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Lysophosphatidic acid stimulates thrombomodulin lectin-like domain shedding in human endothelial cells

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Abstract

Thrombomodulin (TM) is an anticoagulant glycoprotein highly expressed on endothelial cell surfaces. Increased levels of soluble TM in circulation have been widely accepted as an indicator of endothelial damage or dysfunction. Previous studies indicated that various proinflammatory factors stimulate TM shedding in various cell types such as smooth muscle cells and epithelial cells. Lysophosphatidic acid (LPA) is a bioactive lipid mediator present in biological fluids during endothelial damage or injury. In the present study, we first observed that LPA triggered TM shedding in human umbilical vein endothelial cells (HUVECs). By Cyflow analysis, we showed that the LPA-induced accessibility of antibodies to the endothelial growth factor (EGF)-like domain of TM is independent of matrix metalloproteinases (MMPs), while LPA-induced TM lectin-like domain shedding is MMP-dependent. Furthermore, a stable cell line expressing TM without its lectin-like domain exhibited a higher cell proliferation rate than a stable cell line expressing full-length TM. These results imply that LPA induces TM lectin-like domain shedding, which might contribute to the exposure of its EGF-like domain for EGF receptor (EGFR) binding, thereby stimulating subsequent cell proliferation. Based on our findings, we propose a novel mechanism for the exposure of TM EGF-like domain, which possibly mediates LPA-induced EGFR transactivation.

Keywords: Thrombomodulin; Lysophosphatidic acid; Endothelial cells

Thrombomodulin (TM) is a transmembrane protein highly expressed on endothelial cell surfaces and is known to act as an anticoagulant factor [1]. It consists of a single polypeptide chain with five distinct domains: an amino-terminal region homologous to that of C-type lectins, a 6-tandem repeated epidermal growth factor (EGF)-like domain, a serine/threonine-rich sequence, a transmembrane domain, and a cytoplasmic tail [2]. Complex cellular functions of TM have recently been revealed. We demonstrated that the lectin-like domain of TM serves an essential role in cell-to-cell adhesion [3]. In addition, the EGF-like domain of TM has also been suggested as being a novel angiogenic factor [4]. Human recombinant TM containing EGF-like domains plus a serine/threonine-rich domain (TMD23) significantly enhanced the angiogenic response *in vitro* and *in vivo*, suggesting that TM may play a role in regulating new vessel formation [4].

Raised levels of soluble TM can be detected during endothelial cell damages, and are widely accepted as an indicator of endothelial damage or dysfunction [5]. Various symptoms correlated to various endothelial injuries such as atrial fibrillation [6], burn injury [7], and acute lung injury [8] result in elevated levels of soluble TM. TM shed from

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endothelial cell membranes widely exists in plasma, urine, and synovial fluid [5,9]. Soluble TM is detected in a variety of molecular weights, implying that membrane-associated TM may be cleaved into various-sized fragments by proteases [9]. Various proteases including rhomboids, elastase, proteinase 3, and cathepsin G have been reported to affect TM shedding [5,10]. However, the mechanism triggering protease-mediated TM shedding remains unclear.

A recent study demonstrated that proinflammatory factors, including tumor necrosis factor (TNF)-α, interleukin-1 (IL-1)β, and interferon (IFN)-γ enhance TM shedding from alveolar epithelial cells. Moreover, these enhancing effects were profoundly inhibited by the matrix metalloproteinase (MMP) inhibitor, GM 6001 [11]. These results suggested that proinflammatory factor-induced TM shedding is mediated through an MMP-dependent proteolysis process. Lysophosphatidic acid (LPA) is a bioactive lipid mediator present in human plasma, biological fluids, and tissues during endothelial injury [12]. LPA has been reported to be a proinflammatory factor, which regulates various endothelial cell functions [13]. However, the role of LPA in TM shedding in human endothelial cells has not been investigated. In the present study, we demonstrate that in human endothelial cells, LPA induces TM lectin-like domain shedding through MMPs. In addition, LPA stimulates the accessibility of TM EGF-like domain to the binding of antibodies in an MMP-independent manner. Moreover, we confirm that the fragment of TM shed by LPA treatment is the lectin-like domain of TM. Furthermore, a stable cell line expressing TM with deletion of its lectin-like domain exhibited a higher cell proliferation rate than a stable cell line expressing full-length TM. This is the first study indicating that LPA might be a regulator of TM shedding, thus modulating endothelial cell proliferation.

Materials

Reagents and antibodies. LPA and Ki16425 were purchased from Sigma–Aldrich (St. Louis, MO). The monoclonal anti-lectin-like domain of the TM antibody (clone D-3) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). A monoclonal mouse antibody to the EGF5–EGF6 domain of TM immunoglobulin G_1 antibody was purchased from American Diagnostica (Greenwich, CT). Anti-green fluorescence protein (GFP) mouse monoclonal antibody was purchased from Becton Dickinson (San Jose, CA). Dulbecco's modified Eagle's medium (DMEM) and trypsin were purchased from Gibco-BRL (Grand Island, NY). The rabbit anti-human factor VIII antibody and GM 6001 were purchased from Chemicon (Temecula, CA). AG1478 was purchased from Calbiochem (La Jolla, CA).

Cell culture. HUVECs were isolated from fresh umbilical cords (IRB: 9561709146, National Taiwan University Hospital) with previously described method [13]. HEK293 cells (ATCC CRL-1573) were maintained in DMEM supplemented with 10% FBS. HEK293 cells grown to 40–60% confluence were transfected with constructs encoding a GFP-tagged lectin-like domain-deleted fragment of TM, or full-length TM as described previously [3], by Lipofectin reagent (Invitrogen, Carlsbad, CA) to generate cell lines named TMGΔL and TMG, respectively. After transfection, cells were maintained in DMEM supplemented with 400 μg/ml neomycin (Invitrogen). Clonal expression was examined initially by fluorescence microscopy, and clones for further study were selected and expanded.

Immunoprecipitation. Immunoprecipitation was preformed as described previously [14]. Immunoprecipitated products were mixed with reducing sample buffer and processed for Western blotting.

Western blotting. SDS–PAGE was performed with previously described procedure [3] using a 10% separating gel under reduced conditions.

Immunofluorescence staining. Immunofluorescence staining was preformed as described previously [4]. Anti-human lectin-like domain of the TM antibody or anti-human factor VIII antibody was applied to the samples. After three PBS washes, cells were incubated for 1 h at room temperature with fluorescein isothiocyanate (FITC)- or Cy3-conjugated secondary antibodies (DAKO, Carpinteria, CA). Cell nuclei were counterstained with 4′,6′-diamidino-2-phenylindole (Sigma–Aldrich). Glass coverslips were washed, mounted, and examined using a confocal microscope (Leica Microsystems, Bensheim, Germany).

Cyflow analysis. Sub-confluent HUVECs were starved for 16 h and treated as indicated. Suspensions of 10^6 cells in 200 μl PBS with 0.1% fatty acid-free BSA received 2 μl of the anti-lectin-like domain or anti-EGF5–EGF6 domain of the TM antibody and were then incubated for 1 h at 4 °C. Antibody-isolated cells were washed with PBS three times and incubated with goat FITC-conjugated anti-mouse IgG (Pierce Chemical, Rockford, IL) for 2 h at 4 °C. Fluorescent signals were determined by CyFlow® SL (Partec, Münster, Germany) and analyzed by WinMDI version 2.8 software.

Statistical analysis. Significant differences between groups were tested using ANOVA followed by Duncan's new multiple-range test (StatView; Abacus Concept, Berkeley, CA). Each experiment was repeated at least 3 times. A value of $p < 0.05$ was considered statistically significant.

Results

LPA stimulates TM lectin-like domain shedding in human endothelial cells

To verify if LPA stimulates TM lectin-like domain shedding in human endothelial cells, HUVECs were treated with 5 μM LPA for various time durations as indicated (Fig. 1A). Treated cells were fixed and stained with an antibody specific to the TM lectin-like domain and subjected to Cyflow and immunofluorescence analyses. In the Cyflow analysis, levels of the cell-surface TM lectin-like domain in HUVECs were reduced after LPA treatment for 5 min. The reduced effects peaked at 10 min, and then were sustained for at least 60 min (Fig. 1A). In the immunofluorescence analysis, 5 μM of LPA treatment for 30 min profoundly suppressed TM lectin-like domain levels located at the surface of HUVECs (Fig. 1B). The expression patterns of factor VIII, an endothelial cell marker, did not significantly differ in untreated and LPA-treated samples. In addition, we also observed that LPA induced TM lectin-like domain shedding in HUVECs in a concentration-dependent manner (Fig. 1C). These results suggest that LPA induces TM lectin-like domain shedding in HUVECs.

LPA-stimulated TM lectin-like domain shedding is MMP-dependent, while accessibility of the TM EGF-like domain to the antibody is independent of MMPs in human endothelial cells

Since LAP stimulates TM lectin-like domain shedding in human endothelial cells (Fig. 1), we further determined if MMPs, which are proteases that regulate the wound

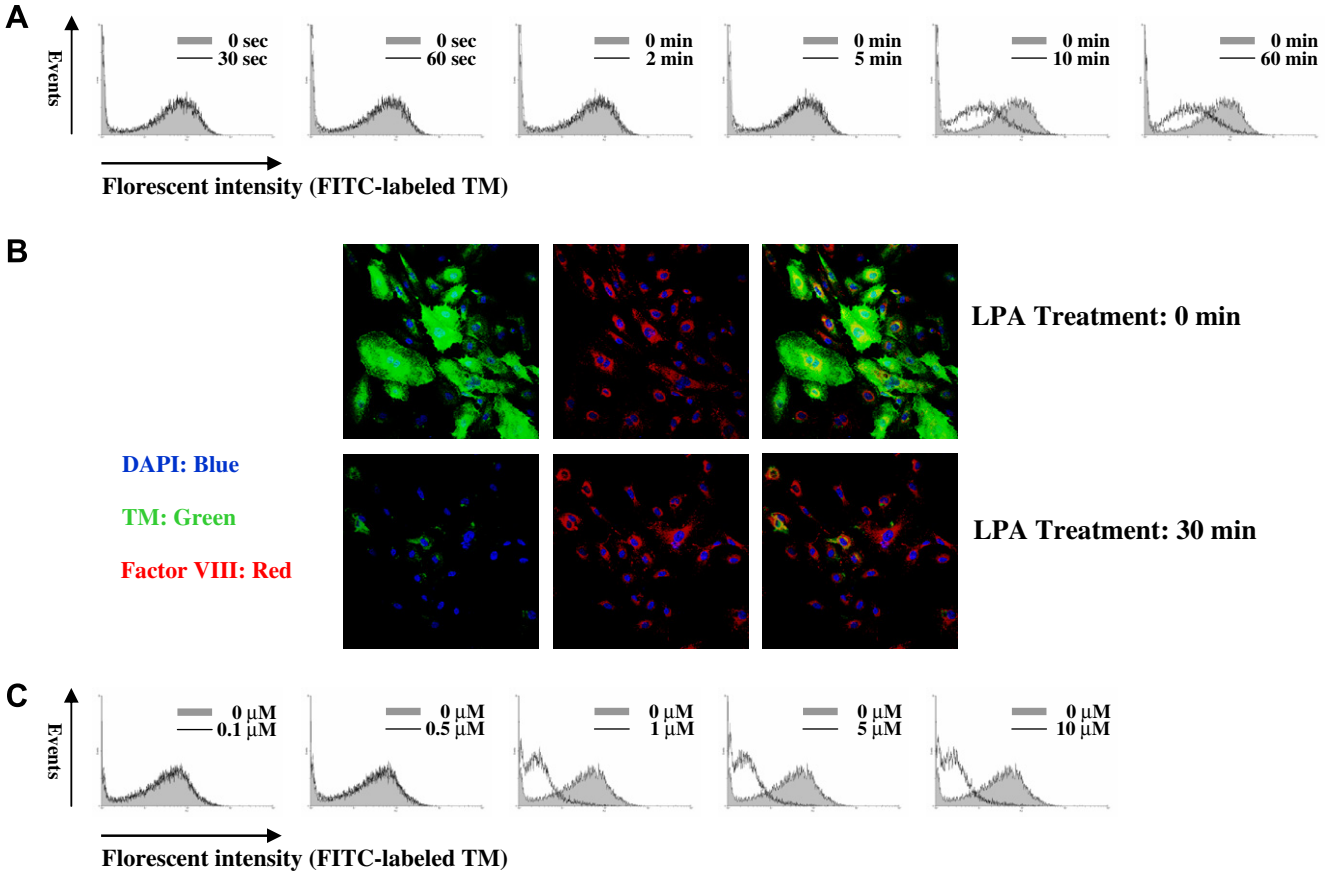

Fig. 1. Effects of LPA on the shedding of lectin-like domain of TM in HUVECs. (A) HUVECs were treated with LPA (5 μM) at various times as indicated. Cells were dissociated by trypsinization and incubated with the anti-lectin-like domain of a TM antibody for 1 h at 4 °C, then treated with a FITC-conjugated secondary antibody and analyzed by Cyflow analysis. (B) HUVECs were treated with LPA (5 μM) for 0 or 30 min. Cells were fixed and stained with the anti-lectin-like domain of the TM antibody and a rabbit anti-human factor VIII antibody, then treated with FITC- and Cy3-conjugated secondary antibodies and analyzed by confocal microscopy. (C) HUVECs were treated with LPA for 30 min at various concentrations as indicated. Cells were dissociated by trypsinization and subjected to Cyflow analysis.

healing process during endothelial cell damage, mediate LPA-stimulated TM lectin-like domain shedding in human endothelial cells. HUVECs were treated with 5 μM of LPA for 30 min. Treated cells were stained with antibodies against the lectin-like or EGF-like domain of TM and subjected to Cyflow analysis. LPA treatments for 30 min reduced the total amount of TM lectin-like domain levels on the cell surface as demonstrated by the anti-lectin-like domain antibody. In addition, this deletion effect was blocked by pretreatment with GM 6001 (Fig. 2A). On the contrary, detectable levels of the TM EGF-like domain on the cell surface increased after LPA treatment for 30 min. However, accessibility of the LPA-induced TM EGF-like domain for antibody binding was not blocked by GM 6001 (Fig. 2A). Furthermore, we showed that LPA-induced TM lectin-like domain shedding in HUVECs could be clocked by Ki16425, an antagonist of LPA_1 and LPA_3 (Fig. 2B). Our results indicate that LPA-induced TM lectin-like domain shedding in human endothelial cells is MMP-dependent. Moreover, LPA-induced accessibility of the TM EGF-like domain to antibody binding occurred in an MMP-independent manner. Our results imply that upon LPA treatment, the EGF-like domain of TM is accessible to antibody binding. Subsequently, the lectin-like domain is removed in an MMP-dependent manner.

LPA-induced TM lectin-like domain cleavage in TM-transfected cells

Since LPA stimulated TM lectin-like domain shedding in human endothelial cells in an MMP-dependent manner (Fig. 2), we further investigated whether the TM fragment shed upon LPA treatment is the lectin-like domain. HEK293 cells, which express low levels of endogenous TM, were transfected to express full-length TM (TMG) or the lectin-like domain-deleted fragment of TM (TMGΔL). The generated stable cell lines were treated with 5 μM LPA for 30 min, and cell lysates were immunoprecipitated with the anti-GFP antibody and subjected to immunoblotting with the anti-GFP antibody. LPA treatment generated a 94-kDa band in TMG cells which corresponds to the size of the deleted lectin domain, TMG, as TMGΔL. Re-probing with an antibody against the TM lectin-like domain on the same blot did not reveal this band

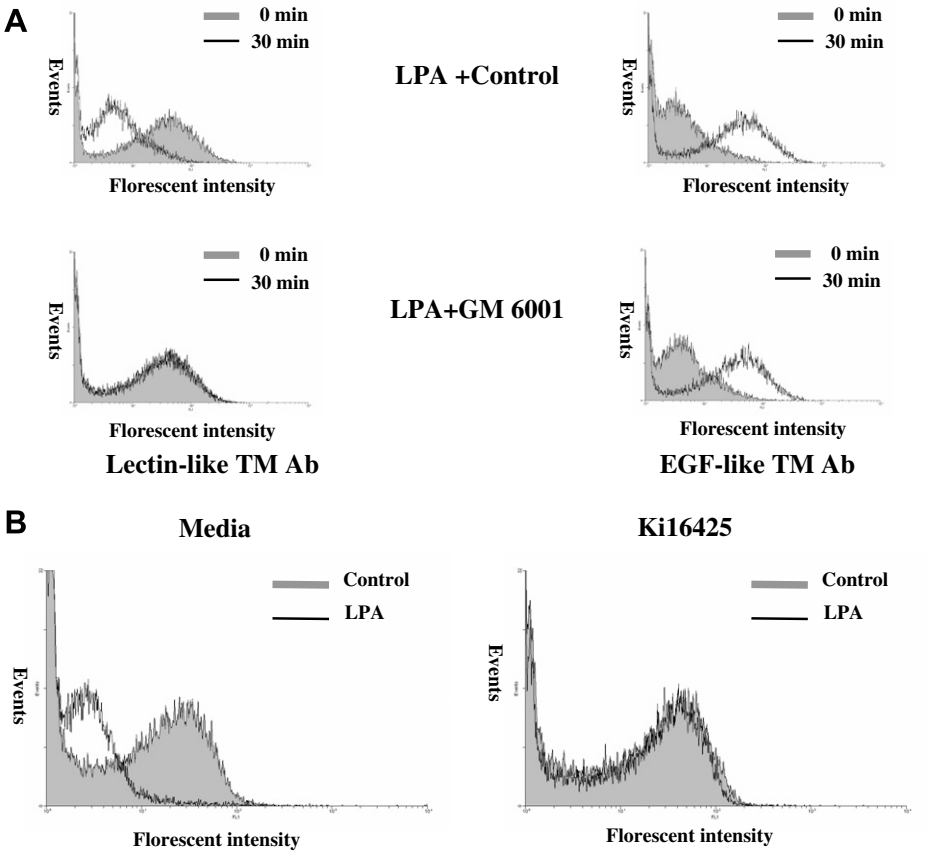

Fig. 2. LPA-induced shedding of the lectin-like domain of TM in HUVECs is MMP-dependent. (A) HUVECs were pre-incubated with GM 6001 (10 μM) and treated with LPA (5 μM) for 30 min. Cells were dissociated by trypsinization and incubated with an anti-lectin-like or EGF-like domain of the TM antibody for 1 h, then treated with FITC-conjugated secondary antibody and analyzed by Cyflow analysis. (B) HUVECs were pre-incubated with media or Ki16425 (10 μM) and treated with LPA (5 μM) for 30 min. Cells were dissociated by trypsinization and incubated with an anti-lectin-like domain of a TM antibody and subjected to Cyflow analysis.

(Fig. 3). These results indicate that the shed fragment in response to LPA is the lectin-like domain of TM.

Lectin-like domain shedding of TM positively regulates cell proliferation

LPA has been reported to regulate endothelial cell proliferation, thereby promoting the wound healing process during endothelial cell damage [15]. In this study, we observed that LPA enhanced TM lectin-like domain shedding and accessibility of the EGF-like domain to antibody binding in HUVECs (Fig. 2), which may trigger EGF receptor (EGFR) transactivation, thereby modulating cell proliferation. To verify whether the forced deletion of the lectin-like domain of TM is responsible for cell proliferation, 1×10^4 TMG or TMGΔL cells were cultured for 0, 24, 48, and 72 h and subjected to cell counting. Our results showed that TMGΔL cells exhibited a higher proliferation rate than TMG cells (Fig. 4A). This observation suggests that removal of the lectin-like domain liberates the endogenous EGF-like domain of TM, which might positively regulate cell proliferation. Moreover, we showed that treatment with 5 μM LPA stimulated cell proliferation in HUVECs, and pretreatment with GM 6001 abolished these enhancement effects, indicating that LPA induces cell proliferation through an MMP-dependent mechanism in HUVECs (Fig. 4B). Pretreatment with AG1478, an EGFR kinase inhibitor, also significantly inhibited LPA-induced cell proliferation in HUVECs, indicating that LPA enhances cell proliferation in HUVECs in an EGFR transactivation-dependent manner.

Discussion

The EGF-like domain of TM has been reported to stimulate cell proliferation in smooth muscle cells, adipocytes, and a hepatoma cell line [16–18]. Moreover, the TM EGF-like domain induces endothelial cell tube formation *in vitro* and *in vivo* [4]. On the contrary, the lectin-like domain of TM exerts anti-inflammatory properties by inhibiting NF-κB activation and mitogen-activated protein kinase pathways [19]. Our results also demonstrated that the LPA-induced EGF-like domain is accessible to antibody binding and TM lectin-like domain shedding (Fig. 4A), which is consistent with previous studies which showed that the TM EGF-like domain exhibits mitogenic

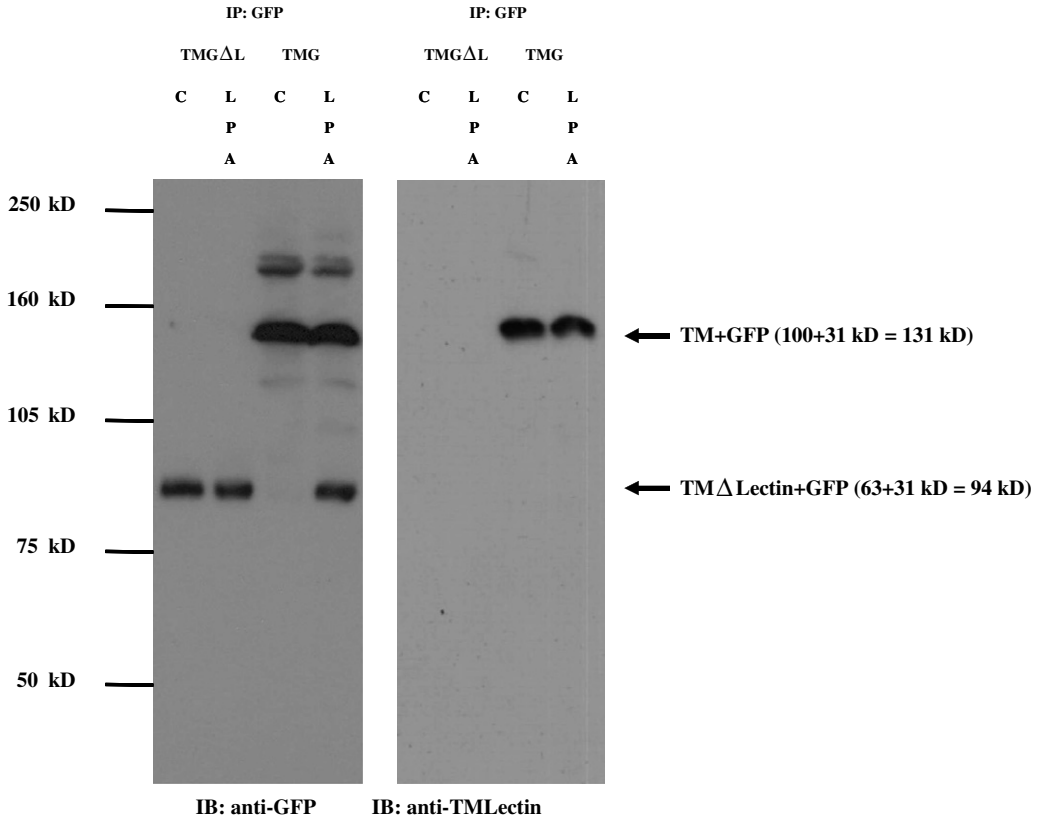

Fig. 3. LPA induces TM lectin-like domain cleavage. TMGΔL or TMG cells was treated with control media or LPA (5 μM) for 30 min. Total cell lysates were isolated and then immunoprecipitated with an anti-GFP antibody and immunoblotted with an anti-GFP antibody. Blots were re-probed against the TM lectin-like domain to evaluate the protein amounts of the TM lectin-like domain.

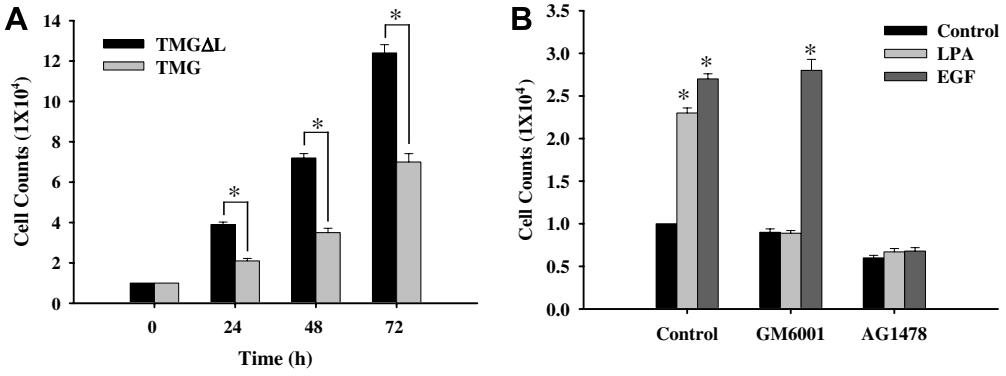

Fig. 4. Deletion of the lectin-like domain of TM stimulates cell proliferation. (A) TMGΔL or TMG cells at 1×10^4 were counted at 0, 24, 48, and 72 h after plating, and a representative result is shown in the figure. The histogram represents cell counts ($^*p < 0.05$). The data are representative of 3 experiments and are expressed as means ± S.E. (B) HUVECs at 1×10^4 were pre-incubated with control media, GM 6001 (10 μM), or AG1478 (1 μM) for 1 h and then treated with control media, LPA (5 μM), or EGF (10 ng/ml) for 72 h. The histogram represents cell counts ($^*p < 0.05$), and a representative result is shown in the figure. The data are representative of three experiments and are expressed as means ± S.E.

activity, while the lectin-like domain shows suppressive effects on cell mitogenesis.

Two forms of soluble TM fragments have been detected in human circulating plasma or urine [9]. These fragments are 63/57- and 35-kDa soluble TM polypeptides, both of which are derived from the N-terminal extracellular region of TM. The molecular weights of these peptides correspond to the size of the lectin-like domain and EGF-like domain containing TM. These TM fragments are likely produced by proteolytic cleavage of cellular TM after endothelial cell damage [20]. Salomaa et al. reported that a high level of soluble TM was associated with a decreased risk of atherosclerosis and coronary heart disease [21]. On the contrary, high soluble TM levels are recognized as an indicator of a severe inflammatory response in atrial fibrillation and vasculopathy [6]. These controversial results suggest that solu-

ble TM containing different domains might be generated during the inflammation process. Soluble TM exhibiting anti-inflammatory activity might contain the lectin-like domain of TM, while soluble TM exerting proinflammatory activity might contain the EGF-like domain of TM. A high concentration of LPA was produced during platelet activation at the sites of vessel endothelial injury [12]. In addition, LPA has also been reported to enhance endothelial cell proliferation, thereby promoting the wound healing process during endothelial cell damage [15]. Our study demonstrated that LPA enhanced TM lectin-like domain shedding in HUVECs (Fig. 1), and the shed TM lectin-like domain released into the circulation might negatively mediate subsequent inflammation processes as a negative feedback regulator. Meanwhile, the LPA-induced TM EGF-like domain being available for EGFR binding followed by lectin-like domain shedding might play a positive role in facilitating inflammatory processes. Since LPA is a proinflammatory agonist and lectin-like domain containing soluble TM level was profoundly elevated during inflammation process, we proposed that LPA might exhibit physiological function on TM fragment shedding *in vivo*. We will investigate if the injection of LPA into animal induces TM lectin-like domain shedding into circulation in the future.

A previous study demonstrated that the proinflammatory factors, TNF-α, IL-1β, and IFN-γ, induce TM shedding in an MMP-dependent manner in alveolar epithelium cells [11]. We further observed that LPA stimulated TM lectin-like domain shedding in an MMP-dependent manner in HUVECs (Fig. 2). However, our data also indicated that LPA stimulated TM EGF-like domain accessibility to antibody binding independent of MMPs (Fig. 2). In addition, LPA-enhanced cell proliferation in HUVECs was suppressed by GM 6001 treatment, further demonstrating that accessibility of the TM EGF-like domain to antibodies might not be sufficient for interaction with the EGFR (Fig. 4B). According to these observations, we hypothesize that LPA might induce TM EGF-like domain accessibility to antibody binding. Subsequently, the lectin-like domain is removed by the action of MMPs. Moreover, lectin-like domain shedding results in the TM EGF-like domain being fully exposed and capable of binding to the EGFR. The availability of the LPA-induced TM EGF-like domain for antibody binding might be due to a conformational change in TM. On the contrary, the availability of the LPA-induced TM EGF-like domain for EGFR binding depends on lectin-like domain shedding by TM.

Our results demonstrated that LPA induced TM lectin-like domain shedding in both HUVECs (Fig. 1) and HEK293 cells (Fig. 3). Moreover, LPA-induced TM lectin-like domain shedding in HUVECs is LPA_1- and LPA_3-dependent (Fig. 2B). Since HUVECs dominantly express LPA_1 and LPA_3 [13] and HEK293 cells express LPA_1 only [22], we proposed that LPA_1 might be the most essential LPA receptor in modulating TM lectin-like domain shedding. Our previous study demonstrated that LPA enhanced MMP-2 expression and activity in human endothelial cells [23]. These results implied that LPA-affected MMP-2 expression and activity might be correlated to LPA-induced TM fragment shedding in human endothelial cells.

A previous study showed that the EGFR tyrosine kinase inhibitor significantly inhibited TM EGF-like domain-induced mitogenic activity in vascular smooth muscle cells [18]. Those results imply that the TM EGF-like domain might stimulate EGFR activation and subsequent cell proliferation. Our study further demonstrated that LPA induced TM EGF-like domain exposure in HUVECs (Fig. 2), and this exposure possibly stimulates cell proliferation (Fig. 4). These findings suggest that LPA-enhanced TM EGF-like domain exposure in human endothelial cells might contribute to EGFR activation and EGFR-mediated cell proliferation. The mechanism of LPA-triggered EGFR transactivation is well established [14]. Through processing heparin-binding epidermal growth factor-like growth factor (HB-EGF) extracellularly in an MMP-dependent mechanism, LPA triggers EGFR transactivation thereby stimulating cell proliferation [14]. Our observation suggests a novel mechanism of LPA on EGFR transactivation: LPA stimulates TM lectin-like domain shedding, which fully exposes the EGF-like domain of TM, thereby contributing to EGFR transactivation in HUVECs.

In summary, our results indicate that LPA induces EGF-like accessibility to antibody binding, which is MMP-independent. However, LPA induces TM lectin-like domain shedding in human endothelial cells in an MMP-dependent manner. Subsequently, TM lectin-like domain shedding leads to full exposure of the EGF-like domain, which is capable of EGFR binding, thus inducing EGFR transactivation and subsequent cell proliferation. LPA might be an agonist, which causes the soluble TM lectin-like domain to move into the circulation during endothelial cell damage. Our study also proposes a novel mechanism of TM possibly being a key regulator of LPA-stimulated EGFR transactivation.

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