PBS, pH 7.4 and cultured in serum free media for 24 h at 37°C. The cells were incubated with Hcy at concentrations 6, 12, 20, 40 μM for 24 h. The culture medium was dialyzed in PBS (pH 7.4) overnight and total protein was concentrated with static concentrator Amicon B-15 (Millipore, Beverly, MA). Total protein was estimated by BIO-RAD dye binding assay. Metalloproteinase activity was performed by gelatin gel zymography as described [Tyagi et al., 1993]. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) containing 1% gelatin was used as impregnated substrate for metalloproteinase. Identical amounts of total protein were loaded onto each lane. Purified MMP-9 was used as standard. The gels were stained by coomassie blue and lytic activity was scanned by ODYSSEY LI-COR densitometer.

Western Blot Analysis of TIMP-1, -4, ADAM-12, and β-1 Integrin

Extracellular culture medium was concentrated in static concentrator Minicon B-15 (Millipore). Identical amounts of total protein were loaded onto 10% SDS–PAGE under reducing conditions. The protein was transferred on to nitro-cellulose membrane by a Bio-Rad transblotter. The TIMP-4 was blotted with anti-TIMP-4 antibody (1:200, Sigma Chemical Co., St. Louis, MO). Purified TIMP-4 was used as standard. ADAM was blotted with an anti-ADAM-12 antibody (1:200, Chemicon). The secondary antibody, anti–rabbit IgG alkaline phosphatase conjugate (1:200, Sigma) was used for the analysis of TIMP-4 and ADAM. TIMP-1 was measured by anti-TIMP-1 monoclonal mouse antibody (1:200, Calbiochem) and secondary antibody anti-mouse alkaline phosphatase conjugate (1:200, Sigma). The β-1 integrin in the extracellular medium was measured by anti-β-1 integrin antibody (1:200, Chemicon

Corp.). To determine the levels of actin in the cell extract, beta-actin antibody (Sigma, 1:500 dilution) was used in Western blots. The secondary antibody, anti-rabbit IgG alkaline phosphatase conjugate (1:200, Sigma) was used. The bands in Western blots were scanned by ODYSSEY LI-COR densitometer.

Co-incubation of MVEC With Hcy Plus TIMP-4

To determine whether TIMP-4 ameliorated β-1 shedding, TIMP-4 was isolated and purified from mouse hearts as described [Tummalapalli et al., 2001]. MVEC were co-incubated with 20 μM Hcy plus 10 μM TIMP-4. The medium was analyzed for β-1 integrin by Western blot analysis.

Statistical Analysis

The results are mean ± SEM from six experiments. A paired Student *t*-test was used when comparing the data in the presence and absence of Hcy.

RESULTS

MVEC

Primary MVEC cultures were confluent in 10–14 days in 10% (v/v) FCS containing MEM. The light transmission micrograph of a MVEC mono-layer is shown in Figure 1A. The results suggest that the MVEC grow closely apposed to each other in a monolayer. To characterize MVEC, immuno-labeling was performed using anti-CD-31 (PECAM-1, an endothelial cell marker). The MVEC were positive for CD-31 antigen (Fig. 1B). The results suggested that 95% MVEC were positive for CD-31.

MMPs and TIMPs

Hcy increased MMP-9 activity at 92 kDa in dose-dependent manner in MVEC (Fig. 2A,B). In control experiments, incubation of cell medium with various concentrations of Hcy did not activated MMPs. Lane 1 in Figure 2 was medium from cells without Hcy treatment, suggesting that MMP-9 was not presented constitutively, however, it was induced by Hcy. Under these conditions we observed most of the MMP-9 in active form. There were differential expression of TIMP-1 and -4 in Hcy-activated MVEC, measured by Western blot analysis (Fig. 3A). The levels of TIMP-1 were increased with increasing concentrations of Hcy. The levels of TIMP-4 were decreased with increasing concentrations of Hcy ($P < 0.01$, when compared

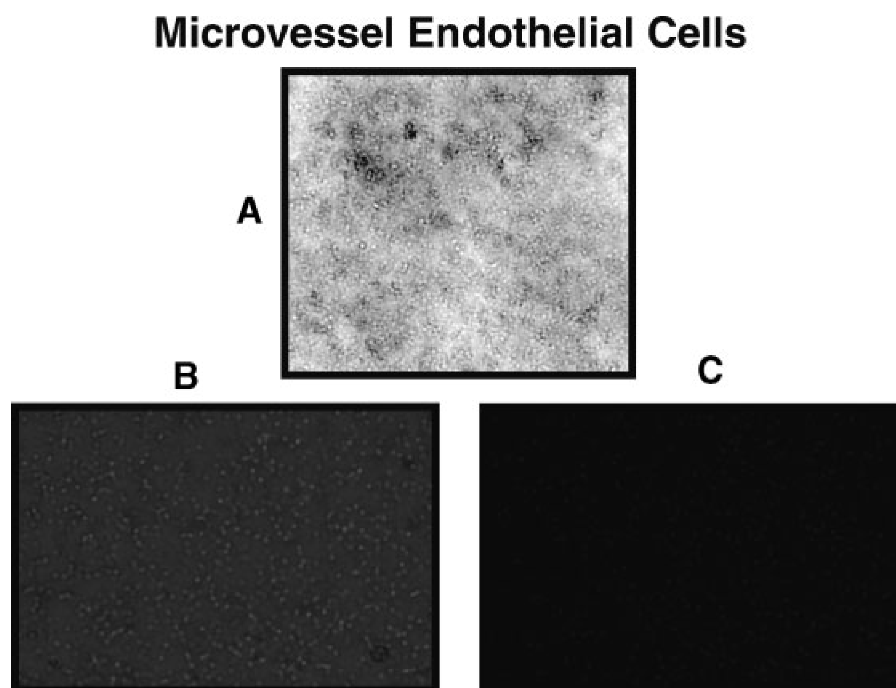

Fig. 1. **A**: Light transmission micrograph of confluent monolayer microvessel endothelial cells (MVEC) isolated from mouse brain. The cells were grown to confluence in 10% FCS. The image was recorded at 10× magnification. **B**: Characterization of MVEC by immuno-labeling using CD-31 (an endothelial cell specific antigen). Cells were grown on cover-slips. The permeabilized cells were labeled with anti-CD-31-fluorescein. The blue fluorescence was recorded by a digital microscope. **C**: Control without primary antibody. The immuno-labeled micrographs were also recorded at 10× magnification.

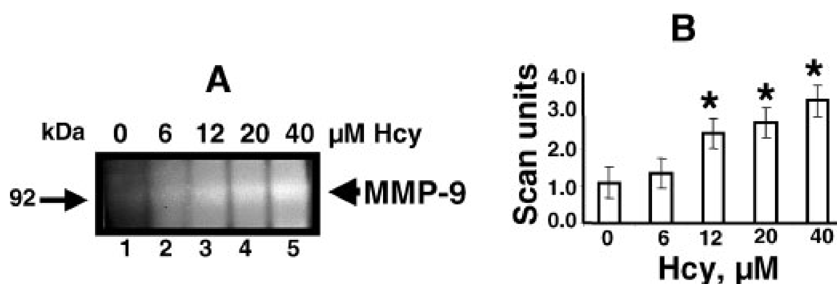

Fig. 2. Zymographic analysis of MMP activity. **A**: Confluent cells were incubated with different dosages of Hcy in serum-free MEM for 24 h. The MMP activity in the medium was measured by 1% gelatin gel zymography. **Lane 1**, cells without Hcy; **lane 2**, cells with 6 μM Hcy; **lane 3**, cells with 12 μM Hcy; **lane 4**, cells with 20 μM Hcy; and **lane 5**, cells treated with 40 μM Hcy. Molecular weight is reported on the left. **B**: Accumulative histographic presentation of the scanned data obtained from different samples under identical amounts of total protein loading onto the gels. The bars represent mean ± SEM from six experiments. *, represents $P < 0.01$ when compared with experiments without Hcy.

with levels without Hcy). Figure 3 also showed a decrease in TIMP-4 only from 0 to 12 μM Hcy, whereas a small increase was seen towards 40 μM Hcy.

ADAM-12 and β-1 Integrin

The levels of ADAM-12 and β-1 integrin were increased in the medium of MVEC cultured with increasing concentrations of Hcy, measured by Western blot analysis (Fig. 4A). There was 1.5-fold increase in the levels of ADAM-12 in the presence of 40 μM Hcy when compared with the levels without Hcy (Fig. 4B). Figure 4 also showed an increase in β-1 integrin only from 0 to 6 μM Hcy, whereas a small decrease was seen towards 40 μM Hcy.

TIMP-4 Ameliorates Hcy-Mediated β-1 Release Into the Medium

To determine whether the TIMP-4 decreased the β-1 integrin shedding, MVEC were co-incubated with Hcy plusTIMP-4. The results suggested that addition of TIMP-4 decreased the release of β-1 into the culture medium (Fig. 5), indicating that Hcy activated the MVEC by releasing the β-1 integrin through activation of metalloproteinases, and TIMP-4 decreased the β-1 release. TIMP-1 did not decrease the levels of beta-1 in the medium.

DISCUSSION

There was Hcy-dose dependent increase in MMP-9 activity in micro vessel endothelial cells

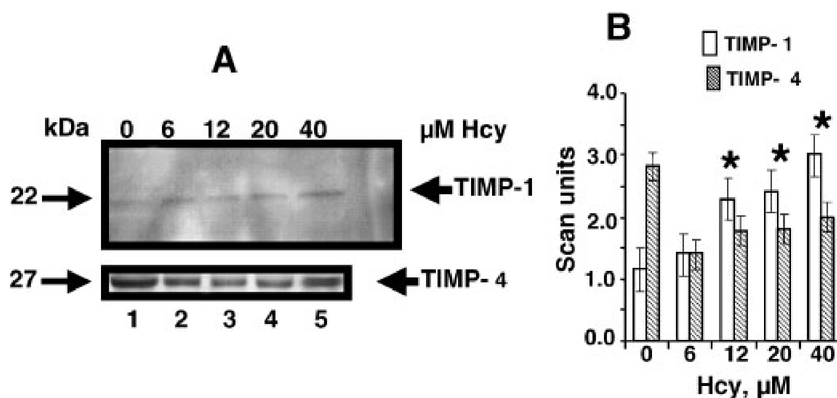

Fig. 3. Western blot analysis of TIMP-1 and -4. **A**: Confluent cells were incubated with different dosages of Hcy in serum-free MEM for 24 h. The identical amounts of total protein were loaded onto each lane in SDS–PAGE. **Lane 1**, cells without Hcy; **lane 2**, cells with 6 μM Hcy; **lane 3**, cells with 12 μM Hcy; **lane 4**, cells with 20 μM Hcy; and **lane 5**, cells treated with 40 μM Hcy. The gels were transferred to nitrocellulose membranes and blotted for TIMP-1 and -4. Molecular weights are reported on the left. **B**: Accumulative histographic scanned data obtained from different samples under identical amounts of total protein loading onto the each lane. The bars represent mean ± SEM from six experiments. *, represents $P < 0.01$ when compared with experiments without Hcy. Because the levels of TIMP-1 and -4 are measured in the secreted cell medium no actin standard is used. However, identical amount of total (10 μg) protein was loaded onto each lane.

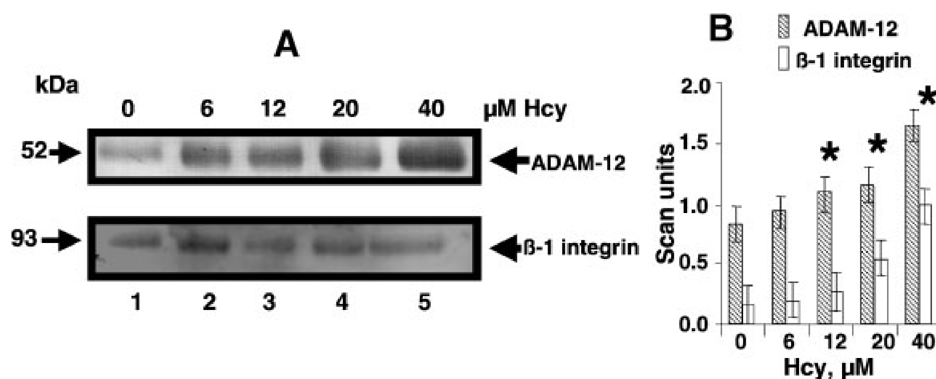

Fig. 4. Western blot analysis of ADAM-12 and β-1 integrin. **A**: Confluent cells were incubated with different dosages of Hcy in serum-free MEM for 24 h. The identical amounts of total protein were loaded onto each lane in SDS–PAGE. **Lane 1**, cells without Hcy; **lane 2**, cells with 6 μM Hcy; **lane 3**, cells with 12 μM Hcy; **lane 4**, cells with 20 μM Hcy; and **lane 5**, cells treated with 40 μM Hcy. The gels were transferred to nitrocellulose membranes and blotted with ADAM-12 and β-1 integrin antibodies. Molecular weights are reported on the left. **B**: Accumulative histographic scanned data obtained from different samples under identical amounts of total protein loading onto the gels. The bars represent mean ± SEM from six experiments. *, represents $P < 0.01$ when compared with experiments without Hcy. Although the band in lane 2 is intense, whereas the band in lane 5 apparently is weak, the accumulative data suggest an increase in overall protein levels of beta-1 when compared with untreated cells.

(Fig. 2A,B). MMP-9 activity increased in mononuclear cells isolated from hyperhomocysteinaemic subjects [Holven et al., 2003]. Horstmann et al. [2003] have reported that MMP-2 and -9 increased during ischemic stroke. Increased micro-vascular permeability instigates interstitial edema, interstitial pressure, and stroke [Laine, 1988]. This may suggest a role of Hcy in mediation of microvessel permeability by increasing metalloproteinase and increasing interstitial edema, fibrosis, and pressure. There are four TIMPs [Visse and Nagase, 2003]. TIMP-2 is constitutively expressed. TIMP-3 is apoptotic to vascular cells. TIMP-1 is induced by Hcy [Yang and Zou, 2003], and TIMP-4 is decreased by Hcy. Therefore, in this study to determine whether the Hcy-mediated decreased in TIMP-4 contributes to integrin shedding, we measured TIMP-4. We observed decreased levels of TIMP-4 in MVEC in the presence of elevated levels of Hcy (Fig. 3). In a previous study, we have shown that the levels of TIMP-4 were increased in compensatory phase and decreased in decompensatory heart failure [Mujumdar and Tyagi, 1999]. This may suggest decompensation by Hcy. Hcy has been shown to increase TIMP-1 in rat mesangial cells. An increase in TIMP-1 level is observed in Hcy-induced oxidative stress [Yang and Zou, 2003]. We observed increased expression of TIMP-1 by MVEC by increasing Hcy concentrations. It was reported that increased levels of

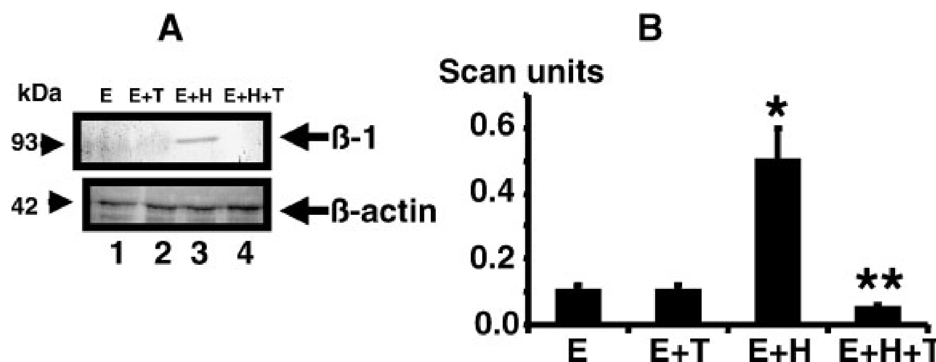

Fig. 5. TIMP-4 inhibits β1-integrin release in MVEC-culture medium. **A**: 10^6 MVEC cultured alone (E), with 10 μM TIMP-4 (T), with 20 μM Hcy (H), and with Hcy and TIMP-4 (E + H + T). After 24 h cell medium was collected and analyzed for β1-integrin by Western blots. The corresponding actin bands from cell homogenates are shown. **B**: The bands in Western blots were scanned and normalized with actin. Each bar represents mean ± SEM from six different experiments. *$P < 0.01$ compared with E alone. **$P < 0.01$ compared with E + H.

TIMP-1 were associated with enhanced MMP-9 activity in amyotrophic lateral sclerosis patients [Beuche et al., 2000], as well as TIMP-1 elevated levels were associated with increased MMP-9 activity in synovial fluid of patients with bacterial meningitis [Leppert et al., 2000]. Young et al. reported increase in TIMP-1 and TIMP-3 expression in brain arteriovenous maltransformations which causes stroke in young adults [Hashimoto et al., 2003]. Although MMP-9 could be induced at the gene levels, here we report that release of MMP-9 in the medium is correlated with the up-regulation of ADAM-12. In addition, Hcy induces ADAM (a metalloprotease [Loechel et al., 1998]) during HHcy in smooth muscle cells [Mujumdar et al., 2002]. Our results suggest differential role of TIMP-1 and -4 in regulation of MMP-9 and ADAM-12, respectively, by Hcy.

The cell–extracellular matrix (ECM) adhesion and cell–cell contacts in endothelial cells are mediated by integral membrane glycoproteins. The integrins are composed of non-covalently linked and denominated subunits chains [Lampugnani et al., 1991]. Previous studies [Frisch and Francis, 1994; Re et al., 1994; Basbaum and Werb, 1996] have reported that disruption of interring mediated survival signals either by depriving cells of ECM or destroying their ECM microenvironment by abnormal expression of MMP leads to apoptosis. An increase in MMP-9 activity disengages the integrins from the cell membrane [Levkau et al., 2002], leading to disrupt matrix–cell interaction causing cell death. The increase in β-1 integrin in the extracellular medium indicates shedding of cell adhesive molecules and signal transduction in MVEC (Fig. 4). The data on the incubation of MVEC with TIMP-4 suggests that β-1 integrin shedding is due, in part, to the decreased levels of TIMP-4 by Hcy (Fig. 5).

In conclusion, we have demonstrated that elevated levels of Hcy induce metalloproteinases that could shed the integrin molecules. The differential regulation of TIMP-1 and -4 may tip the balance to shedding and mitogenic activity in MVEC, as TIMP-1 induces endothelial cell proliferation [Tyagi et al., 1996]. Although the results suggest in vitro role of Hcy in the integrin shedding, it does not depict the role in vivo. The experiments to demonstrate the role of Hcy in integrin shedding in vivo are in progress.

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