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Research Article

TGFβ-induced protein mediates lymphatic endothelial cell adhesion to the extracellular matrix under low oxygen conditions

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Abstract. TGFβ-induced protein (TGFBI) is an extracellular protein that mediates cell adhesion to collagen, laminin and fibronectin through its interaction with different β integrins. We had previously reported that hypoxia-induced TGFBI mRNA expression in lymphatic endothelial cells (LEC). Here, we demonstrate that TGFBI can contribute to hypoxia-induced increases in LEC adhesion to the ECM. We show that while there are no changes in α_1, α_4, αv, β_1, β_2, β_3, $\alpha_5\beta_1$, $\alpha v\beta_3$, $\alpha v\beta_5$ integrin expression on the LEC surface after hypoxia exposure, there exists an accumulation of TGFBI adaptor protein in LEC supernatants. We also demonstrate that hypoxia driven TGBFI expression is dependent on TGFβ production by LEC. Furthermore, we show that TGFBI mediated LEC adhesion and migration through the ECM by its binding to the β_3 integrin.

The identification of the specific mechanisms regulating LEC–ECM interactions may help us design new terapeutic applications for diseases in which lymphatic vessel function is compromised.

Keywords. Lymphatic endothelium, hypoxia, TGFβ, integrin, adhesion.

Introduction

The lymphatic vasculature comprises a tree-like hierarchy of vessels that transports extravasated fluid and macromolecules in a single direction from tissues back to the blood circulation [1]. It exerts important functions in immune surveillance, tissue fluid homeostasis and fat absorption, being involved in wound healing and some pathological processes, such as lymphedema and cancer metastasis [2]. This vasculature constitutes a highly permeable system for cell trafficking since it is composed of a single thin, non-fenestrated endothelial-cell layer, not covered by pericytes or smooth muscle cells. Furthermore, its basement membrane is incomplete in such a way that lymphatic endothelial cells (LEC) are anchored to the extracellular matrix through elastic fibers that keep the vessels from collapsing when changes in the interstitial pressure occur [2]. Some of the lymphatic-associated diseases (e.g., in lymphedema, wound healing, and tumor metastasis) are closely related to alterations in the interaction of these cells with the ECM [3]. For example, it has been described that

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during tumor lymphangiogenesis the endothelial cells increased their interactions with the ECM by over-expressing integrins, a family of matrix-recognizing receptor molecules [4, 5].

The integrin receptors are composed of two monomers of α and β subunits. There exist 18 α and 8 β different monomers and, from combinations between them, 25 different integrins that have been described in mammalian cells emerged. These proteins are expressed in all tissues where they mediate cell binding to different ECM proteins, such as vitronectin, fibronectin, laminin, collagen, fibrinogen, and von Willebrand factor [6]. Considerable data suggest that the interaction of endothelial cells with the interstitial matrix is relevant to maintain their morphology as well as to provide vessel stabilization during stress conditions [7]. Indeed, it has been described that LEC grown on collagen type I in the absence of exogenous growth factors exhibited a significantly higher survival rate than those grown without ECM [8]. Furthermore, a recent study has shown that there is an association between the expression of the integrin subunits α5 and β1 and the activation of VEGFR-3, the receptor for the lymphangiogenic factors VEGF C and D that are crucial for LEC cell survival [9].

In a previously published report, we demonstrated that lymphatic endothelial cells (LEC) responded to hypoxia by increasing their adhesion to the extracellular matrix [10]. We also described the hypoxia-induced expression of several genes related to ECM adhesion and remodeling, TGFBI (transforming growth factor-β induced or βig-h3) being among them. TGFBI codifies for an extracelullar matrix protein expressed ubiquitously [11]. It associates with ECM molecules, including collagens, fibronectin, laminin and glycosaminoglycans. To date, integrins are the sole cell surface receptors identified for TGFBI, and its interaction with them is related to important cellular functions such as cell proliferation, adhesion, migration, and differentiation [11].

In this paper, we describe how TGFBI contributes to hypoxia-induced increases in LEC adhesion to the ECM. Succinctly, here we report that 1) while there is no change in integrin expression on the LEC surface after hypoxia exposure, TGFBI, an integrin adaptor protein accumulates in LEC extracellular media in this condition, 2) TGBFI over-expression is dependent on TGFβ production and the subsequent activation of the SMAD 2/3 signaling pathway and, 3) TGFBI mediates LEC adhesion and migration through the ECM by binding to the β_3 integrin.

We thus propose TGFBI as a TGFβ-induced molecule that contributes to LEC increased adhesion under stress conditions. The identification of the specific mechanisms regulating LEC–ECM interactions may help us to design new therapeutic applications for diseases in which lymphatic vessel function is compromised.

Materials and Methods

Cell culture. Cryopreserved primary human lymphatic endothelial cells (LEC) were obtained from Clonetics (HMVEC-dLyAd – Human Dermal Lymphatic Microvascular Endothelial, Lonza, Barcelona, Spain). They were cultured on collagen-coated 10 cm^2 dishes in endothelial complete medium (EGM, Clonetics-Lonza) consisting of EBM2 (endothelial basal medium 2) supplemented with EGM-2 MV SingleQuots (Clonetics-Lonza) composed of FBS, hydrocortisone, EGF, VEGF, FGF-2, insulin-like growth factor 1, ascorbic acid and gentamicin/amphotericin at the concentrations supplied by the manufacturer (not specified). Cell cultures were maintained at 37 °C in a humidified 5 % CO_2 incubator.

Hypoxic exposure. 5×10^5 LEC cells were cultured on collagen covered 10 cm^2 dishes in EGM medium. When the cells reached 70 % confluence, the cell medium was replaced by EGM without FGF-2 and VEGF, and the cells were cultured under this condition for 24 h. LEC cells were then exposed to hypoxia (1 % O_2) for different time periods in a multigas incubator MCO-18M from Sanyo (Leicestershire, UK).

RNA Isolation cDNA synthesis and quantitative Real-Time PCR. Total RNA was extracted with Trizol (Gibco, Carlsbad, USA) following manufacturer's instructions and further purified by use of the RNeasy total RNA isolation kit (Qiagen, Maryland, USA).

One microgram from each RNA sample was reverse-transcribed into cDNA using 200 units of the Superscript II reverse transcriptase (Promega, Madison, USA) in a final volume of 20 µl.

For gene expression analysis the sequences of the primers used were the following: β-actin sense: 5′-TCTACAATGAGCTGCGTGTG-3, β-actin antisense: 5′-GGTGAGGATCTTCATGAGGT-3, and TGFBI sense: 5′-GGACTGACGGAGACCCTCAA-3′, TGFBI antisense: 5′-GCATCTCTCCCAAGAGTCTGCT-3′. Real-time PCR was performed using the SYBR green PCR master mix and the iCycler real-time PCR instrument from Applied Biosystems, (Warrington, UK). Cycling conditions were: 1 x (50 °C 2 min and 95 °C 2 min), 40 × (95 °C 15 s and 60 °C 1 min) and 1 × (95 °C 15 s, 60 °C 1 min and 95 °C 15 s). All samples were run in triplicate, in a total reaction volume of 25 µl that included 1 µl of cDNA

and 20 μM of each gene-specific primer. Fold changes in the expression of each target mRNA relative to β-actin were calculated based on the threshold cycle (Ct) as $2^{-\Delta(\Delta Ct)}$, where $\Delta Ct = Ct$ target – Ct β-actin and $\Delta(\Delta Ct) = \Delta Ct_{1\%} - \Delta Ct_{21\%}$.

Lymphatic endothelial cells adhesion assays. To measure LEC adhesion to several extracellular matrix proteins, 1×10^6 LEC cells/ml were labelled in adhesion-medium (RPMI 0.5 % BSA, 20 mM HEPES) with 10 μM calcein-AM (Fluka, Steinheim, Germany) for 20 min at 37 °C. 96-well plates (TPP, St. Louis MO, USA) were coated with 50 μg/ml type I rat tail collagen (Becton Dickinson, New Jersey, USA), 50 μg/ml collagen type IV (Sigma), 10 μg/ml fibronectin (Sigma), 5 μg/ml of human recombinant TGFBI (R&D Systems, Inc., Minneapolis, USA), or 3 % BSA as negative control (Sigma) for 2 h at 37 °C. Before starting the adhesion experiments, the 96 well plates were blocked by incubating them in a solution of 1 % BSA in RPMI for 1 h at 37 °C. Afterwards the wells were rinsed with PBS and 1×10^4 LEC cells were seeded per well and allowed to attach for 30 min at 37 °C. For integrin blocking experiments, 1 μg/ml of the following monoclonal anti-integrin antibodies were added 30 min before the LEC adhesion experiments were performed: anti-α_1 integrin (MAB1973Z, Chemicon International, California, USA), anti-β_3 integrin (MAB2023Z, Chemicon International), anti-α_4 integrin (340976, Becton Dickinson, New Jersey, USA), anti-integrins αv, β_1, β_2, $\alpha_5\beta_1$, $\alpha v\beta_3$, $\alpha v\beta_5$ (Integrin Classics Kit, ECM435, Chemicon International) and a nonspecific IgG (MAB004, R&D Systems). Non-adherent cells were removed by gentle washing. Quantification of adherent cells was performed on a Polarstar Galaxy, BMG (Offenburg, Germany) plate reader, using 485 excitation and 530 emission filters. Six replicates were made for each condition.

For the inhibition of TGFβ pathway in the adhesion experiments, LEC were incubated under hypoxia in the presence of either 1 μg/ml of an anti-TGFBI antibody (60007-1-1 g, Proteintech Group, Inc, Illinois, USA), or 200 μg/ml of a TGFβ1 blocking peptide (P144) [12] or 1 μg/ml of a non-specific IgG (MAB004, R & D Systems) for 24 h. After that time, LEC were trypsinized and used in the adhesion experiments as described above.

Flow cytometry analysis. LEC were cultured and exposed to hypoxia for 24 h as described above. After that time, the endothelial cells were harvested and resuspended in 100 μl of staining buffer (5 % FBS, EDTA 2 mM in PBS) and incubated for 10 min at room temperature with a concentration of 1 μg/ml of the following monoclonal antibodies: anti-α_1 integrin (MAB1973Z, Chemicon International), anti-β_3 integrin (MAB2023Z, Chemicon International), anti-α_4 integrin (340976, Becton Dickinson), anti-integrins αv, β_1, β_2, $\alpha_5\beta_1$, $\alpha v\beta_3$, $\alpha v\beta_5$ (Integrin Classics Kit, ECM435, Chemicon Interational) or a nonspecific IgG (MAB004, R&D Systems). Afterwards, the cells were washed with staining buffer and subsequently incubated with 1:500 green-fluorescent Alexa Fluor 488 anti-mouse-secondary antibodies (Molecular Probes) at room temperature for 10 min. Cells were washed twice with staining buffer and analyzed on a FACScan flow cytometer using Cellquest software (BD-PharMingen).

Migration assay. LEC migration was assayed using Boyden chambers (BD Biosciences, San Diego, CA) by measuring the spontaneous migration of endothelial cells through 8-μm pore size filters. A total of 4×10^4 LEC were seeded in 500 μl of migration medium (RPMI supplemented with 0,5 % BSA) on the top membrane of transwell chambers pre-coated with 50 μg/ml of type I rat tail collagen (Becton Dickinson, New Jersey, USA), 50 μg/ml of collagen type IV (Sigma), 10 μg/ml of fibronectin (Sigma), 5 μg/ml of recombinant human TGFBI or BSA at a concentration w/v of 3 % as control for nonspecific binding. For blocking experiments, 1 μg/ml of an anti-TGFBI antibody (60007-1-1 g, Proteintech Group, Inc, Illinois, USA), 200 μg/ml of a TGFβ1 blocking peptide (P144) [12], 1 μg/ml of several anti-integrin antibodies (see above) or a nonspecific IgG (see above) were added to LEC 30 min before starting the migration experiments. LEC migration was allowed to occur during 12 h at 37 °C. After that time the upper sides of the filters were wiped with cotton swabs and rinsed twice with PBS. Cells on the lower surface of the filter were fixated in formaldehyde 4 % for 2 h and stained during 10 min with a solution of crystal violet stain. The quantification of migrated cells was performed by inverted microscopy inspection. At least four high-powered fields were counted per sample. All the experiments were performed in triplicates.

Western blot analysis. LEC were cultured on 10 cm^2 plates and maintained for 3, 6, 12 and 24 h under 1 % pO_2 (hypoxia) or treated with TGFβ at a final concentration of 2 ng/ml for 24 h. To reoxygenate LEC, the cells were cultured under hypoxia (1 % pO_2) for 24 h followed by a six hour period of normoxia exposure (21 % pO2).

To obtain the extracellular protein extracts, LEC supernatants were concentrated using Amicon Ultra-15 5KDa concentration columns (Millipore, Massachussetts, USA) at 3500 x g for 2 h. Total protein

extracts were obtained by cell lysis on ice in a buffer containing 1% NP-40, 0.1% SDS, 0.5% deoxycholic acid, 2 mM sodium orthovanadate, 0.2 mM glycerol 2-phosphate and protease inhibitors (Complete mini EDTA-free; Roche, Indianapolis, USA) in PBS. Protein content was measured using the BCA protein assay kit from Pierce (Illinois, USA), 10 μg of each sample being separated by electrophoresis in 12% denaturing polyacrylamide-SDS gels, and subsequently transferred to nitrocellulose membranes (Amersham-Pharmacia). The membranes were blocked afterwards by incubating them overnight in a solution of 5% non-fat dry milk in PBS-T or with 5% BSA in PBS-T for TGFBI determination. Primary antibody incubation was performed for 1 h at room temperature. The primary antibodies used were: anti-TGFβ 1:300, (MAB1835, R&D, Systems, Inc., Minneapolis, USA), anti-TGFBRII 1:600 (AF-241-NA, R&D), anti-phospho-SMAD 2/3 1:2000 (Chemicon International), all of them added in a solution of 5% non-fat dry milk in PBS-T. Anti-SMAD 2/3 1:2000 (Cell Signaling, Massachusetts, USA) and anti-TGFBI 1:2000 (Proteintech Group, Inc, Illinois, USA) were both tested in a solution of 5% BSA in PBST. The western blot membranes were washed three times with PBST and incubated with HRP-conjugated anti-mouse IgG (NA931V, GE Healthcare, Wisconsin, USA) or HRP-conjugated anti-rabbit IgG (sc-2030, Santa Cruz Biotechnology, California, USA) secondary antibody in 5% non-fat dry milk in PBST or in 5% BSA in PBST for TGFBI. The membranes were developed using the Lumi-light^{PLUS} Western Blotting Kit (Roche Molecular Biochemicals, Mannheim, Germany) and exposed to Amersham Hyperfilm ECL (GE Healthcare).

Results

Lymphatic endothelial cells respond to hypoxia with increased adhesion to the ECM. Interactions between endothelium and extracellular matrix are of great importance for the maintenance of vessel structure and integrity. In an attempt to study the influence of stress situations on the lymphatic endothelium, we measured LEC adhesion to the extracellular matrix proteins collagen I, collagen IV and fibronectin, under three different conditions. First, LEC were grown in normoxia (21% PO_2), second, they were exposed to hypoxia (1% PO_2) and, third, LEC cells were grown under hypoxia (1% PO_2) for 24 h followed by a 6 h period of normoxia (21% PO_2). As is shown in Figure 1, LEC responded to hypoxia by increasing their adhesion to all the extracellular matrix proteins tested. The increases observed in cell adhesion were approximately 1.6 fold over the adhesion found in normoxia. The specificity of the hypoxia driven reaction was demonstrated, since LEC adhesion to the ECM returned to baseline levels when the cells were reoxygenated.

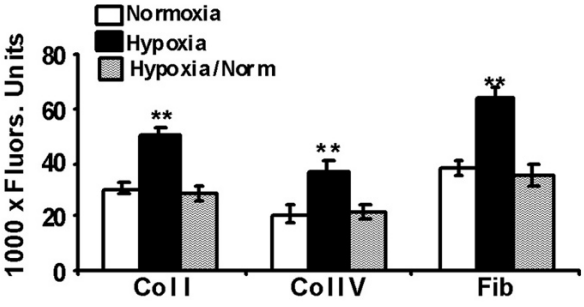

Figure 1. LEC responded to hypoxia with an increased adhesion to the ECM. To measure LEC adhesion to the ECM, endothelial cells were trypsinized 24 h after being grown in normoxia (21% pO_2, white columns), hypoxia (black columns, 1% pO_2), or grown under hypoxia for 24 h and further reoxygenated for an additional 6 h (lined columns). LEC cells were labeled by incorporation of calcein, and allowed to adhere for 30 min onto collagen I, collagen IV and fibronectin coated wells. The statistical comparisons between cell adhesion in the different treatments were performed using an ANOVA test, $^{**}P < 0.005$.

Integrins are the main receptors implicated in cell adhesion to the extracellular matrix. In order to determine which subtype of integrin may be involved in LEC adhesion to the extracellular matrix, we used specific monoclonal antibodies raised against several integrin subunits to block LEC adhesion to collagens and fibronectin. LEC adhesion to fibronectin was blocked when cells were incubated in the presence of antibodies to all integrins tested except for anti ITG-α_1 and ITG-α_4, which have never been described as receptors for this extracellular matrix protein. The degree of cell adhesion inhibition ranged from 35 to 42% with respect to basal cell adhesion levels (Figure 2). When studying LEC adhesion to collagen I and IV, we observed that it only depended on integrin α_1 binding. We were not able to find any significant difference in the degree of adhesion blockage between normoxia and hypoxia exposed LEC; the inhibition of cell adhesion was of about 35–42% from basal adhesion levels for all the integrins implicated.

Hypoxia did not increase integrin expression in LEC but up-regulated the production of the adapter molecule TGFBI. In order to fully discount the alterations in integrin expression as being responsible for the differences observed in LEC cell adhesion under hypoxia, we analyzed, by flow cytometry, their presence on the LEC surface 24 h after treatment. As

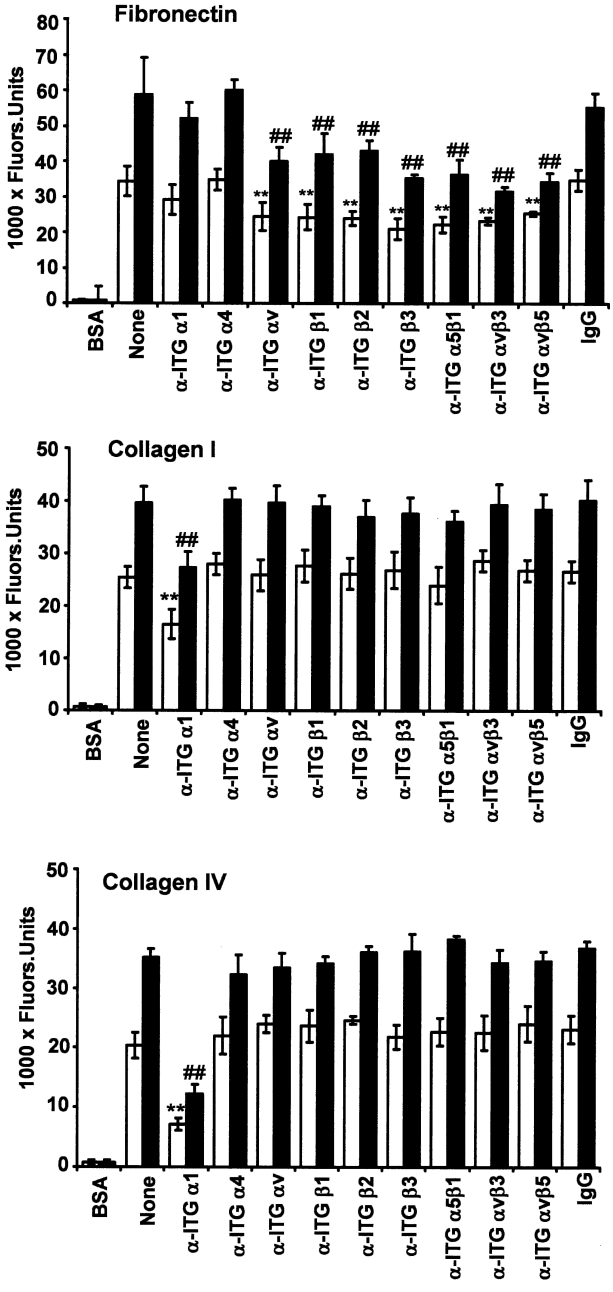

Figure 2. LEC increased adhesion by hypoxia is not specifically blocked by specific anti-integrin antibodies. In order to study the involvement of the integrins α_1, α_4, αv, β_1, β_2, β_3, $\alpha_5\beta_1$, $\alpha v\beta_3$ and $\alpha v\beta_5$ in LEC adhesion of ECM proteins, LEC adhesion experiments were performed as mentioned in Figure 1 in the presence of anti-integrin blocking antibodies (1 μg/ml). An irrelevant IgG was used as non-specific antibody and BSA as non-specific substrate. Statistical comparisons between cell adhesion between in the absence and in the presence of each specific antibody in normoxia (white columns), and hypoxia (black columns) is shown. Statistical analysis was performed using an ANOVA test $P < 0.005$, significant differences in cell binding as compared to cell adhesion in the absence of blocking antibodies under normoxia (**) or hypoxia (##) exposed LEC.

depicted in Figure 3, we could not detect any change in the expression of any of the integrin tested by flow cytometry. In fact, the integrin α_4, which did not render any specific binding in the adhesion blocking experiments, was not expressed by LEC. Therefore, since the increases observed in cell adhesion were not due to an up-regulation of integrin expression on LEC surface, we hypothesized that increased binding to the ECM could be mediated by an integrin adaptor protein.

To test this possibility, we reanalyzed our previously published microarray analysis on LEC differential gene expression under hypoxic conditions [10] to search for ECM binding molecules other than integrins. Among all of the differentially induced genes analyzed, we detected TGFBI, an integrin-extracellular matrix adaptor molecule, as a very reasonable candidate, since it had been described as mediating vascular cell adhesion [11] and it was the only integrin adaptor protein over-expressed in hypoxia exposed LEC.

Therefore, we checked whether there was an accumulation of TGFBI protein in the supernatants of LEC grown during 24 h under different conditions: normoxia, hypoxia, or exposed to hypoxia and further reoxygenated for an additional 6 h. As can be seen in Figure 4, TGFBI protein accumulates in the supernatant of LEC 24 h after hypoxia exposure but it returned to basal levels when the cells were reoxygenated.

This result suggests that TGFBI might be involved in LEC adhesion under low oxygen conditions.

TGFBI-induced expression by hypoxia in LEC is mediated by TGFβ secretion. The next step was to determine whether TGFβ was induced in LEC cells exposed to hypoxia, and its possible role as activator of TGFBI expression. As can be observed in Figure 5A, TGFβ accumulated in LEC extracellular media as early as three hours after hypoxic exposure and was detectable by western blot during the entire experimental procedure. At the same time, we observed a slight decrease in the amount of TGFβ protein in the cell supernatants 24 h after hypoxia exposure, probably due to TGFβ binding to its receptor on the cell surface. In fact, Figure 5B shows TGFBRII expression is augmented throughout the experiment, confirming that this was the case.

Once we determined that LEC secreted TGFβ under hypoxia, we then analyzed whether this cytokine was functional in our cells. We therefore measured the phosphorylation of SMAD 2/3 protein, a known TGFβ signal transducing molecule. We observed that SMAD 2/3 was phosphorylated in hypoxia exposed LEC, reaching a peak at 3 h that decreased

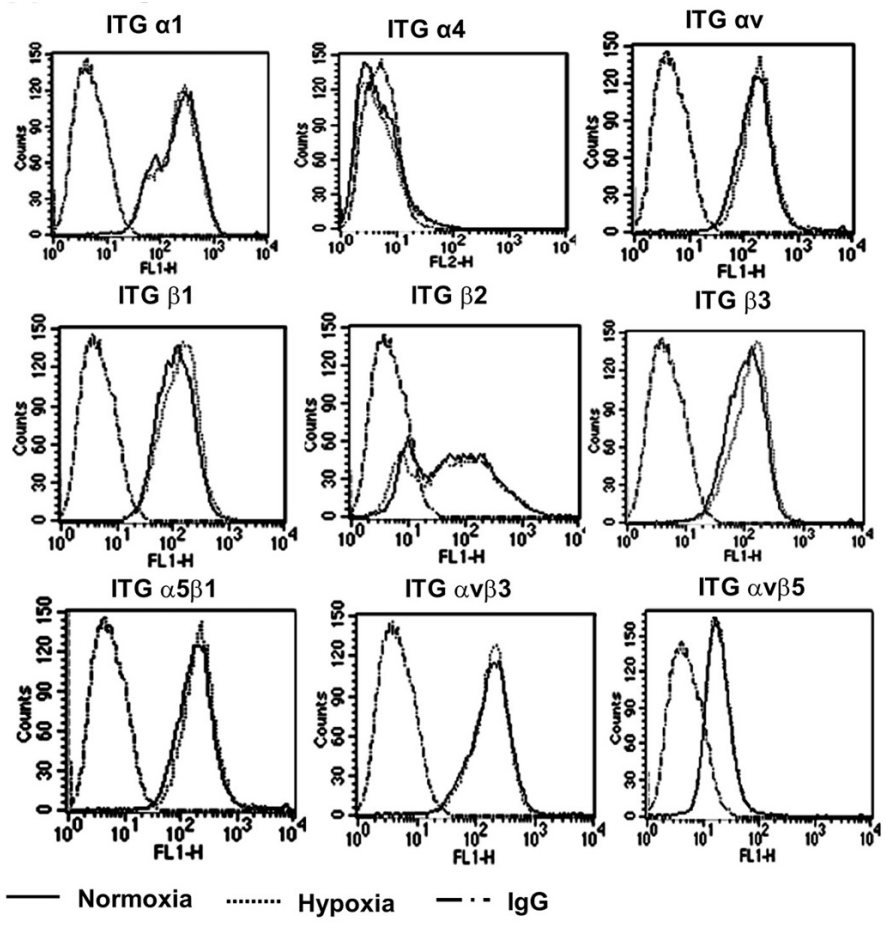

Figure 3. Integrin expression on the cell surface is not altered after hypoxic exposure in LEC. The expression levels of the integrins (ITG) α_1, α_4, αv, β_1, β_2, β_3, $\alpha_5\beta_1$, $\alpha v\beta_3$ and $\alpha v\beta_5$ on LEC cell surface was tested by flow cytometry 24 h after being grown in normoxia or hypoxia. IgG was used as isotype control.

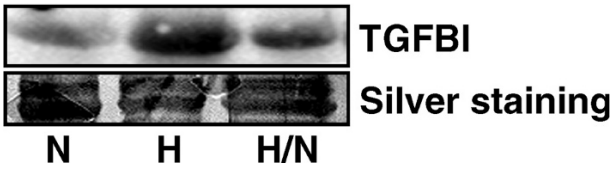

Figure 4. The integrin-extracellular matrix adaptor molecule TGFBI accumulates in the supernatant of hypoxia exposed LEC. Western blot detection of TGFBI in the extracellular media of LEC grown for 24 h under normoxia (N), hypoxia (H) or hypoxia and reoxygenation (H/N) for 6 h. One result representative of at least three different experiments is shown. Silver staining of the membranes was used as loading control.

back to basal levels at 12 h (Fig. 5C). This diminution in SMAD 2/3 phosphorylation could be a consequence of a negative TGFβ feedback regulatory mechanism, as has recently been described [13].

TGFBI was initially described as TGFβ inducible protein [11]. In order to study whether TGFβ was inducing TGFBI secretion to the extracellular media, LEC were incubated 24 h in the presence of increasing amounts of TGFβ. As can be seen in Figure 6A, TGFBI is induced by TGFβ in a dose-dependent manner, as previously described in other cell systems. Next we studied the relationship between TGFβ secretion, SMAD 2/3 phosphorylation and TGFBI production by LEC under hypoxia. We analyzed TGFBI mRNA and protein expression in LEC supernatants at different time intervals after exposure to hypoxia or TGFβ and compare them to the levels found in normoxic conditions. We observed that, as happened with TGFβ, and SMAD2/3 activation, TGFBI mRNA and protein expression reached a peak at 3 h after hypoxic exposure, and remained high throughout the experiment (Fig. 6B and C).

Then, we wanted to ascertain whether blocking TGFβ binding to its receptor could impede TGFBI secretion under hypoxia. To achieve this, we incubated LEC with a TGFβ blocking peptide (P144, 200 μg/ml) while exposing them to hypoxia and analyzed TGFBI expression and accumulation in the extracellular media. Treatment with the TGFβ blocking peptide P144 abrogated both TGFBI mRNA synthesis and protein secretion (Figs. 6D and E). These results demonstrate that TGFBI-induced expression by hypoxia is mediated by the presence of active TGFβ.

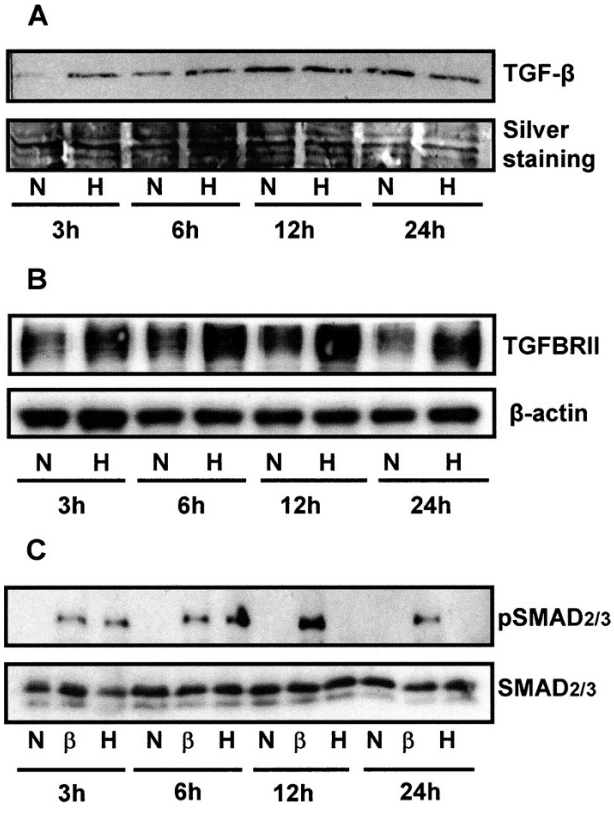

Figure 5. The TGFβ pathway is activated in LEC exposed to low oxygen conditions. *A*) Time course detection of TGFβ expression by Western-blotting in the supernatants of hypoxia (H) and normoxia (N) grown LEC. Silver staining of the membranes was used as a loading control. *B*) Detection of TGFBRII expression in total protein extracts from LEC exposed to hypoxia, (H), normoxia (N). As control for protein load detection β-actin protein content was detected. *C*) Expression and activation of P-SMAD 2/3 in normoxia (N), TGFβ treated (2 ng/ml) (β), or hypoxia (H) exposed cells analyzed by western blot. One result representative of at least three different experiments is shown. The expression of SMAD 2/3 was used to ascertain total SMAD content.

LEC increased adhesion in hypoxia is dependent on TGFβ and TGFBI. To address the question of whether TGFBI could mediate LEC adhesion to the ECM under hypoxia, we performed the same adhesion experiments as described in Figure 1, pre-incubating LEC with either the TGFβ inhibitor peptide P144 or with a commercially available anti-TGFBI monoclonal antibody. The results presented in Figure 7A, show that both of them reduced cell adhesion back to the levels detected under normoxic conditions, indicating that TGFBI is implicated in hypoxia-induced cell adhesion. Noteworthy is the fact that basal cell adhesion was also reduced when anti-TGFBI antibodies or P144 were used in normoxia, suggesting that this protein could also be involved in cell adhesion under physiological conditions.

To ascertain whether LEC adhesion was dependent on the amount of TGFBI protein present in the ECM we performed LEC adhesion assays on 96-well plates covered with different amounts of recombinant TGFBI. As can be seen in Figure 7B, LEC adhered to TGFBI in a dose-dependent manner: the higher the concentration of TGFBI, the stronger adhesion, except for the highest concentration of recombinant protein (10 μg/ml), at which cell adhesion decreased slightly. This could be due to an accumulation of pro-apoptotic peptides derived from TGFBI proteolitic fragments [27].

TGFBI facilitates LEC adhesion and migration through the ECM by its interaction with β_3 integrin. Since it was first described, TGFBI has been associated not only with cell adhesion but also with cell migration [28]. To test if TGFBI was also implicated in LEC migration, we performed migration assays on Boyden chambers covered with collagen I, IV or with fibronectin. The results shown (Figs. 7C and D) confirmed that TGFBI facilitates LEC spontaneous migration, since the use of an anti-TGFBI antibody significantly reduced the number of migrated cells, while this effect was not detected when using a non-specific antibody.

Given that integrins are the only cellular receptors described for TGFBI, we wanted to elucidate which integrin subtype was mediating LEC binding to TGFBI. To accomplish this, we measured LEC adhesion to recombinant TGFBI (5 μg/ml) covered plates in the presence of several anti-integrin blocking antibodies. As shown in Figure 8A, LEC adhesion to TGFBI was inhibited only when blocking antibodies to the $\alpha v\beta_3$ or β_3 integrins were used, demonstrating that the β_3 integrin receptor is important for LEC binding to TGFBI.

We finally wanted to test whether the same integrin participated in TGFBI dependent LEC migration. We therefore performed migration assays of LEC monolayers on TGFBI (5 μg/ml) or 3% BSA coated Boyden chambers in the presence or absence of an anti-β_3 integrin antibody. From the results presented in Figure 8B and C, we inferred that LEC showed significantly higher migration when the migration assays were performed on TGFBI than BSA coated Boyden Chambers. This migration was reduced in both cases by anti-β_3 or anti-$\alpha v\beta_3$ integrin antibodies, but the use of non-specific IgG did not block it. This result demonstrates that LEC migration through TGFBI protein is also mediated by its binding to the β_3 integrin. Together, these data demonstrate for the first time that the β_3 integrin is the receptor of TGFBI in LEC.

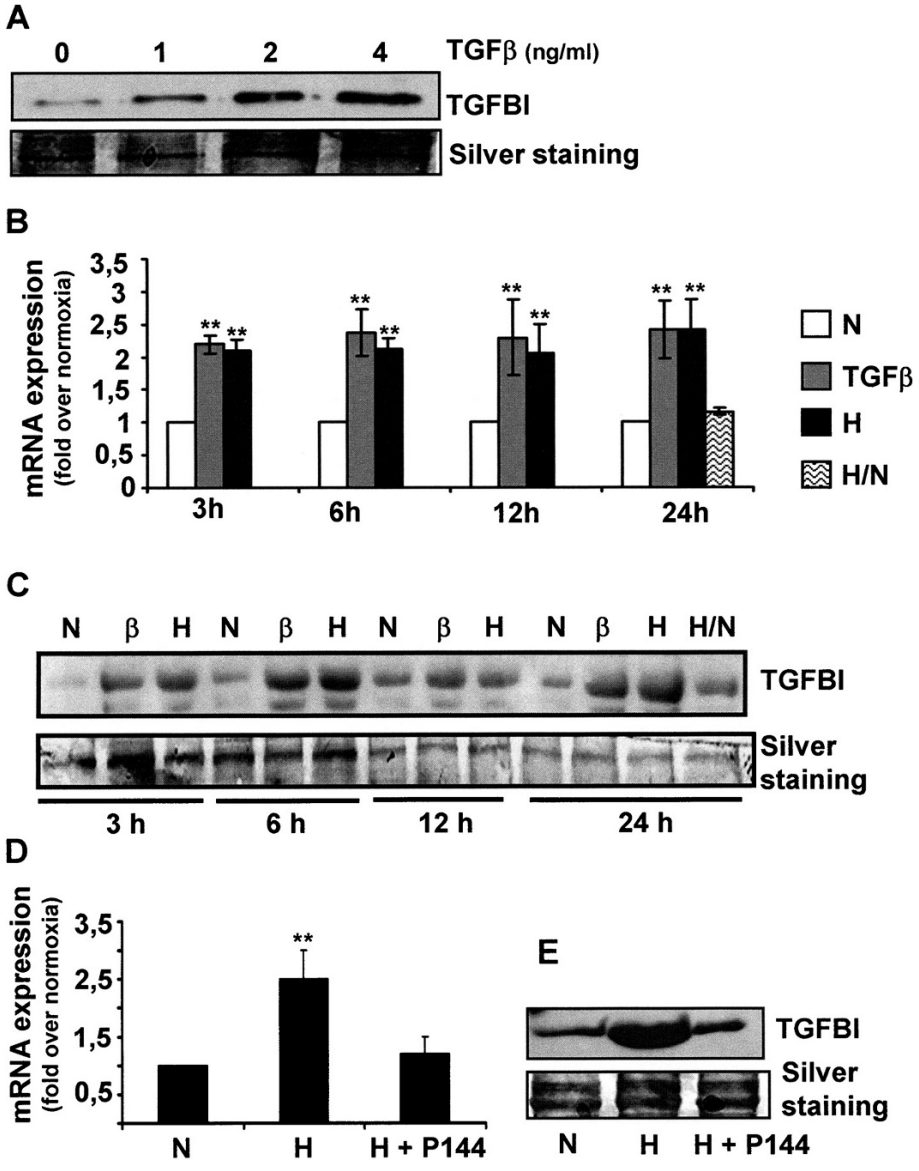

Figure 6. TGFBI expression is induced by hypoxia in LEC in a TGFβ dependent manner. *A*) TGFβ-induced accumulation of TGFBI in the extracellular media of LEC treated for 24 h with different concentrations of this cytokine, also measured by western blotting. *B*) Real time PCR analysis of TGFBI mRNA expression in LEC grown under normoxia (N, white columns), hypoxia (H, black columns, 1 % pO_2) or TGFβ treated (T, grey columns, 2 ng/ml) for different time intervals, or grown for 24 h under hypoxia and further reoxygenated for 6 h (H/N, lined columns). *C*) Western blot analysis of TGFBI expression in the supernatant of LEC cultured under normoxia (N), hypoxia (H) treated with 2 ng/ml of TGFβ (β) or grown under hypoxia for 24 h and further reoxygenated for 6 h (H/N). D) Real time PCR analysis of TGFBI expression in LEC grown for 24 h under normoxia (N), Hypoxia (H) or hypoxia in the presence of the TGFβ inhibitory peptide P144, (H + P144). E) Western blot detection of TGFBI expression in the supernatants of LEC cultured for 24 h in normoxia (N), hypoxia (H) and hypoxia in the presence of a TGFβ inhibitory peptide P144 (200 μg/ml). Comparisons in TGBFI expression were made using an ANOVA test, $^{**}P < 0.005$.

Discussion

Chronic hypoxia takes place at the beginning of pathogenic processes such as systemic sclerosis [14], pulmonary hypertension and pulmonary vascular remodeling [15]. It is also present in cancer development, since tumor growth usually surpasses that of the adjacent blood vessels leading to defective oxygen supply in their surroundings [16]. Alterations in blood endothelial cell function by hypoxia have been deeply studied both in terms of cell physiology and gene expression [17, 18]. In fact, it has been shown that hypoxia increases endothelial cell stiffness and adhesion to several ECM components [19]. But, while there is abundant data on the response of blood endothelial cells to hypoxia, the underlying mechanisms governing lymphatic endothelial cell response to low oxygen conditions remain unclear. The study of the lymphatic endothelium response under stress is not trivial because this endothelium has been involved in several pathological conditions in which ECM also plays an important role, such as lymphedema, psoriasis, diabetes and cancer [20].

To shed light on this subject, we previously described the transcriptional and functional response of LEC to chronic hypoxia [10]. In that paper, we demonstrated that, under low oxygen conditions, LEC augmented their adhesion to collagen I and IV and fibronectin and increased the expression of some genes related to matrix composition such as fibronectin and TGFBI. In the present work, we have gone a step further, showing that LEC adhere to the collagen I and IV by the integrin α_1, and to fibronectin by the integrins, α_v, β_3, $\alpha_5\beta_1$, $\alpha v\beta_3$ and $\alpha v\beta_5$, similar to what happens in

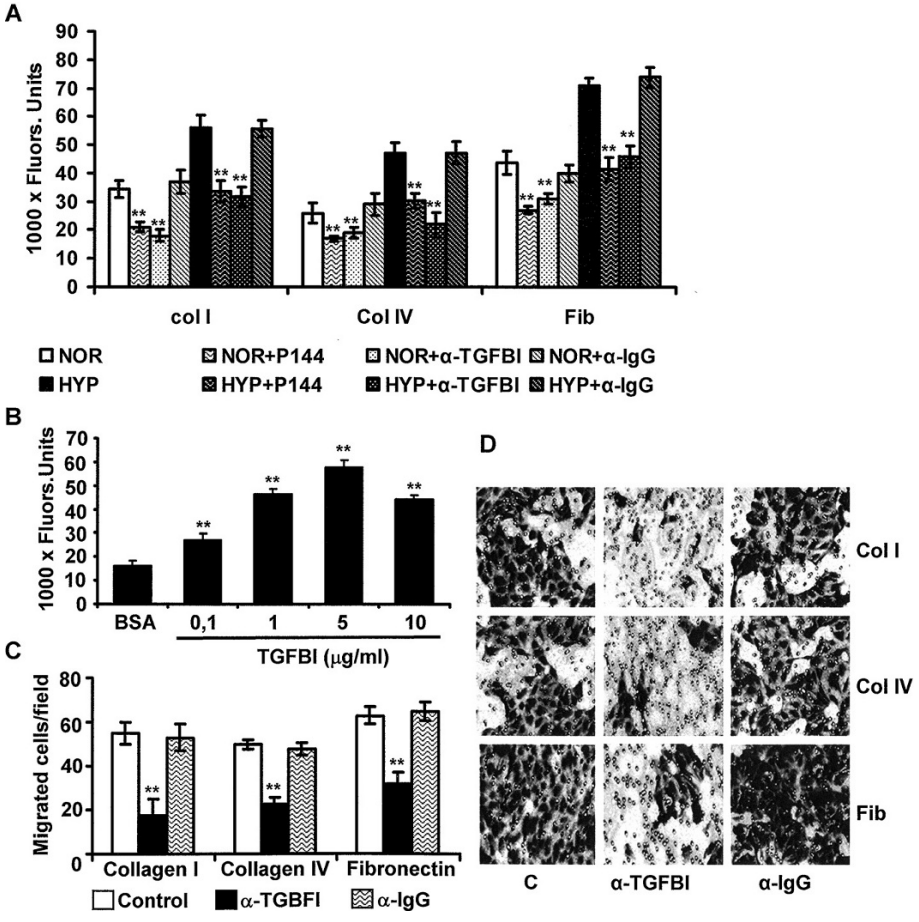

Figure 7. LEC adhesion to the ECM is abrogated by the use of a TGFβ inhibitory peptide or of an anti-TGFBI antibody. *A*) LEC adhesion to collagen I, IV and to fibronectin was measured after the cells had been grown for 24 h under normoxia or hypoxia in the absence or presence either of a TGFβ–blocking peptide (P144, 200 μg/ml) or of an anti-TGFBI antibody (1 μg/ml). As negative control, LEC adhesion experiments were performed in the presence of an irrelevant antibody (IgG). *B*) Dose-response analysis of LEC adhesion to increasing amounts of recombinant TGFBI. *C*) Quantification of LEC transmigration through collagen I, IV and, fibronectin covered Boyden chambers in presence or absence of an anti-TGFBI (α-TGFBI) antibody. An irrelevant IgG (α-IgG) was used as control for non-specific blockage. *D*) Crystal violet staining of LEC cells migrated through ECM coated Boyden chambers. Comparisons between cell adhesion were performed using an ANOVA statistical test, $^{**}P < 0.005$.

blood endothelium. We observed higher adhesion rates after the endothelial cells had been exposed to hypoxia. Surprisingly, we could not detect any increase in the cell surface expression of any of the integrins studied. Therefore we hypothesize that another mechanism might be responsible of cell adhesion in these circumstances.

Integrins can bind to the ECM through two different mechanisms; either direct adhesion or through adaptor proteins. For example, recently a group of adaptor proteins have been described, the CCN family of proteins, that binds to both integrin and to the ECM through two different structural domains [21]. The fact that we did not find higher integrin expression after hypoxia exposure leads us to suspect that this second mechanism might contribute to the increases observed in LEC binding to the ECM. In this work, we described that TGFBI, an integrin adaptor molecule [11] accumulates in the extracellular media of hypoxia-exposed LEC. TGFBI is an extracellular matrix protein that is induced by transforming growth factor-β in several cell types [22]. It seems to have many different functions depending on the cell type in which it is expressed, which varies from cell adhesion (the main function described) to cell growth, differentiation and apoptosis [23, 24]. Moreover, TGFBI has been involved in some vascular diseases such as corneal dystrophy, atherosclerosis and in tumor angiogenesis [25–27].

We demonstrate here that the increase observed in TGFBI under hypoxia contributes to LEC cell adhesion to the ECM. This result is further confirmed by the fact that incubation of hypoxia exposed LEC with an anti-TGFBI antibody returned LEC adhesion to the levels obtained under normoxia.

According to the results obtained in other tissues, TGFBI production by LEC was preceded by the secretion of TGFβ and the activation of the SMAD 2/3 pathway. In fact, we demonstrated that the induction of TGFBI depended on TGFβ production, since the use of a TGFβ blocking peptide abrogated it. TGFβ induction under hypoxia has already been described in blood endothelial cells, where it exerts a relevant role in matrix deposition and cell adhesion to the ECM [29]. It is well established that TGFβ signaling is related to vascular diseases such as atherosclerosis, hypertension [30], and to inflammatory diseases such as systemic sclerosis [31]. Interestingly, there are several reports that illustrate the association of inflammatory diseases, such as psoriatic

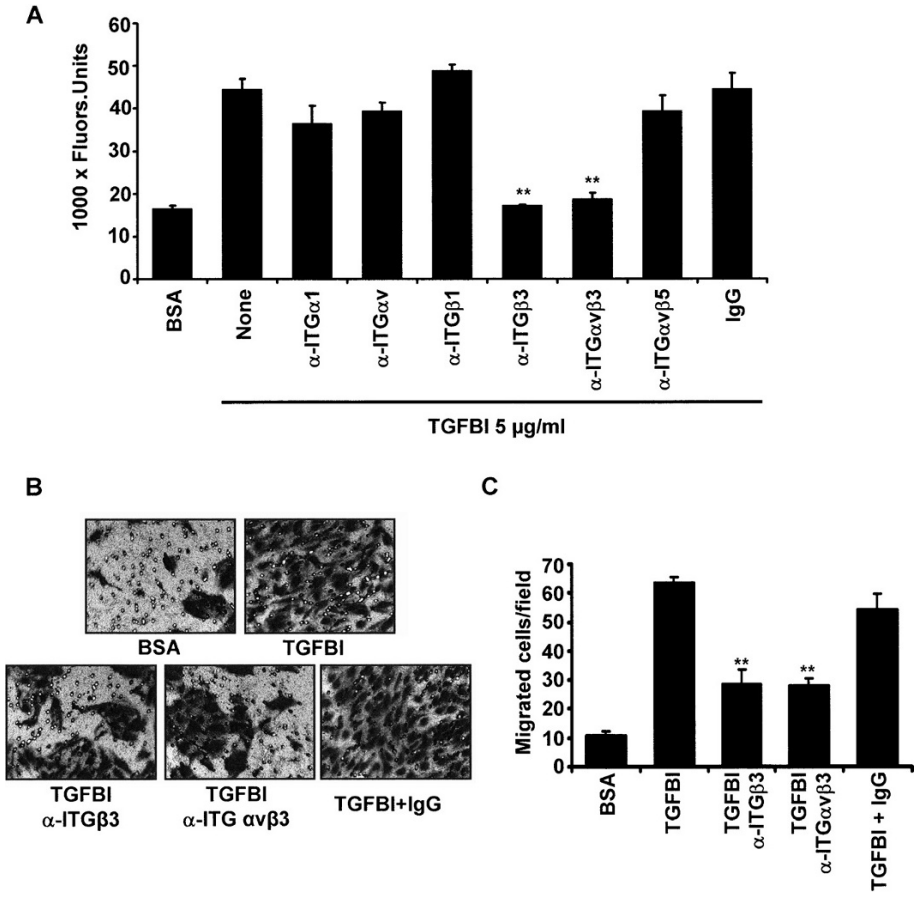

Figure 8. TGFBI mediates LEC adhesion and migration through its interaction with the β_3 integrin. *A*) Cell adhesion to recombinant TGFBI (5 µg/ml) was assayed in the presence of anti- α_1, αv, β_1, β_3, $\alpha v\beta_3$ or $\alpha v\beta_5$ integrin (α-ITG) blocking antibodies (1 µg/ml). BSA was used as control for basal cell adhesion to ECM and an irrelevant IgG as non-specific antibody. *B*) To study the implication of the β_3 integrins in TGFBI mediated LEC migration, Boyden chambers were coated with recombinant TGFBI or BSA and LEC transmigration was allowed to occur for 12 h in the presence or in the absence of an anti-integrin (α-ITG) β_3 or anti-integrin $\alpha v\beta_3$ blocking antibodies. Control IgG was used as control for non-specific inhibition (all at 5 ng/ml). *C*) Crystal violet staining of LEC cells migrated through Boyden chambers. Comparisons between cell adhesion were performed using an ANOVA statistical test, $^{**}P < 0.005$.

lesions [32] and rheumatoid arthritis [33] with lymphatic dysfunction, providing a possible functional link between altered TGFβ expression and lymphatic disorders. Even more, a report published while this paper was being evaluated shows how this cytokine regulates the formation of lymphatic vessels during embryonic development, tumor growth and in chronic inflammation, which highlights the importance of the study of TGFβ mediated responses in this system [45].

TGFBI is involved in cell adhesion through its binding to the integrins on the cell surface. This binding occurs through an RGD region of TGFBI located in its C-terminus. There are several integrins that can bind TGFBI. Depending on the cell type, the integrin involved in cell adhesion and the intracellular signaling pathways triggered within the cell differs [11]. For example, TGFBI mediated binding of vascular smooth muscle cells to the ECM occurs through the $\alpha_v\beta_5$ integrin and leads to Akt, Erk and FAK phosphorylation [34]. At the same time it has been described that TGFBI binding to the ECM occurs through the $\alpha_3\beta_1$ integrin in keratinocytes and human corneal epithelial cells [35, 36]. Besides, it is the interaction of TGFBI with $\alpha_v\beta_3$ that elicits a migratory response on HUVEC cells [37]. In the present work, we show that TGFBI dependent LEC adhesion and migration through the ECM is due to LEC binding to recombinant TGFBI protein through the β_3 integrin subunit.

The β_3 integrin is not a direct receptor for collagen I and IV, but since TGFBI acts as a β_3 integrin adaptor protein that binds to different matrix proteins through its RGD domain, it makes possible for the integrins to indirectly bind to ECM proteins that are not their direct ligands.

Since β_3 integrin mediates hypoxia-induced LEC adhesion through its binding to TGFBI, the use of monoclonal blocking antibodies should have abolished the augment in LEC adhesion to the ECM observed after hypoxia. This was not the case in our hands. We presume that it might be due to the rapid TGFBI binding to its integrin receptor after being secreted, in such a way that TGFBI mediated adhesion cannot be abolished once they are bound. One possible experiment that will clarify this point is to perform LEC adhesion experiments after the cells have been exposed to hypoxia in the presence of anti-β3-blocking antibodies during the entire incubation period.

Finally, it has been described that chronic hypoxia leads to vascular alterations [38, 39]. In fact, a recently published report has shown that hypoxia/re-oxygenation induce endothelial cell permeability through PI3K-dependent dispersion of adherents junctions [40]. Interestingly, Ma et al. [41] have published that TGFBI over-expression by colon carcinoma cells leads to higher endothelial cell permeability by disruption of VE-cadherin in the adjacent endothelium through its binding to their $\alpha v\beta_5$ integrins and the subsequent activation of the src kinase activity. But this does not seem to be the case in our system. Firstly, because we observed increased lymphatic endothelial cell adhesion after hypoxia exposure, and, secondly, because LEC binding to TGFBI under hypoxia is not mediated by the $\alpha v\beta_5$ integrin but by $\alpha v\beta_3$. In fact, it has been published that PI3K/Akt signaling through the $\alpha v\beta_3$ integrin in blood endothelial is inhibited by the $\alpha v\beta_5$ mediated activation of src kinase activity [42]. These results show again the heterogeneity between blood and lymphatic endothelial cells in terms of receptor expression and response to stress stimuli [43, 44]. It would be of great interest to discriminate which kinase activity is activated after TGFBI binding to hypoxia exposed LEC.

In summary, in this paper we have demonstrated that TGFBI protein is expressed by LEC under hypoxia in a TGFβ-dependent way. This protein participates in LEC adhesion and migration through its interaction with the β3 integrin.

The study of the molecular pathways involved in the LEC-ECM relationship offers new data in the growing field of lymphatic research. This knowledge should contribute to the design of therapies for lymphatic related diseases.

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1 Witte,M. H., Bernas, M. J., Martin, C. P. and Witte, C. L. (2001) Lymphangiogenesis and lymphangiodysplasia: from molecular to clinical lymphology. Microscopy Research and Technique 55, 122–145.
2 Saharinen, P., Tammela, T., Karkkainen, M. J. and Alitalo, K. (2004) Lymphatic vasculature: development, molecular regulation and role in tumor metastasis and inflammation. Trends in Immunology 25, 387–395.
3 Ji, R. C. (2005) Characteristics of lymphatic endothelial cells in physiological and pathological conditions. Histology and Histopathology 20,155–175.
4 Kajiya K., Hirakawa S., Ma, B., Drinnenberg I. and Detmar M. (2005) Hepatocyte growth factor promotes lymphatic vessel formation and function. The EMBO journal 24, 2885–2895.
5 Vlahakis N. E., Young B. A., Atakilit A and Sheppard D. (2005) The lymphangiogenic vascular endothelial growth factors VEGF-C and -D are ligands for the integrin α9beta1. J Biol Chem 280, 4544–4552.
6 Eliceiri B. P.and Cheresh, D. A. (2006) Role of α v integrins during angiogenesis. Cancer Journal 6, 245–249.
7 Davis, G. E. and Senger, D. R. (2005). Endothelial extracellular matrix: biosynthesis, remodeling, and functions during vascular morphogenesis and neovessel stabilization. Circulation Research 97, 1093–1107.
8 Podgrabinska, S., Brau,n P., Velasco, P., Kloos B., Pepper, M. S. and Skobe, M. (2002). Molecular characterization of lymphatic endothelial cells. Proceedings of the National Academy of Science of the United States of America 99, 16069–16074.
9 Zhang X, Groopman JE, Wang JF. (2005) Extracellular matrix regulates endothelial functions through interaction of VEGFR-3 and integrin α 5β1. Journal of Cellular Physiology 202, 205–214.
10 Irigoyen, M., Ansó, E., Martínez, E., Garayoa, M., Martínez-Irujo, J. J. and Rouzaut. A. (2007) Hypoxia alters the adhesive properties of lymphatic endothelial cells. A transcriptional and functional study. Biochimica and Biophysica Acta 1773, 880–890.
11 Thapa, N., Lee, B. H. and Kim, I. S. (2007) TGFBIp/betaig-h3 protein: A versatile matrix molecule induced by TGFβ. The International Journal of Biochemistry and Cell Biology 39, 2183–2194.
12 Dotor, J., López-Vázquez, A. B., Lasarte, J. J., Sarobe, P., García-Granero, M., Riezu-Boj, J. I., Martínez, A., Feijoó, E., López-Sagaseta, J., Hermida, J., Prieto, J., and Borrás-Cuesta. F (2007) Identification of peptide inhibitors of transforming growth factor β 1 using a phage-displayed peptide library. Cytokine 39, 106–115.
13 Ho, J., Cocolakis, E., Dumas, V. M., Posner, B. I., Laporte, S. A. and Lebrun, J. J. (2005) The G protein-coupled receptor kinase-2 is a TGFβ-inducible antagonist of TGFβ signal transduction. EMBO J. 24, 3247–3258.
14 Abraham, D. and Distler, O. (2007) How does endothelial cell injury start? The role of endothelin in systemic sclerosis. Arthritis Research and Therapy 9 Suppl 2:S2.
15 Pak, O., Aldashev, A., Welsh, D. and Peacock, A. (2007) The effects of hypoxia on the cells of the pulmonary vasculature. The European Respiratory Journal 30, 364–372.
16 Fukumura, D. and Jain, R. K. (2007) Tumor microenvironment abnormalities: causes, consequences, and strategies to normalize. Journal of Cellular Biochemistry 101, 937–949.
17 Stenmark, K. R., Fagan, K. A. and Frid, M. G. (2006) Hypoxia-induced pulmonary vascular remodeling: cellular and molecular mechanisms. Circulation Research 99, 675–691.
18 Manalo, D. J., Rowan, A., Lavoie, T., Natarajan, L., Kelly, B. D., Ye, S. Q., Garcia, J. G., and. Semenza G. L (2005) Transcriptional regulation of vascular endothelial cell responses to hypoxia by HIF-1. Blood 105, 659–669.
19 An, S. S., Pennella, C. M., Gonnabathula, A., Chen, J., Wang, N., Gaestel, M., Hassoun, P. M., Fredberg, J. J. and Kayyali, U.S (2005) Hypoxia alters biophysical properties of endothelial cells via p38 MAPK
and Rho kinase-dependent pathways. American Journal of Physiology. Cell Physiology 289, 521–530.
20 Liersch, R. and Detmar, M. (2007) Lymphangiogenesis in development and disease. Thrombosis and Haemostasis 98, 304–310.
21 Leask, A. and Abraham, D.J (2006) All in the CCN family: essential matricellular signaling modulators emerge from the bunker. J Cell Sci. 119, 4803–4810.
22 Kawamoto, T., Noshiro, M., Shen, M., Nakamasu, K., Hashimoto, K., Kawashima-Ohya, Y., Gotoh, O. and Kato, Y (1998) Structural and phylogenetic analyses of RGD-CAP/beta ig-h3,

a fasciclin-like adhesion protein expressed in chick chondrocytes. Biochimica and Biophysica Acta 1395, 288–292.
23 Skonier, J., Bennett, K., Rothwell, V., Kosowski, S., Plowman, G., Wallace, P., Edelhoff, S., Disteche, C., Neubauer, M. and Marquardt, H (1994). ßig-h3: a transforming growth factor-ß-responsive gene encoding a secreted protein that inhibits cell attachment in vitro and suppresses the growth of CHO cells in nude mice. DNA and Cell Biology 13, 571–584.
24 Kim, J.-E., Park, R.-W., Choi, J.-Y., Bae, Y.-C., Kim, K.-S., Joo, C.-K. and Kim, I.-S. (2002) Molecular properties of wild-type and mutant betaIG-H3 proteins. Investigative ophthalmology and isual science 43, 656–661.
25 Munier, F. L., Munier, F. L., Korvatska, E., Djemaï, A., Le Paslier, D., Zografos, L., Pescia, G. and Schorderet, D.F (1997) Kerato-epithelin mutations in four 5q31-linked corneal dystrophies. Nature Genetics 15, 247–251.
26 O'Brien, E. R., Bennett. K. L., Garvin, M. R., Zderic, T. W., Hinohara, T., Simpson, J. B., Kimura, T., Nobuyosh,i M., Mizgala, H., Purchio, A. and Schwartz, S.M (1996) ßig-h3, a transforming growth factor-ß-inducible gene, is overexpressed in atherosclerotic and restenotic human vascular lesions. Arteriosclerosis, Thrombosis and Vascular Biology 16, 576–584.
27 Nam, J. O., Jeong, H. W., Lee, B. H., Park, R. W. and Kim, I. S. (2005) Regulation of tumor angiogenesis by fastatin, the fourth FAS1 domain of betaig-h3, via α vβ3 integrin. Cancer Research 65, 4153–4161.
28 Kim, J. E., Kim, S. J., Lee, B. H., Park, R. W., Kim, K. S. and, Kim, I. S., (2000) Identification of motifs for cell adhesion within the repeated domains of transforming growth factor-beta-induced gene, betaig-h3. J Biol Chem. 275,30907–30915.
29 Zhang, H., Akman, H. O., Smith, E. L., Zhao, J, Murphy-Ullrich J. E. and Batuman, O. A. (2003) Cellular response to hypoxia involves signaling via Smad proteins. Blood 101, 2253–2260.
30 Dijke, P. and Arthur, H. M. (2007) Extracellular control of TGFβ signalling in vascular development and disease. Nature reviews. Molecular Cell Biology 8, 857–869.
31 Tamby, M. C., Chanseaud, Y., Guillevin, L. and Mouthon, L. (2003) New insights into the pathogenesis of systemic sclerosis. Autoimmunity Reviews 2, 152–157.
32 Kaiserling, E., Krober, S. and Geleff, S. (2003). Lymphatic vessels in the colonic mucosa in ulcerative colitis. Lymphology 36, 52–61.
33 Paavonen, K., Mandelin, J., Partanen, T., Jussila, L., Li, T. F., Ristimaki, A., Alitalo, K. and Konttinen, Y.T (2002). Vascular endothelial growth factors C and D and their VEGFR-2 and 3 receptors in blood and lymphatic vessels in healthy and arthritic synovium. The Journal of Rheumatology 29, 39–45.
34 Lee, B. H., Bae, J. S., Park, R. W., Kim, J. E., Park, J. Y. and Kim, I. S. (2006) Betaig-h3 triggers signaling pathways mediating adhesion and migration of vascular smooth muscle cells through α vβ5 integrin. Experimental and Molecular Medicine 38, 153–161.
35 Bae, J.S, Lee, S. H., Kim, J. E., Choi, J. Y., Park, R. W., Yong Park, J., Park, H. S., Sohn, Y. S., Lee, D. S., Bae Lee, E. and Kim, I.S (2002) ßig-h3 supports keratinocyte adhesion, migration, and proliferation through α 3ß1 integrin. Biochemical and Biophysical Research Communications 294, 940–948.
36 Jeong HW, Kim IS. TGFβ1 enhances betaig-h3-mediated keratinocyte cell migration through the α 3β1 integrin and PI3K. J Cell Biochem. 2004 Jul 1;92(4):770–780.
37 Nam, J. O., Kim, J. E., Jeong, H. W., Lee, S. J., Lee, B. H., Choi, J. Y., Park, R. W., Park, J. Y. and Kim, I.S (2003) Identification of the α vβ3 integrin-interacting motif of betaig-h3 and its anti-angiogenic effect. J. Biol.Chem 278, 25902–25909.
38 Semenza, G.L (2005) Involvement of hypoxia-inducible factor 1 in pulmonary pathophysiology. Chest. 128(6 Suppl), 592S-594S.
39 Stupack, D. G. and Cheresh, D. A. (2004) Integrins and angiogenesis. Current topics in developmental biology 64, 207–238.
40 Wojciak-Stothard, B., Tsang, L. Y., Paleolog, E., Hall, S. M. and Haworth, S.G (2006) Rac1 and RhoA as regulators of endothelial phenotype and barrier function in hypoxia-induced neonatal pulmonary hypertension. American J Physiol Lung Cell Mol Physiol. 290, L1173–1182.
41 Ma, C., Rong, Y., Radiloff, D. R., Datto, M. B., Centeno, B., Bao, S., Cheng, A. W., Lin, F., Jiang, S., Yeatman, T. J. and Wang, X. F. (2008) Extracellular matrix protein betaig-h3/TGFBI promotes metastasis of colon cancer by enhancing cell extravasation. Genes Dev. 22(3), 308–321.
42 Dormond, O., Ponsonnet, L., Hasmim, M., Foletti, A. and Rüegg, C. (2004) Manganese-induced integrin affinity maturation promotes recruitment of α v β3 integrin to focal adhesions in endothelial cells: evidence for a role of phosphatidylinositol 3-kinase and Src. Thromb Haemost. 92, 151–161.
43 Nilsson, I., Shibuya, M. and Wennström, S. (2004) Differential activation of vascular genes by hypoxia in primary endothelial cells. Exp Cell Res. 299, 476–485.
44 Johnson, L. A., Clasper, S., Holt, A. P., Lalor, P.F,, Baban, D. and Jackson, D. G. (2006) An inflammation-induced mechanism for leukocyte transmigration across lymphatic vessel endothelium. J Exp Med. 203, 2763–2777.
45 Oka, M., Iwata, C., Suzuki, H. I., Kiyono, K., Morishita, Y., Watabe, T., Komuro, A., Kano, M. R. and Miyazono, K. (2008) Inhibition of endogenous TGF-β signaling enhances lymphangiogenesis. Blood 111, 4571–4579.

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