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Endothelial cell–laminin interaction: modulation of LDH expression involves $\alpha_6\beta_4$ integrin–FAK–p38MAPK pathway

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Abstract One of the possible mechanisms of the angiogenic effect of laminin (Ln) involves modulation of the biological activity of VEGF by regulating poly ADP ribosylation (PAR). PAR modification of VEGF was found to be related with the changes in NAD^+ associated with a shift in LDH isoenzymes. Further investigations on LDH gene expression in HUVECs suggested that the effect of Ln was mediated through $\alpha_6\beta_4$ integrin–FAK–src-p38 MAPK pathway. This was evidenced by (a) co-immunoprecipitation of β_4 integrin with α_6 subunit, (b) activation by tyrosine phosphorylation of β_4 integrin and FAK, (c) co-immunoprecipitation of FAK with β_4 and with adapter protein, *src*, (d) increased phosphorylation of p38 MAPK in cells maintained on Ln and (e) blocking of effect of Ln on LDH-B gene expression by inhibition of p38 MAPK. Increase in serine phosphorylation of c-fos and c-jun and higher levels of heterodimers of AP-1 in the nucleus in cells maintained on Ln suggested activation of AP-1 transcription factor. These results provide evidence for modulation of endothelial cell function relevant to angiogenesis by Ln through $\alpha_6\beta_4$ integrin.

Keywords Laminin · LDH · AP-1 Transcription factor · $\alpha_6\beta_4$ integrin · FAK · p38 MAPK

Introduction

The formation of new capillary blood vessels from pre-existing vasculature, termed angiogenesis, involves complex interactions among the different types of cells, particularly endothelial cells (ECs), extracellular matrix (ECM) and soluble factors such as cytokines which lead to EC proliferation, migration and tube formation [1]. The ECM regulates angiogenesis by providing support for cell adhesion and migration and plays a critical role in establishing endothelial cell polarity and stabilizing capillary structures [2]. It can also serve as a reservoir and modulator for growth factors by sequestering angiogenic factors such as FGF-2 and VEGF.

Role of the ECM in EC function has been studied using individual components of the ECM and by molecular dissection of each component. Individual components of the ECM have been shown to promote EC adhesion, growth and migration [3]. *In vivo*, the ECs that line the lumen of blood vessels are in contact with the underlying basement membrane (BM). Laminin (Ln), the major adhesive glycoprotein of the BM, mediates diverse biological effects including assembly of the basement membrane and angiogenesis [4–7]. It promotes adhesion, migration and survival of ECs, which are critical in neovascularization [4–7] and plays a fundamental role in angiogenesis by directly affecting gene and protein expression profiles in ECs [8]. Studies carried out in our laboratory using HUVEC model system demonstrated that Ln promotes angiogenesis by modulating endothelial cell functions relevant to angiogenesis [9].

One of the mechanisms of the pro-angiogenic effect of Ln is found to be the modulation of the biological activity of VEGF by regulating its post-translational poly ADP ribosylation (PAR) modification [9]; less PAR modified VEGF is biologically more active [10]. Reduced PAR modification of VEGF is due to a decrease in the levels of NAD^+ (one of the substrates for poly ADP ribosylation reaction) in the [NAD^+/NADH] pool. This shift towards NADH is due to change in the activity of NAD^+ dependent

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dehydrogenases, particularly lactate dehydrogenase (LDH) [9]. Ln modulates the expression of LDH isoenzymes causing a shift from the A rich forms to B rich forms, which efficiently catalyzes the conversion of lactate to pyruvate and the reduction of NAD^+ to NADH [9] resulting in decreased PAR modification of VEGF and increase in its angiogenic potential.

Interaction of ECs with Ln is mediated by cell surface receptors, particularly integrin receptors [11, 12]. Ln–integrin interaction generates signals through multiple signal transduction pathways involving different components such as focal adhesion kinase (FAK), mitogen activated protein kinase (MAPK), small GTPase of the Rho family and cytoskeletal components [13]. Endothelial cells express at least 11 integrin receptors composed of different α and β subunits [14] of which $\alpha_6\beta_1$ and $\alpha_6\beta_4$ integrins are the major Ln receptors. $\alpha_6\beta_1$ integrin plays an important role in mediating adhesion of a variety of cells to Ln [15–17]. Use of blocking antibodies suggested that the mechanisms of the modulation of LDH isoenzymes in cells by Ln involved α_6 integrin dependent downstream signaling, which may contribute to the proangiogenic effects of Ln [9]. But the nature of the β subunit of the integrin and the downstream signaling were not fully understood. This study was therefore carried out to identify the Ln receptor, downstream signaling pathways recruited by it and the transcription factors responsible for modulating LDH isoenzymic pattern. The results suggested that the Ln effect is mediated through $\alpha_6\beta_4$ integrin–FAK–src-p38 MAPK pathway.

Materials and methods

Materials

MCDB131 medium, antibiotic–antimycotic solution, *o*-phenylene diamine dihydrochloride, diamino benzidine, protease inhibitor cocktail, bovine serum albumin, protein A sepharose, collagen type I, polylysine, monoclonal antibodies against protein kinase B (Akt), phospho-Thr, c-Fos, c-Jun, polyclonal phospho p38 MAPK antibody and HRP-conjugated secondary antibody were purchased from M/s Sigma Aldrich Co USA. Polyclonal antibodies against human LDH A and B forms were raised in rabbits in the laboratory. NC membrane was from BioRad, USA. Laminin-1 from EHS tumor was kindly provided by late Dr. R. Timpl, Martinsreid, Munich, Germany. Monoclonal antibodies against α_6 integrin, β_4 integrin, FAK and src (Pharmingen, USA) were kindly provided by Dr. Anil Kumar, London, UK.

Methods

Isolation and culture of HUVECs

Endothelial cells were isolated by collagenase perfusion of the umbilical vein [18, 19]. Cell preparations with viability over 90% as determined by trypan blue exclusion were used. Culture plates were coated passively overnight at 37°C with various ECM proteins (50 μg/ml) and washed with 1 M NaCl, followed by PBS. Cells in serum free MCDB131 medium were seeded in 60 mm NUNC plates pre-coated with various matrix substrata, allowed to attach for 5 h, unattached cells were removed and attached cells were maintained in culture at 37°C in a 95% air and 5% CO_2 atmosphere in a Sanyo carbon dioxide incubator. The cultures were immunostained for the expression of endothelial cell specific activities such as CD31 and vWF.

Immunoprecipitation, electrophoresis and Western blot

After exposure to different culture conditions, media and cells were collected and lysed in RIPA buffer [0.01 M Tris, pH 8.0, 0.14 M NaCl, 1% sodium deoxycholate, 0.1% SDS, (sodium ortho vanadate (1 mM) and sodium fluoride (20 mM) were added in experiments where phosphorylation was detected)] containing protease inhibitor cocktail and subjected to immunoprecipitation as described earlier [19]. Protein was estimated by the method of Lowry *et al*. [20]. For assessing phosphorylation, equivalent amount of proteins as determined by ELISA [21] were used for immunoblotting.

Proteins immunoprecipitated from cell extracts and media were subjected to SDS-PAGE [22] and transferred onto nitrocellulose membranes [23]. Immunoblot analyses were performed using desired primary antibody at a dilution of 1:1,000. The membrane was then washed and incubated with the secondary antibody conjugated to horseradish peroxidase (dilution of 1:2,000). The bands were detected by staining with DAB and the relative intensity of bands was quantitated using BioRad Quantity One version 4.5 software in a BioRad gel doc as described before [19].

Preparation of nuclear fractions of HUVEC

Nuclear fraction of HUVECs was prepared as described before [24]. Medium was removed and cell layers were washed with Ca^{2+}/Mg^{2+}-free PBS, harvested and centrifuged at 3,000 rpm for 5 min. The pellets were washed twice with ice-cold PBS, resuspended in 1 ml of fresh buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1.5 mM $MgCl_2$, 0.2% Igepal, 1.0 mM dithiothreitol,

and 0.5 mM phenylmethylsulfonyl fluoride), incubated for 5 min on ice with occasional stirring, and then centrifuged at 300 rpm for 5 min. Nuclear extracts were then released by resuspending the pellets in buffer B (20 mM HEPES, 420 mM NaCl, 0.1 mM EDTA, 1.5 mM $MgCl_2$, 25% glycerol, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 1.0 μg/l leupeptin and pepstatin) and incubating the tubes on ice for 10 min with intermittent gentle shaking. Nuclear extracts were stored at −80°C until further use.

Statistical analysis

All the data are expressed as mean with standard error of mean. The statistical significance of difference was analyzed by Duncan's one-way analysis of variance (ANOVA) using SPSS 11.0 Software. A value of $P<0.05$ was considered significant.

Results

Change in LDH isoenzymes with the progression of the culture

Zymographic analysis showed that freshly isolated cells expressed predominantly A_2B_2 and the relative amount of the different forms was in the order $A_2B_2>AB_3>B_4$ and was in the ratio 70:24:6. As the cells adhere to Ln substratum, there was a shift into B rich form. After 48 h in culture, the relative distribution of LDH isoenzymes in cells maintained on Ln substratum was in the order $B_4>AB_3>A_2B_2$ in the ratio 48:37:15 confirming our earlier report [9]. The B rich form has high affinity to lactate favouring the conversion of lactate to pyruvate and has low affinity to pyruvate [25]. The rate of conversion of lactate to pyruvate by lysates of cells maintained on Ln increased with the progress of the culture. To confirm the modulatory effect of Ln on the expression of LDH, HUVECs were maintained in culture on Ln-coated substratum and the levels of LDH subunits were determined by western blotting. After 24 h the relative level of A subunit of LDH in cells maintained on Ln was more, while after 48 h, there was a shift from A rich form to B rich form. A two fold increase in the levels of B subunit was observed with the progression of culture suggesting that the shift in isoenzymes was due to change in the level of B subunit.

β_4 subunit is associated with the α_6 integrin subunit

Immunoblot analysis of cell lysates confirmed the expression of α_6 integrin on ECs and showed that HUVECs maintained on Ln matrix substratum expressed significantly high levels of α_6 integrin, when compared to those maintained on Col I and polylysine substrata (Fig. 1A). To identify the subunit of integrin associated with α_6 integrin, α_6 subunit was immunoprecipitated from cell lysates, separated by SDS PAGE, blotted onto NC membrane and probed with antibodies against different β subunits. Results showed that the β_4 subunit was predominantly associated with the α_6 integrin subunit, (Fig. 1B) the level of which was three-fold high when compared to β_1 subunit in cells maintained on Ln substratum.

Further, the activation of the integrins was studied by immunoprecipitating β_4 and β_1 subunits from cells maintained on Ln matrix substratum, blotting onto NC membrane and probing with anti pTyr antibody. There was significant phosphorylation of Tyr of β_4 subunit, while no significant phosphorylation of β_1 subunit was observed suggesting activation of $\alpha_6\beta_4$ integrin in cells maintained on Ln (Fig. 1C).

Activated $\alpha_6\beta_4$ integrin recruits focal adhesion kinase

To examine if focal adhesion sites are formed upon activation of $\alpha_6\beta_4$ integrin in HUVECs maintained on Ln matrix substratum, β_4 subunit was immunoprecipitated from lysates of cells maintained in culture on Ln matrix substratum, electrophoresed, blotted and probed with monoclonal FAK antibody (Fig. 1D). Co-immunoprecipi-

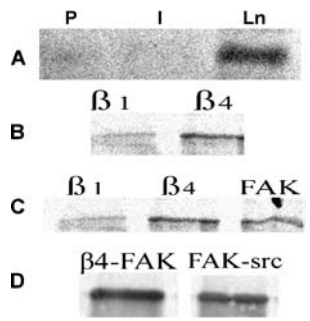

Fig. 1 α_6 integrin-generated focal adhesion signaling complex: (*A*) Freshly isolated HUVECs were maintained in culture on various substrata for 48 h and at the end of the experiment, equivalent amounts of cell protein were subjected to immunoblot using anti-α_6 integrin. *P* Polylysine, *I* Col I and *Ln* laminin. (*B*) α_6 integrin immunoprecipitated from cells cultured on Ln matrix substratum was separated by SDS PAGE, transferred to NC membrane and probed with anti β_1 integrin (β_1) and anti β_4 integrin (β_4) antibodies. (*C*) β_1 integrin (β_1), β_4 integrin (β_4) and FAK (*FAK*) were immunoprecipitated from cells maintained on Ln matrix substratum, separated by SDS PAGE, transferred to NC membrane and probed with anti pTyr antibody. (*D*) β_4 integrin immunoprecipitated from cells maintained on Ln matrix substratum was subjected to western blotting and probed with anti FAK antibody (β_4-*FAK*). FAK immunoprecipitated from cells maintained on Ln matrix substratum was subjected to western blotting and probed with anti src antibody (*FAK-src*)

tation of FAK with β_4 integrin suggested the association of FAK with the β_4 integrin subunit in cells adhered onto Ln. Further, western blotting of immunoprecipitated FAK followed by probing with monoclonal anti phospho tyrosine antibody revealed tyrosine phosphorylation of FAK suggesting FAK activation (Fig. 1C). To identify the adapter protein at the focal adhesion sites, FAK was immunoprecipitated, separated by SDS PAGE, transferred to NC membrane and probed with anti *src* antibody. Co-immunoprecipitation with FAK suggested the association and recruitment of *src* to the focal adhesion complex (Fig. 1D).

Activation of Akt and p38 MAPK

To identify the signaling pathways that are activated downstream of the focal adhesion kinase, the activation of p38 MAPK and Akt, which are two major pathways involved in the modulation of EC functions, was analyzed. As p38 MAPK activation involved phosphorylation of Ser and Thr residues, the change in the phosphorylation on p38 MAPK was studied. Equivalent amounts of p38 MAPK, as estimated by ELISA, from cells maintained on various matrix substrata were immunoprecipitated using specific p38 MAPK antibody, separated by SDS PAGE, subjected to western blotting and probed with monoclonal anti phospho serine/threonine p38 MAPK antibody. The level of Ser/Thr phosphorylation of p38 MAPK was two-fold higher in cells maintained on Ln matrix substratum when compared with those maintained on Col I and polylysine substrata indicating activation of p38 MAPK in cells maintained on Ln matrix substratum (Fig. 2).

Akt signaling pathway is a general cell survival signaling pathway. As Thr phosphorylation is involved in activation of Akt pathway, the phosphorylation of Thr on Akt in cells maintained on different matrix substrata was studied by immunoprecipitation of Akt followed by probing with pThr antibody and the results are shown in Fig. 2. In HUVECs maintained on different matrix substrata, phosphorylation of Thr of Akt was observed almost to the same extent.

p38 MAPK activates AP-1 transcription factor

Our earlier results on RT-PCR analysis showed upregulation of LDH-B gene in cells maintained on Ln substratum [9]. This effect of Ln was reversed on inhibiting p38 MAPK suggesting p38 MAPK dependent signaling pathway in the regulation of expression of LDH-B gene. Attachment of cells to Ln has been reported to induce mRNA expressions of c-fos and c-jun in different cell types [26, 27]. To examine whether adhesion of HUVECs to Ln influences the activation and nuclear translocation of AP-1

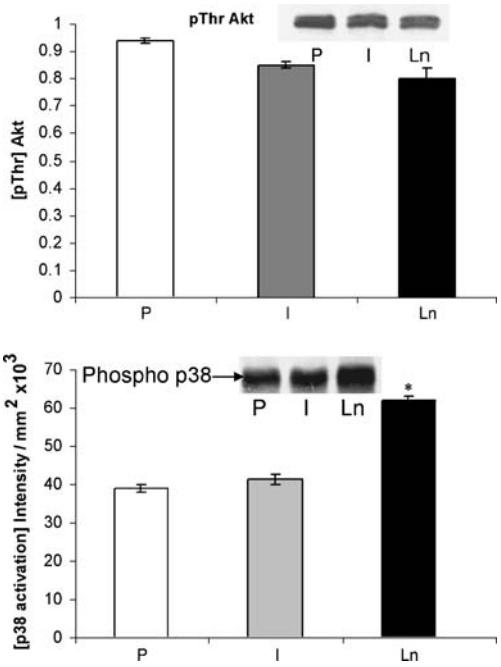

Fig. 2 Activation of Akt and p38 MAPK: HUVECs were maintained in culture on various substrata for 48 h and the activation of Akt and p38 MAPK assessed. Equivalent amounts of Akt and p38 MAPK as estimated by ELISA were subjected to immunoprecipitation with the respective antibodies, separated by SDS PAGE, transferred to NC membrane and probed with anti pThr antibodies to detect Akt (*inset in top panel*) and pSer/pThr-p38 MAPK antibody to detect p38 MAPK phosphorylation (*inset in bottom panel*). *P* Polylysine, *I* Col I and *Ln* laminin. The intensity of the bands was quantified using BioRad gel doc (pThr Akt—*top panel*; phospho p38 MAPK—*bottom panel*). The results presented are average of quadruplicate experiments±SEM. *Statistically significant compared to *P* ($P<0.05$)

transcription factor, which is reported to act downstream of p38 MAPK, the levels and distribution of c-fos and c-jun were studied. The components of the AP-1 transcription factor, c-fos and c-jun have been reported to be activated by Ser phosphorylation. Therefore, Ser phosphorylation was also studied, by immunoprecipitating c-fos and c-jun from lysates of cells maintained on various matrix substrata, western blotting and probing with anti pSer antibody. The extent of phosphorylation of both c-fos and c-jun was two-fold higher in cells maintained on Ln matrix substratum when compared with those from cells maintained on Col I substratum (Fig. 3). Analysis of the nuclear and cytoplasmic fractions of the cells maintained on various matrix substrata revealed nuclear distribution of c-fos and c-jun indicating AP-1 translocation into the nucleus (Fig. 3). Co-immunoprecipitation followed by immunoblot analysis showed almost equivalent amounts of c-fos and c-jun in cells maintained on Ln matrix substratum. But in cells maintained on Col I and polylysine substrata, relatively high amount of c-jun was observed in the nuclear fraction (Fig. 3).

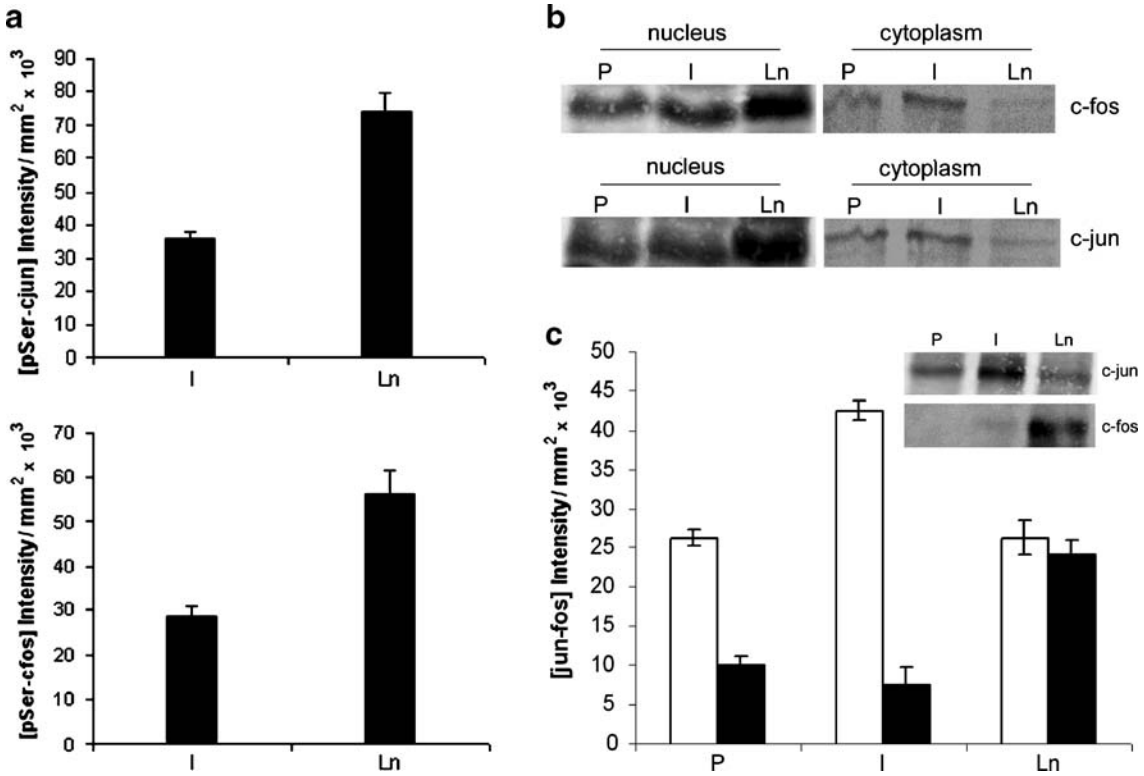

Fig. 3 (**a**) Activation of components of AP-1 transcription factor by phosphorylation: HUVECs were maintained in culture on various substrata for 48 h, the cells were harvested, equivalent amounts of c-jun (*top panel*) and c-fos (*bottom panel*) as estimated by ELISA were immunoprecipitated, separated by SDS PAGE, transferred to NC membrane and probed with monoclonal anti phospho serine antibody. *I* Col I and *Ln* laminin. The intensity of the bands was quantified using BioRad gel doc. The results presented are average of quadruplicate experiments±SEM. (**b**) Nuclear translocation of c-jun and c-fos: HUVECs were maintained in culture on various substrata for 48 h, the cells were harvested, nuclear and cytoplasmic fractions separated and analysed for the presence of c-jun (*lower panel*) and c-fos (*upper panel*) subunits of AP-1. Nuclear and cytoplasmic fractions were separated by SDS PAGE, transferred to NC membrane and probed with monoclonal anti c-jun and c-fos antibodies. *P* Polylysine, *I* Col I and *Ln* laminin. (**c**) Analysis of dimers of AP-1: HUVECs were maintained in culture on various substrata for 48 h, the cells were harvested, nuclear fraction separated and c-jun from protein equivalent samples were immunoprecipitated, separated by SDS PAGE, transferred to NC membrane and probed with monoclonal anti c-jun and c-fos antibodies (*inset*). *P* Polylysine, *I* Col I and *Ln* laminin. The intensity of the bands (*filled square*—c-fos, *empty square*—c-jun) was quantified using BioRad gel doc. The results presented are average of quadruplicate experiments±SEM

Discussion

Endothelial cells express multiple integrin receptors of which $\alpha_6\beta_4$ and $\alpha_6\beta_1$ integrins are reported to be the major receptors for Ln. Results presented here demonstrated that the principal receptor for Ln that mediates its effect relevant to angiogenesis is $\alpha_6\beta_4$ integrin. This was suggested by the following observations under conditions where Ln promoted angiogenesis. (a) Co-immunoprecipitation showed that HUVECs maintained in culture on Ln substratum expressed significantly higher amounts of β_4 integrin than those maintained on other matrix protein substrata such as Col I (Fig. 1). (b) Co-immunoprecipitation of α_6 integrin subunit with β_4 subunit from lysates of cells maintained on Ln substratum suggests that β_4 subunit is associated with α_6 subunit. (c) The relative amount of $\alpha_6\beta_4$ integrin was more than $\alpha_6\beta_1$ integrin in cells maintained on Ln substratum (Fig. 1). The importance of β_4 subunit in ECs adhering to Ln is further indicated by tyrosine phosphorylation of β_4 subunit, which is reported to undergo activation by tyrosine phosphorylation [28, 29] on binding of ligands.

Results presented here further demonstrated the role of $\alpha_6\beta_4$ integrin receptor in modulating the functions of ECs relevant to angiogenesis by Ln. This was suggested by the modulation of the expression of genes that code for lactate dehydrogenase (LDH), a key enzyme involved in the regulation of aerobic metabolism of cells, through $\alpha_6\beta_4$ integrin dependent signaling pathways. LDH is a tetrameric enzyme composed of polypeptide subunits encoded by two structurally distinct genes, LDH-A and LDH-B [30]. It is a NAD^+-dependent enzyme that catalyses the interconversion of lactate to pyruvate through oxidation–reduction reaction. Five isoenzymic forms designated as A_4, A_3B, A_2B_2, AB_3 and B_4 formed by association of A and B subunits, are

distributed in different tissues. Difference in the kinetic properties of these isoenzymes contributes to difference in the rate of utilization of pyruvate and lactate in different tissues. A_4 isoenzyme has a high V_{max} for pyruvate and thus the A rich isoenzyme is well-adapted to convert pyruvate to lactate. On the other hand, B_4 isoenzyme has a low V_{max} for reducing pyruvate to lactate and favors conversion of lactate to pyruvate. Our data suggest that Ln causes a shift from A rich form to B rich isoenzymes in endothelial cells. Immunoblotting confirmed our earlier finding and suggests that this shift in LDH isoenzymes is due to an increase in the level of B subunits due to upregulation of the expression of LDH-B gene.

Ln appears to cause upregulation of LDH-B gene in ECs through $\alpha_6\beta_4$ integrin dependent downstream signaling involving FAK-src-p38 MAPK pathway. This was suggested by (a) activation of $\alpha_6\beta_4$ integrin by Tyr phosphorylation of β_4 integrin as cells adhered to Ln (Fig. 1), (b) co-immunoprecipitation of FAK with β_4 integrin subunit and Tyr phosphorylation of FAK suggest association of FAK with $\alpha_6\beta_4$ integrin and activation of FAK (Fig. 1), (c) recruitment of src to the focal adhesion complex (Fig. 1) and (d) reversal of the effect of Ln on LDH isoenzyme pattern in cells by blocking antibody against α_6 integrin and SB202190, an inhibitor of p38 MAPK [9]. The role of p38 MAPK was further confirmed by increased activation of p38 MAPK by phosphorylation in cells maintained on Ln substratum (Fig. 2).

Our results on the activation of β_4 integrin as cells adhere to Ln are consistent with earlier reports [28, 29], which showed that ligation of $\alpha_6\beta_4$ integrin, induced tyrosine phosphorylation of the cytoplasmic domain of β_4 subunit, followed by recruitment of the adapter proteins and activation of signaling pathways. In addition to signaling function, the cytoplasmic tail of β_4 plays a crucial role in the assembly of hemidesmosomes. Integrins have been reported to activate focal adhesion signaling by tyrosine phosphorylation of the focal adhesion proteins FAK, paxillin, and p130cas, [31, 32] events that appear to be mediated by stretch-induced activation of c-Src [32]. The phosphorylation of FAK is critical to stretch-induced extracellular signal-regulated kinase (ERK) and p38 MAPK activation [33, 34]. Results presented here suggest that the engagement of $\alpha_6\beta_4$ integrin by laminin causes the recruitment and activation of FAK, which in turn recruits c-Src to the focal adhesion site. The activation of FAK caused the activation of two major pathways viz. Akt and p38 MAPK as evidenced by their phosphorylation mediated activation. Akt is a general cell survival pathway, which was seen to be activated to the same level in cells maintained on different matrix substrata. The activation of p38 MAPK was, however, significantly high in cells maintained on Ln matrix substratum thus suggesting that the modulation of LDH by Ln is mediated through p38 MAPK.

Although it is not clear how the expression of LDH-A and LDH-B genes is coordinated, expression of LDH-A gene is reported to be modulated by hypoxia inducible factor and cAMP-mediated pathways [35]. But the regulation of expression of LDH-B gene is less understood. A search for specific transcription factor responsive elements revealed that promoter sequence of LDH-B gene has a number of transcription factor binding sequences including sites for AP-1, HNF-1 and c-myb. Of these transcription factors, AP-1 appears to be particularly significant in this context as p38 MAPK is reported to modulate its activity [36]. AP-1 is a group of basic leucine zipper (bZIP) transcription factors consisting of the Fos (c-Fos, FosB, Fra1, and Fra2) and Jun (c-Jun, JunB, and JunD) families [37, 38]. These transcription factors in the cytoplasm are activated by phosphorylation in response to stimuli and translocated into the nucleus, where it modulates the expression of target genes. Activation of AP-1 in HUVECs by Ln was indicated by higher Ser phosphorylation of c-fos and c-jun and their translocation into the nucleus (Fig. 3). Although we could not provide direct proof for the role of AP-1 transcription factor, our data on the activation of p38 MAPK, translocation of AP-1 to nucleus and the reversal of these effects by inhibitor of p38 MAPK, provide indirect evidence for the role of AP-1 in Ln mediated regulation of LDH-B gene expression and shift in LDH isoenzyme pattern in ECs.

Results presented here and that reported earlier [9] suggest that Ln, a basement membrane protein, can affect the aerobic metabolism of endothelial cells by modulating the expression of LDH isoenzymes, thus suggesting that ECM is not a mere scaffold support for the cells, rather, it can regulate the metabolic activity of cells by modulating gene expression. There are reports showing the modulation of expression of isocitrate dehydrogenase isoenzymes in mammary epithelial cells by ECM proteins [39]. The consequence of the shift in LDH isoenzymes in cells maintained on Ln is reflected in the increase in the rate of conversion of lactate to pyruvate and a decrease in the level of NAD^+ consequent on its conversion by reduction to NADH. Decrease in NAD^+ reduces the rate of poly ADP ribosylation of VEGF, making it biologically more active [9]. It therefore appears that one of the possible mechanisms contributing to the pro-angiogenic effect of Ln is the modulation of the angiogenic potency of VEGF through changes in its post-translational modifications consequent on changes in the expression of a key NAD^+-dependent dehydrogenase, viz., LDH by endothelial cells through $\alpha_6\beta_4$ integrin–FAK–src-p38 MAPK dependent signaling pathway. These observations provide further evidence in

support of the role of Ln in the modulation of endothelial cell functions relevant to angiogenesis through $\alpha_6\beta_4$ integrin.

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