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Modulation of Expression of LDH Isoenzymes in Endothelial Cells by Laminin: Implications for Angiogenesis

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Abstract Endothelial cell (EC) matrix interaction is critical in angiogenesis. Although matrix components can regulate the process of angiogenesis by acting as a reservoir of various cytokines, it is not clear if extracellular matrix (ECM) can modulate the production and activity of angiogenic cytokines. Investigations were therefore carried out to study the influence of the basement membrane (BM) protein, laminin (Ln) on the activity of vascular endothelial growth factor (VEGF), the major angiogenic cytokine, using isolated human umbilical vein ECs (HUVECs) in culture. Analysis of the biochemical markers of angiogenesis confirmed proangiogenic effect of Ln. The levels of VEGF protein and mRNA were not different in cells maintained on Ln, collagen I or polylysine substrata. Chorioallantoic membrane assay using VEGF isolated from cell extracts however revealed that Ln increased its angiogenic potency. Immunoblotting and HPLC analysis showed considerable reduction in poly adenosyl ribosylation of VEGF associated with a significant decrease in the levels of NAD^+, in cells maintained on Ln substrata. Further, a shift in the isoenzymic pattern of LDH towards the B rich forms and an upregulation of LDH B gene were observed in cells maintained on Ln. Ln modulates expression of LDH gene through $\alpha_6\beta_4$ integrin mediated downstream signaling involving p38 mitogen activated protein kinases (MAPK) pathway. It thus appears that Ln can affect aerobic metabolism of ECs by modulating the expression of LDH isoenzymes resulting in a decrease in the level of NAD^+ that can cause a reduction in the poly adenosyl ribosylation of VEGF altering its angiogenic potency. J. Cell. Biochem. 103: 1808–1825, 2008. © 2007 Wiley-Liss, Inc.

Key words: HUVECs; Laminin; VEGF; Poly adenosyl ribosylation; Lactate dehydrogenase

Angiogenesis involves the migration, proliferation, and differentiation of endothelial cells (ECs) resulting in capillary tube formation [Risau, 1997]. Adherence of ECs to the extracellular matrix (ECM) is a critical step in the process of neovascularization. Apart from providing mechanical structural support and maintenance of polarity, the ECM supports cellular functions by regulating multiple signaling pathways that in turn modulates cell shape, survival, and apoptosis. ECM exerts its effect by interacting with cells through specific cell surface molecules including signal transducing integrin receptors [Davis and Senger, 2005]. Many of the interstitial and provisional ECM components are potent regulators of EC migration [Dejana et al., 1985; Senger et al., 2002]. Effect of ECM studied using individual components shows that laminin (Ln), the major glycoprotein of the basement membrane (BM) which separates the endothelium from the underlying stroma is proangiogenic in nature [Dixelius et al., 2004] unlike other components such as thrombospondin I and endostatin which are antiangiogenic [Streit et al., 1999; Kim et al., 2000].

The process of angiogenesis is also regulated by cytokines, most important of them being vascular endothelial growth factor (VEGF). It is an EC specific mitogen [Torry et al., 1995; McLaren et al., 1996; Xiong et al., 1998] characterized as a heparin binding angiogenic growth factor. The biological activity of VEGF has been reported to be modulated at the transcriptional, post-transcriptional and post-translational levels in several cell types [Xiong et al., 1998; Neufeld et al., 1999; Semenza,

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2001]. One of the major post-translational modifications reported for VEGF is poly ADP ribosylation (PAR). PAR modification of VEGF has been reported to inhibit its angiogenic activity [Xiong et al., 1998]. Metabolite such as lactate also has been reported to influence the status of PAR modification of VEGF, making it more potent biologically [Xiong et al., 1998; Kumar et al., 2007]. Although ECM influences the availability of cytokines, either by serving as a reservoir to control their release to the cells [Davis and Senger, 2005] or by participating in crosstalk between integrins and cytokine receptor specific pathways [Constant and Colognato, 2004], it is not clear whether ECM can influence the production and activity of cytokines. However, exposure of IBE cells to Ln 1 has been reported to increase the expression of FGF2 [Dixelius et al., 2004].

Ln promotes EC adhesion, migration, proliferation, and multicellular organization during angiogenesis [Aumailley and Smyth, 1998; Gonzalez et al., 2002]. Impaired microvessel formation on deletion of Ln α4 chain and increased vascularization of the tumor in human adeno carcinoma upon overexpression of LN α1 chain demonstrate the critical role of this BM adhesion protein in promoting angiogenesis [De Arcangelis et al., 2001; Thyboll et al., 2002]. Molecular dissection of Ln revealed the presence of multiple active sites for angiogenic regulation [Malinda et al., 1999]. Ln appears to exert its effect through specific integrin mediated downstream signaling [Davis and Senger, 2005]. However, some peptide sequences in Ln bearing the angiogenic regulatory property appear to modulate the angiogenic effect through interaction with other cell surface molecules such as syndecans [Mochizuki et al., 2007]. Though Ln may play a fundamental role in angiogenesis by directly affecting gene and protein expression profile in ECs [Dixelius et al., 2004], its role in switching these cells to angiogenic phenotype is less understood. The present study was aimed to examine whether Ln can modulate the production and biological activity of the major angiogenic cytokine viz. VEGF. For this, human umbilical vein ECs (HUVECs) were maintained on different matrix protein substrata including Ln and the levels of mRNA, protein, and biological activity of VEGF were analyzed. The results suggest that Ln can influence the biological activity of VEGF produced by HUVECs by modulating the expression pattern of lactate dehydrogenase (LDH) isoenzymes.

MATERIALS AND METHODS

Materials

MCDB131 medium, antibiotic–antimycotic solution, *o*-phenylene diamine dihydrochloride, DEPC, diamino benzidine, Tris, glycine, protease inhibitor cocktail, bovine serum albumin, protein A sepharose, collagen type I, polylysine, monoclonal antibodies against VEGF (lyophilized purified immunoglobulin), E-selectin, von Willebrand factor (vWF) (Factor VIII antigen), CD31 (PECAM1), polyclonal phospho p38 mitogen activated protein kinases (MAPK) antibody, HRP-conjugated secondary antibody, and snake venom phosohodiesterase were purchased from M/s Sigma–Aldrich Co. Perfect RNA Mini isolation kits and C- Master RT Plus PCR kits were purchased from Eppendorf, Germany. NC membrane was from BioRad. Ln-1 from EHS tumor was kindly provided by late Dr. R. Timpl, Martinsreid, Munich, Germany. Monoclonal anti-α6 integrin (calbiochem) was kindly provided by Dr. Anil Kumar, London, UK. Tissue culture plastic wares were purchased from NUNC, Denmark. Monoclonal anti-PAR antibody was purchased from Trevigen.

Methods

Isolation and Culture of HUVECs. ECs were isolated by collagenase perfusion of the umbilical vein [Jaffe et al., 1973]. The viability of isolated HUVECs was determined by trypan blue exclusion. 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 thrice. Cells in serum free MCDB131 medium (medium that selectively promotes the growth of ECs) were seeded in 60 mm NUNC plates pre-coated with various matrix substrata, allowed to attach overnight, 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 EC specific activities such as CD31 and vWF.

ELISA. Amount of E-selectin, vWF, and CD31 (PECAM1) were quantitated by ELISA [Engvall and Perlman, 1971] using HRP

conjugated secondary antibody. *o*-phenylene diamine dihydrochloride was used as substrate. Color intensity at 495 nm was read in a multiwell microplate reader (Thermo Multiskan Spectrum).

Isolation of VEGF and Assay of Angiogenic Activity. HUVECs were maintained in culture in 60 mm culture dishes precoated with various matrix substrata, in MCDB131 medium for 48 h as described above. VEGF from the cells and media were affinity isolated on heparin sepharose [Kumar et al., 2007]. Assay of angiogenic activity of the heparin binding fraction was carried out using aliquots of the eluted samples containing equivalent amounts of VEGF as determined by ELISA, in chicken chorioallanotic membrane (CAM) assay model.

Chicken Chorioallanotic membrane (CAM) Assay. Chicken chorioallanotic membrane (CAM) assay was performed as described earlier [Ribatti et al., 1997; Kumar et al., 2007]. Briefly, samples soaked in filter disc were applied on to the CAM of 4 days old chick embryo and further incubated for 8 days. At the end of the incubation period, the CAMs were photographed and hemoglobin was estimated in the CAM using Drabkin's reagent as a measure of vessel density [Ribatti et al., 1997]. CAMs treated with filter discs soaked in TBS served as the vehicle control.

Immunoprecipitation 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 and sodium fluoride were added in experiments where phosphorylation was detected)] containing protease inhibitor cocktail and subjected to immunoprecipitation as described earlier [Kumar et al., 2007]. Protein was estimated by the method of Lowry et al. [1951].

Proteins immunoprecipitated from cell extracts and media were subjected to Western blot analysis [Towbin et al., 1979]. Proteins were separated on a 10% polyacrylamide gel [Laemmli, 1970] and transferred onto nitrocellulose membranes. 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.

Relative Quantitative Reverse Transcription PCR (RT-PCR). Total RNA from HUVECs maintained in culture was isolated using Perfect RNA Mini isolation kit according to manufacturer's instructions. The nucleotide sequence of primer pairs used to determine the levels of human VEGF, VEGF R2, LDH A, LDH B, and GAPDH mRNA were as follows:

VEGF (105 bp)

sense primer-5′-ACGATCGATACAGAAACCACG-3′ and
antisense primer-5′-CTCTGCGCAGAGTCTCCTCT-3′,

VEGF R2 (216 bp)

sense primer-5′-TGCACTGCAGACAGATCTAC-3′ and
antisense primer-5′-GCAGACATAGTCTCCTTGGT-3′

LDH A (407 bp)

sense primer-5′ATACACTTTGGGGGATCCAAAAGGA-3′ and
antisense primer-5′AAAAAATGTTGGACTAGGCATGTTC-3′

LDH B (249 bp)

sense primer-5′-AGTCAGTCTGCCAAGTCATTC-3′ and
antisense primer-5′-AAGTTTCCATTTTAGGGGTTCTTC-3′

GAPDH (680 bp)

sense Primer-5′-CGGAGTCAACGGATTTGGTCGTAT-3′ and
antisense primer-5′-GCAGGTCAGGTCCACCACTGAC-3′.

The primer sequences were selected from NCBI nucleotide database and custom synthesized from Sigma Chemicals, Bangalore. Reverse transcription PCR (RT-PCR) was performed in an Eppendorf thermocycler, as described before [Kumar et al., 2007].

Assay of NAD^+. The concentration of NAD^+ was estimated fluorimetrically as described earlier [Estabrook et al., 1968]. The increase in fluorescence occurring on addition of alcohol dehydrogenase (1,500 units) was determined by excitation at a wavelength of 340 nm and emission at a wavelength of 470 nm in a Perkin-Elmer LS45 luminescence spectrometer [Kumar et al., 2007].

Assay of enzymes. Glyceraldehyde phosphate dehydrogenase (GAPDH) [Krebs, 1955], isocitrate dehydrogenase [Bodansky et al., 1960], and malate dehydrogenase [Lang-Unnasch, 1992] were assayed spectrophotometrically.

Briefly, the enzyme extracts were incubated in appropriate buffers (sodium pyrophosphate buffer pH 8.5 for GAPDH; Tris HCl pH 7.5 for isocitrate dehydrogenase; and phosphate buffer pH 7.4 for malate dehydrogenase) in the presence of various substrates (0.015 M DL-glyceraldehyde-3-phosphate and 7.5 mM NAD for GAPDH; 0.1 M isocitrate and 1 mM NAD for isocitrate dehydrogenase; 35 mM oxaloacetate and 2.5 mM NADH for malate dehydrogenase) and the shift in optical density at 340 nm was recorded. Enzyme activity was expressed in terms of micromoles of NAD or NADH utilized in the reaction.

Zymographic analysis of LDH isoenzymes. LDH (EC 1.1.1.27) isoenzymes were analyzed by separation on native PAGE (7.5% gel) followed by staining for LDH activity. LDH isoenzymes were stained for 10 min in the dark at 37°C in a reaction medium consisting of 50 mM Tris (pH 8.0), 10 mM lactic acid, 0.5 mg/ml NAD^+, 0.2 mg/ml nitro-blue tetrazolium, and 0.05 mg/ml phenazine methosulfate. The bands were scanned using a BioRad gel documentation system. The LDH isoenzyme data were expressed as the proportion of each isoenzyme band relative to the total. Total LDH activity was calculated as the sum of the intensities of all bands in a lane.

Digestion of VEGF bound PAR and quantitation using HPLC. The extent of PAR modification of VEGF was further analyzed by digesting equivalent amounts of VEGF secreted by cells maintained on various matrix substrata with pronase, followed by phosphodiesterase. Briefly, affinity purified VEGF from the culture media were subjected to pronase digestion (1 mg/ml pronase in Tris HCl buffer, pH 7.5) for 1 h [Shima et al., 1970], then the reaction was terminated by adding 15% perchloric acid. The supernatant was neutralized with 1.8 N KOH and the purity of PAR confirmed by measuring the ratio of A_{230} to A_{260}, which was 0.2389 [Kanai et al., 1980]. The supernatant was further subjected to digestion using phosphodiesterase (100 μg snake venom phosphodiesterase/ml of Tris HCl buffer pH 8 containing 1 mM $MgCl_2$) for 1 h. The reaction was stopped by the addition of 15% perchloric acid and the supernatant neutralized with KOH. The major product of phosphodiesterase digestion, phosphoribosyl AMP was quantitated by HPLC with ADP ribose as the standard, as PR-AMP corresponds to φADP ribose [Fujimura et al., 1967]. The amount of PR-AMP liberated was quantitated at 260 nm by isocratic HPLC using a C18 column with a solvent system consisting of potassium phosphate buffer (pH 6): methanol (75:25), in a Shimadzu LC-10AT instrument.

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

Angiogenic Nature of Matrix Substrata

To study the effect of Ln on EC function, particularly relevant to angiogenesis, HUVECs were maintained on petridishes passively coated with Ln and collagen type I (Col I). Cells maintained on polylysine coated petridishes served as controls. EC-constitutive activity such as vWF and angiogenic marker activities such as production of E-selectin and CD31 were analyzed and the results are presented in Figure 1. The level of vWF was not significantly different in cells maintained on various matrix substrata. After 48 h of culture, the level of E-selectin was twofold higher in cells maintained on Ln substratum as compared to those on Col I and 2.6-fold higher than those maintained on polylysine coated plates. The levels of CD31 were threefold higher in cells maintained on Ln substratum as compared to cells on Col I and polylysine coated plates after 48 h in culture.

VEGF and VEGF R2 Expression by HUVECs Cultured on Different Substrata

To examine whether the proangiogenic effect of Ln was due to a change in the production of VEGF, the levels of VEGF mRNA and that of the VEGF protein were quantitated. There was no significant difference in the expression of VEGF mRNA among cells maintained on Ln, Col I, and polylysine coated substrata (Fig. 2). The expression of VEGF receptor 2 (VEGF R2) was 73% more in cells maintained on Ln as compared to those on Col I matrix and threefold greater than those maintained on polylysine coated plates.

Cells maintained on different matrix substrata synthesized and secreted VEGF. Immunoblot analysis of media, with monoclonal anti-VEGF antibody revealed two bands of ~50 and ~20 kDa (Fig. 2B(a)). The amount of VEGF

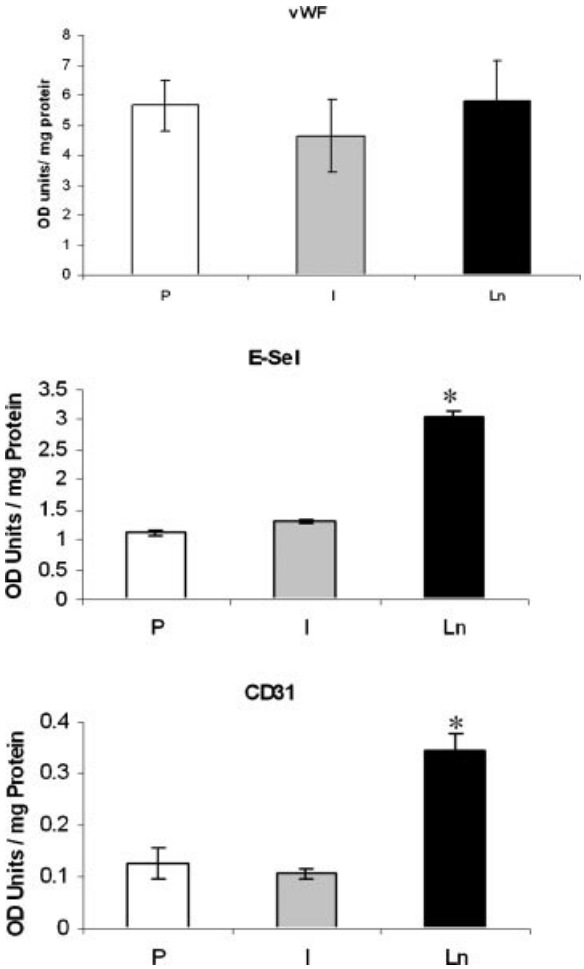


Fig. 1. Analysis of biochemical markers of angiogenesis: isolated HUVECs were maintained in culture on Ln, Col I (I), and polylysine (P) coated plates for 48 h in MCDB131 medium in a carbon dioxide incubator. At the end of the experiment, cells were harvested and the levels of vWF and E-selectin in medium and CD31 in cell layer were determined by ELISA. The values given are the average of quadruplicate experiments ± SEM. *Statistically significant compared to P ($P < 0.05$).

produced by cells maintained on Ln, Col I, and polylysine substrata, determined by ELISA (Fig. 2B(b)) were not significantly different.

Angiogenic Activity of VEGF Isolated From Cells Maintained on Various Matrix Substrata

Since there was no significant difference in the mRNA and protein levels of VEGF, and a significant increase in the expression of VEGF R2 (a specific receptor for VEGF) was observed, the possibility of difference in the biological activity of VEGF produced by cells maintained on the different substrata was examined. VEGF was affinity isolated on heparin-sepharose and its angiogenic potency was tested in CAM angiogenesis model. VEGF isolated from cells maintained on Ln had significantly higher angiogenic potency as compared with VEGF isolated from cells maintained on Col I and polylysine substrata (Fig. 3). The vessel density in terms of hemoglobin was threefold higher in CAMs treated with VEGF isolated from medium and cells maintained on Ln as compared with those on Col I and polylysine substrata. Since equal amounts of VEGF isolated from various groups were applied on to the CAMs and a higher angiogenic response was shown by VEGF from cells maintained on Ln, a shift in the angiogenic potency of VEGF in cells maintained on Ln was evident. The removal of VEGF by immunoprecipitation from the heparin isolated fractions reduced the angiogenic potency of the extracts significantly, suggesting VEGF to be the major angiogenic factor in the extracts.

Post-Translational Modification of VEGF

The possibility of post-translational modification affecting the angiogenic activity of VEGF that is, PAR modification of VEGF was analyzed. Equivalent amounts of VEGF was immunoprecipitated using anti-VEGF antibody from the cell extracts and media of cells maintained in culture on different matrix proteins, separated by SDS PAGE, blotted on to NC and probed with anti-PAR antibody. Two bands corresponding to ~50 and ~20 kDa were identified by anti-PAR antibody (Fig. 4). The intensity of both 50 and 20 kDa bands was significantly low in cells maintained on Ln as compared to cells on other substrata, suggesting that PAR modification of the VEGF produced by cells maintained on Ln decreased significantly when compared to cells maintained on other substrata.

PAR modification of VEGF was confirmed and quantitated by digesting equivalent amounts of VEGF secreted by cells maintained on various matrix substrata successively with pronase and phosphodiesterase, and detection of the reaction product phospho ribosyl AMP (PR-AMP) by HPLC. The amount of PR-AMP was significantly low in VEGF isolated from cells maintained on Ln substratum as compared to that from cells maintained on Col I and polylysine (Fig. 5).

Estimation of NAD^+ Levels

To examine how alteration in PAR modification of VEGF has occurred in cells maintained

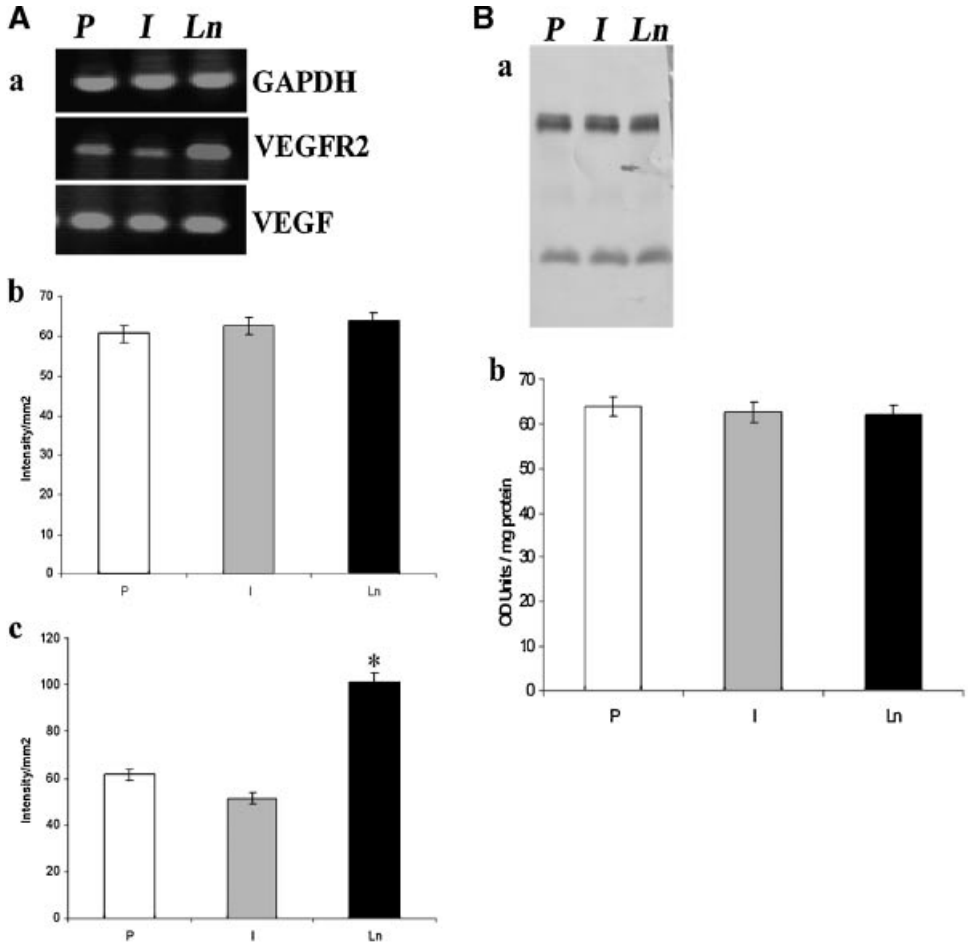


Fig. 2. RT-PCR analysis of VEGF and VEGF R2 expression and estimation of VEGF production: (**A**) isolated HUVECs were maintained in culture on different matrix substrata as described in legends to Figure 1. At the end of the experiment, cells were harvested, total RNA isolated and mRNA levels of VEGF and VEGF R2 were analyzed by semi-quantitative RT-PCR as described in methods. The products of PCR were separated on 1.75% agarose gel and bands visualized by ethidium bromide staining (**a**). **Lane 1**—polylysine, **Lane 2**—Col I, and **Lane 3**—Ln. Relative intensity of bands were quantitated by BioRad Quantity One software and normalized with the intensity of the band for internal control (GAPDH). The results presented are average of quadruplicate experiments ± SEM. VEGF (**b**), VEGF R2 (**c**) *statistically significant compared to control ($P < 0.05$). **B**: For the estimation of VEGF, HUVECs were maintained in MCDB131 medium on various matrix substrata and cell culture medium was collected, VEGF affinity isolated on heparin-sepharose beads, electrophoressed and detected by Western blot analysis using monoclonal anti-VEGF (**a**) **Lane 1**—polylysine (P), **Lane 2**—Col I (I), and **Lane 3**—Ln. The level of VEGF was estimated by ELISA (**b**). The results presented are average of quadruplicate experiments ± SEM.

on different matrix substrata, the level of NAD^+, the principal substrate involved in poly adenosyl ribosylation reaction, was estimated (Fig. 6). The level of NAD^+ in cells maintained on Ln was less than 50% of that in cells maintained on Col I or polylysine substrata, suggesting a shift in the NAD^+/NADH pool towards NADH in cells maintained on Ln.

Modulation of LDH Activity by Matrix Protein Substratum

As the levels of NAD^+ and NADH in the cells are being regulated by various dehydrogenases, the activities of some of the key dehydrogenases were assayed. No significant difference or marginal difference in the activity of GAPDH (Polylysine—$25.2 \pm 2.2 \times 10^{-3}$, Col I—$22.6 \pm 1.26 \times 10^{-3}$, Ln—$23.8 \pm 1.54 \times 10^{-3}$), isocitrate dehydrogenase (Polylysine—$10.6 \pm 0.52 \times 10^{-3}$, Col I—$14.02 \pm 1.2 \times 10^{-3}$, Ln—$13.8 \pm 1.24 \times 10^{-3}$), and malate dehydrogenase (Polylysine—$12.05 \pm 1.065 \times 10^{-3}$, Col I—$11.86 \pm 1.26 \times 10^{-3}$, Ln—$13.23 \pm 1.42 \times 10^{-3}$) was observed in cells maintained on various matrix substrata. The activity of LDH was however significantly low in cells maintained on Ln substratum when

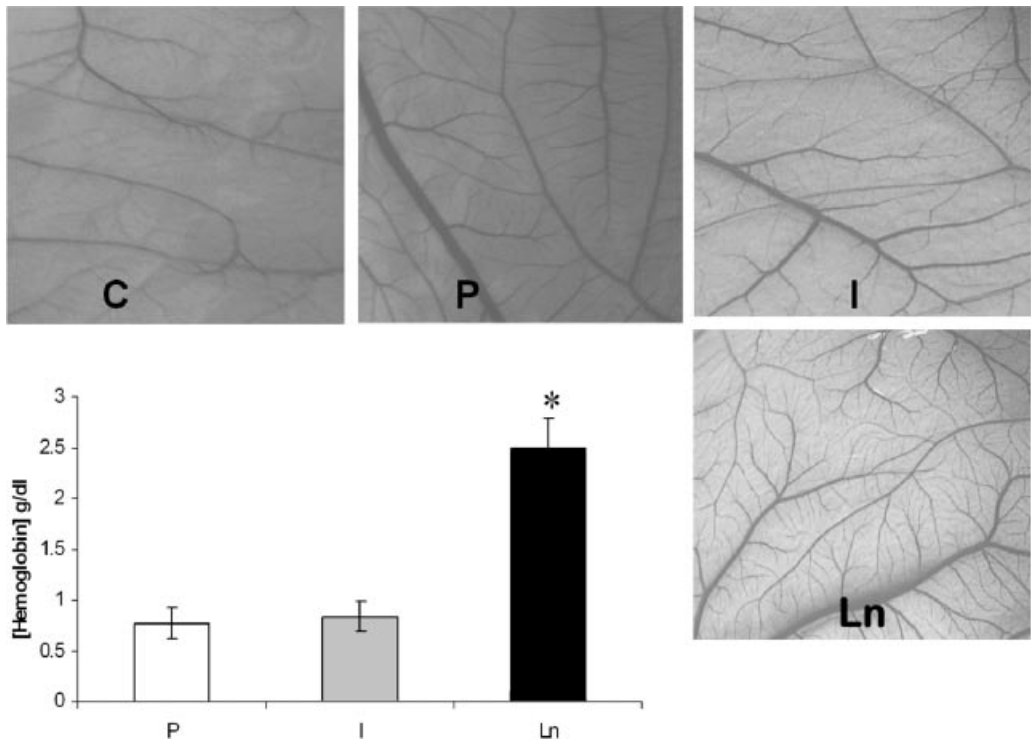


Fig. 3. Angiogenic activity of VEGF isolated from cells cultured on various matrix protein substrata: HUVECs were maintained in MCDB131 medium on various matrix protein substrata. Heparin binding fraction of the cell extracts from all the groups equivalent to same amount of VEGF (as estimated by ELISA) were applied on to the CAMs of 4 days old chick embryos. After 8 days from treatment, the vessel density in the CAM were photographed and quantitated in terms of hemoglobin. The **topmost panel** shows photographs of vessel densities and below is given the levels of hemoglobin, less the vehicle control in each group. Vehicle control (c), heparin binding fraction of extract of cells maintained on polylysine (P), Col I (I), Ln substrata. The results presented are average of quadruplicate experiments ± SEM. *Statistically significant compared to P ($P < 0.05$).

compared to those on Col I and polylysine coated surfaces (Polylysine—$630.3 \pm 20.2 \times 10^{-3}$, Col I—$519.65 \pm 46.3 \times 10^{-3}$, Ln—$300 \pm 36.26 \times 10^{-3}$). The possibility of change in the level of NAD^+ as a result of changes in the activity of LDH was examined by assaying the forward and backward reactions of LDH and the results are presented in Figure 7. After the first 24 h of culture, the distribution of forward and backward reaction of LDH that is, conversion of pyruvate to lactate and lactate to pyruvate was in the ratio 60:40, in cells maintained on Ln, Col I, and polylysine coated plates. The distribution of forward to backward reaction after 48 h of culture was significantly different from that of the first 24 h, in cells maintained on Ln, whereas this was unchanged in cells maintained on other substrata. The activity of the enzyme for forward reaction (pyruvate to lactate) was 29% and that of backward reaction (lactate to pyruvate) was 71% of the total LDH activity in cells maintained on Ln, indicating a shift in the relative rate of utilization of the substrate, lactate which in turn can cause a shift in the NAD^+/NADH pool towards NADH reducing the levels of NAD^+.

Modulation of LDH Isoenzymic Pattern by Ln Substratum

The LDH isoenzymes produced by the cells maintained on various matrix protein substrata were determined by zymography (Fig. 8). The major isoenzymic form in freshly isolated cells was A2B2 isoform. In cells maintained for 24 h in culture on various substrata, the predominant form was still A2B2 with AB3 and B4 as minor forms. The isoenzymic pattern of the cells maintained on Ln however changed by 48 h of cells in culture with a shift from the A2B2 to B4 form. The level of B4 isoenzyme increased from about 17 to 45% in cells maintained on Ln as compared to 10 to 14% in cells maintained on Col I. The levels of B4 isoenzyme increased with

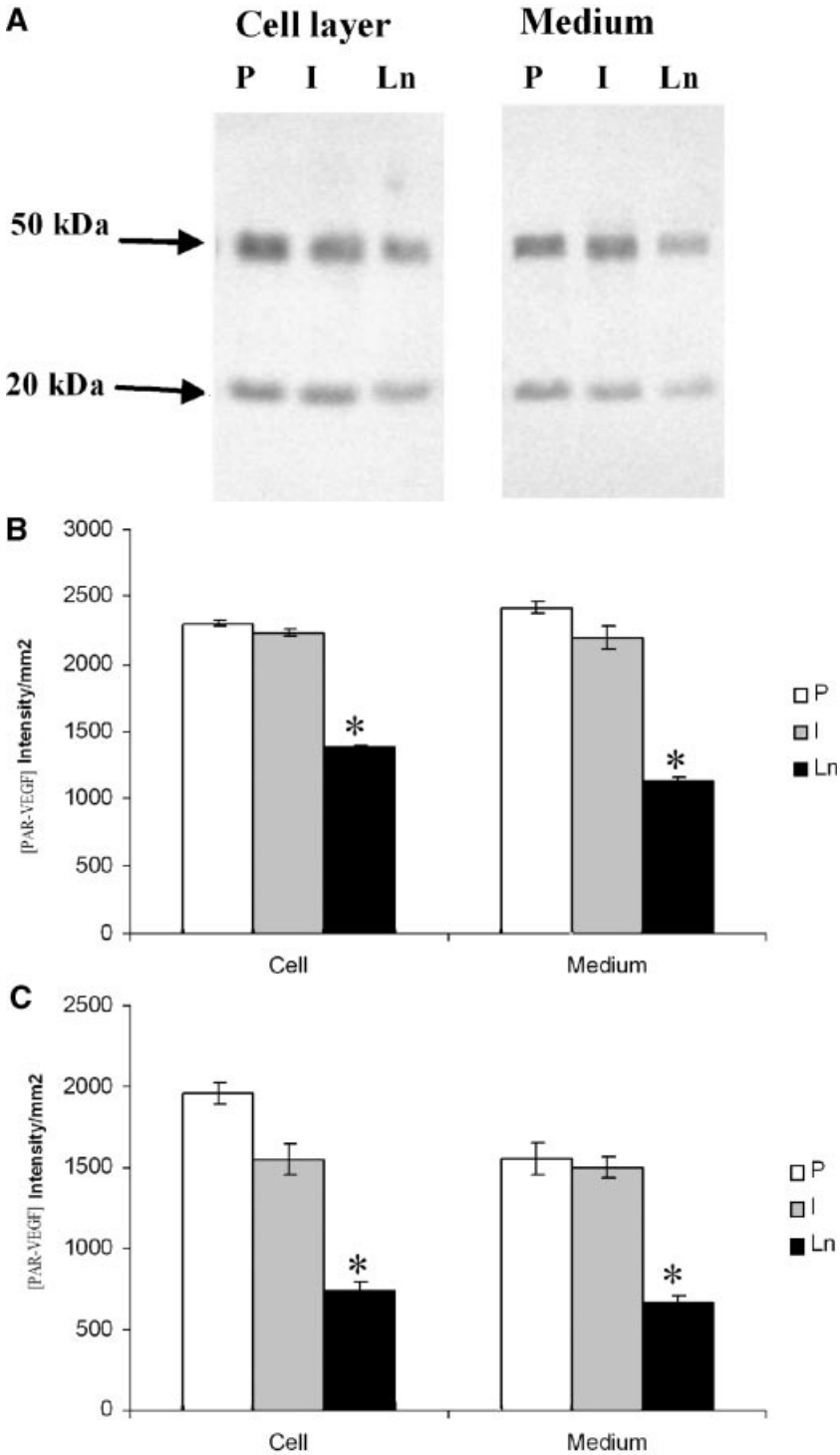
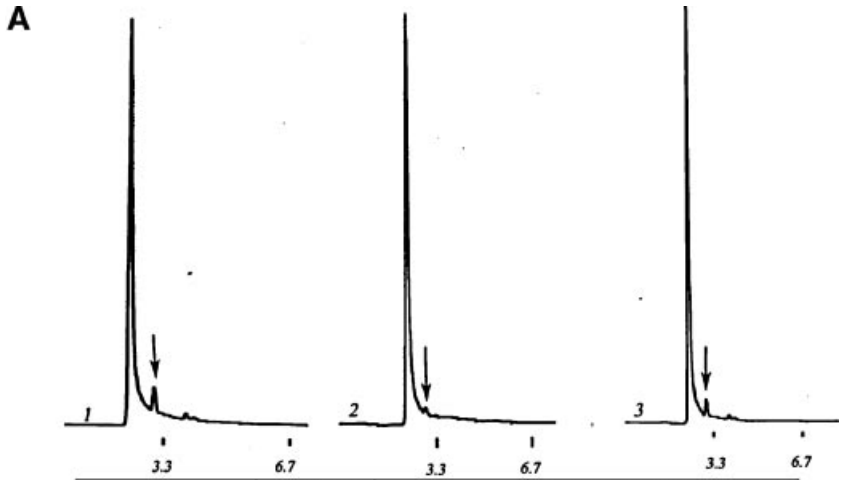


Fig. 4. PAR modification of VEGF: HUVECs were maintained in MCDB131 medium on various matrix protein substrata as described in legends to Figure 1 for 48 h. VEGF, immunoprecipitated from cell extracts and media, were separated by SDS–PAGE, transferred to NC membrane and probed with anti-PAR antibody (**A**). P—polylysine, I—Col I, and Ln—Laminin. The intensity of the bands, ~50 kDa (**B**) and ~20 kDa (**C**) was quantified using BioRad gel doc and plotted. The results presented are average of quadruplicate experiments ± SEM. *Statistically significant compared to *P* ($P < 0.05$).



Sample	Retention Time	Peak area	Concentration of PR-AMP (nM/ µg VEGF)
Poly lysine	3.01	77	520 ± 23
Col I	3.03	225	1487.5 ± 83.2
Ln	3.03	37	259.8 ±17.8
Poly lysine + SB	3.02	100	675.3 ± 12.3
Col I + SB	3.03	232	1566.7 ± 26.8
Ln + SB	3.03	201	1268 ± 68.7

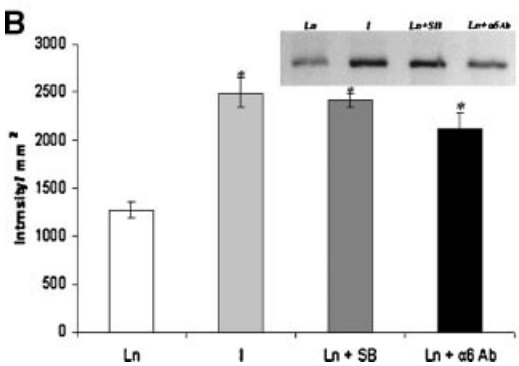


Fig. 5. Changes in the levels of VEGF bound PAR: (**A**) HUVECs were maintained in MCDB131 medium on various matrix substrata as described in legends to Figure 1 for 48 h. Heparin binding fraction of the medium from all the groups equivalent to same amount of VEGF (as estimated by ELISA) were sequentially digested with pronase and phosphodiesterase for 60 min each as described in text. The protein was removed after each digestion using perchloric acid and the amount of PR-AMP in the neutralized supernatant after phosphodiesterase digestion was quantitated by HPLC as described in Section ''Methods.'' 1—Cells maintained on Col I; 2—cells maintained on Ln; and 3—cells maintained on Ln and treated with SB 202190. The values presented in tabular format are mean of duplicate experiments. **B**: HUVECs were maintained in MCDB131 medium on various matrix protein substrata as described in legends to Figure 1 for 48 h. Equivalent amounts of VEGF, immunoprecipitated from media were separated by SDS–PAGE, transferred to NC membrane and probed with anti-PAR antibody. Ln—cells maintained on Ln; I—cells maintained on Col I; Ln + SB—cells maintained on Ln and treated with SB 202190; Ln + α6 Ab—cells maintained on Ln and treated with α6 blocking antibody. The results presented are average of quadruplicate experiments ± SEM. *Statistically significant compared to Ln control ($P < 0.05$).

a concomitant decrease in the relative levels of A2B2 isoforms whereas the levels of AB3 form remained almost unaltered.

Differential Expression of LDH A and LDH B Gene

To examine if the shift in the isoenzymic pattern of LDH was a consequence of shift in the expression pattern of the genes encoding the two chains of LDH that is, LDH A and LDH B, the levels of LDH A and LDH B mRNA were analyzed by semi-quantitative RT-PCR (Fig. 9). HUVECs in culture expressed both LDH A and LDH B genes. After 24 h of cells in culture, LDH A gene expressed by cells maintained on Ln, Col I, and polylysine coated plates was about 60% of the total and LDH B gene expression was 40%.

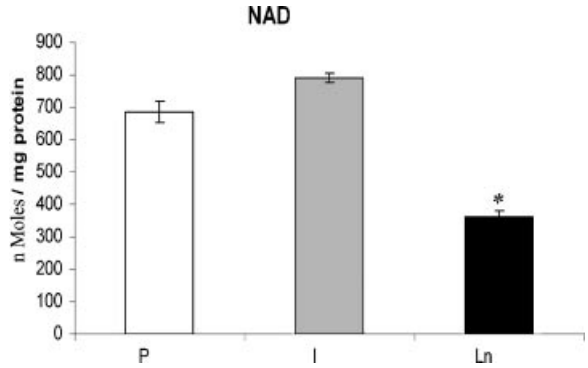


Fig. 6. Levels of NAD^+: HUVECs were maintained in MCDB131 medium on various matrix protein substrata as described in legends to Figure 1 for 48 h and the level of NAD^+ in the cells were estimated flourimetrically. Polylysine (P), Col I (I), and Ln. The values given as nanomoles of NAD^+ are average of quadruplicate experiments ± SEM. *Statistically significant compared to P ($P < 0.05$).

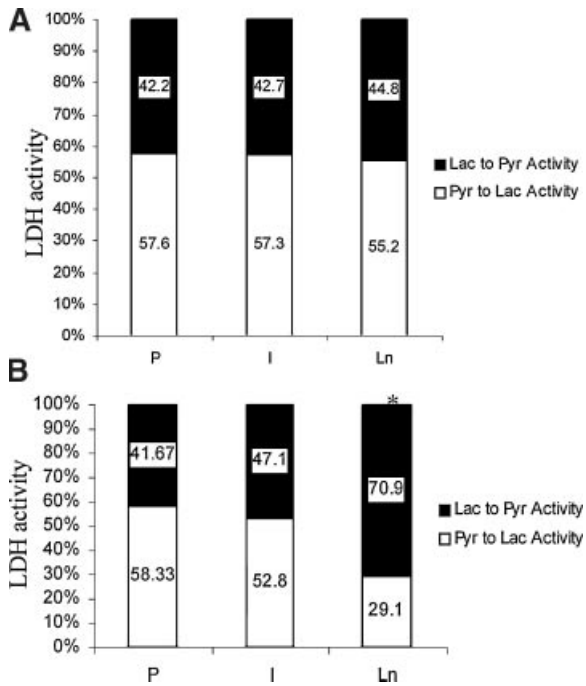


Fig. 7. Matrix dependence of LDH activity in HUVECs: HUVECs were maintained in MCDB131 medium on various matrix protein substrata as described in legends to Figure 1 for 24 h (**A**) and 48 h (**B**). At the end of the experiment, cells were harvested, lysed in PBS-triton-x-100 buffer (0.05% triton X-100) and the clear supernatant used for the activity of LDH in either directions that is, reduction of pyruvate and oxidation of lactate. The relative distribution of the rate of forward and backward reaction is presented as ratio of the total LDH activity. The results presented are average of quadruplicate experiments ± SEM. *Statistically significant compared to control ($P < 0.05$).

The pattern of expression of these genes was almost similar even after maintaining cells in culture for 48 h on Col I and polylysine substrata, but the proportion of LDH A to LDH B mRNA in cells maintained on Ln was 40–60%. A 60% increase in the level of LDH B mRNA was observed in cells maintained on Ln after 48 h of culture as compared to those after 24 h of culture. Although a 10% increase in the level of LDH B mRNA was observed in cells maintained on Col I and polylysine coated plates, it was accompanied by a similar increase in the level of LDH A mRNA as well. In cells maintained on Ln about 17% decrease in the level of LDH A mRNA after 48 h of culture as compared to 24 h was observed suggesting a shift in the expression of LDH A gene to LDH B gene in cells maintained on Ln substratum.

Modulation of LDH Isoenzymic Pattern by Ln Involves Integrin Dependent Downstream Signaling Through p38 MAP Kinase Pathway

The principal integrin receptor that mediates EC–Ln interaction is reported to be α6β4 integrin [Klein et al., 1993]. To examine whether the effect of Ln on LDH isoenzyme production is mediated through α6β4 integrin, cells in culture were treated with antibody against α6 integrin and studied the LDH isoenzymic pattern (Fig. 10). The ratio of A2B2:AB3:B4 shifted from 13:54:33 in cells maintained on Ln to 42:40:18 when cells maintained on Ln were treated with anti-α6 integrin antibody. The ratio of LDH isoenzymes in cells maintained on polylysine substratum was 34:50:16. Association of α6 subunit with β4 subunit was confirmed by immunoprecipitation of cell membrane extracts with antibody against β4 subunit followed by SDS–PAGE and immunoblotting with anti-α6 antibody (data not shown).

To study the pathway acting downstream of α6β4 integrin–Ln interaction, the effect of inhibitors of certain signaling pathways on the production of LDH isoenzymes in cells maintained on Ln was analyzed. On treatment with SB 202190, a specific inhibitor of p38 MAPK, Ln mediated shift in the isoenzymic pattern of LDH was not observed. SB 202190 treatment caused a shift in the ratio of LDHA and LDHB mRNA to 60:40 from 40:60 (Fig. 10). The activation of p38 MAPK in HUVECs maintained on Ln was confirmed by immunoblot analysis. As VEGF is also known to cause activation of p38 MAPK pathway [Rousseau et al., 1997; Issbrücker

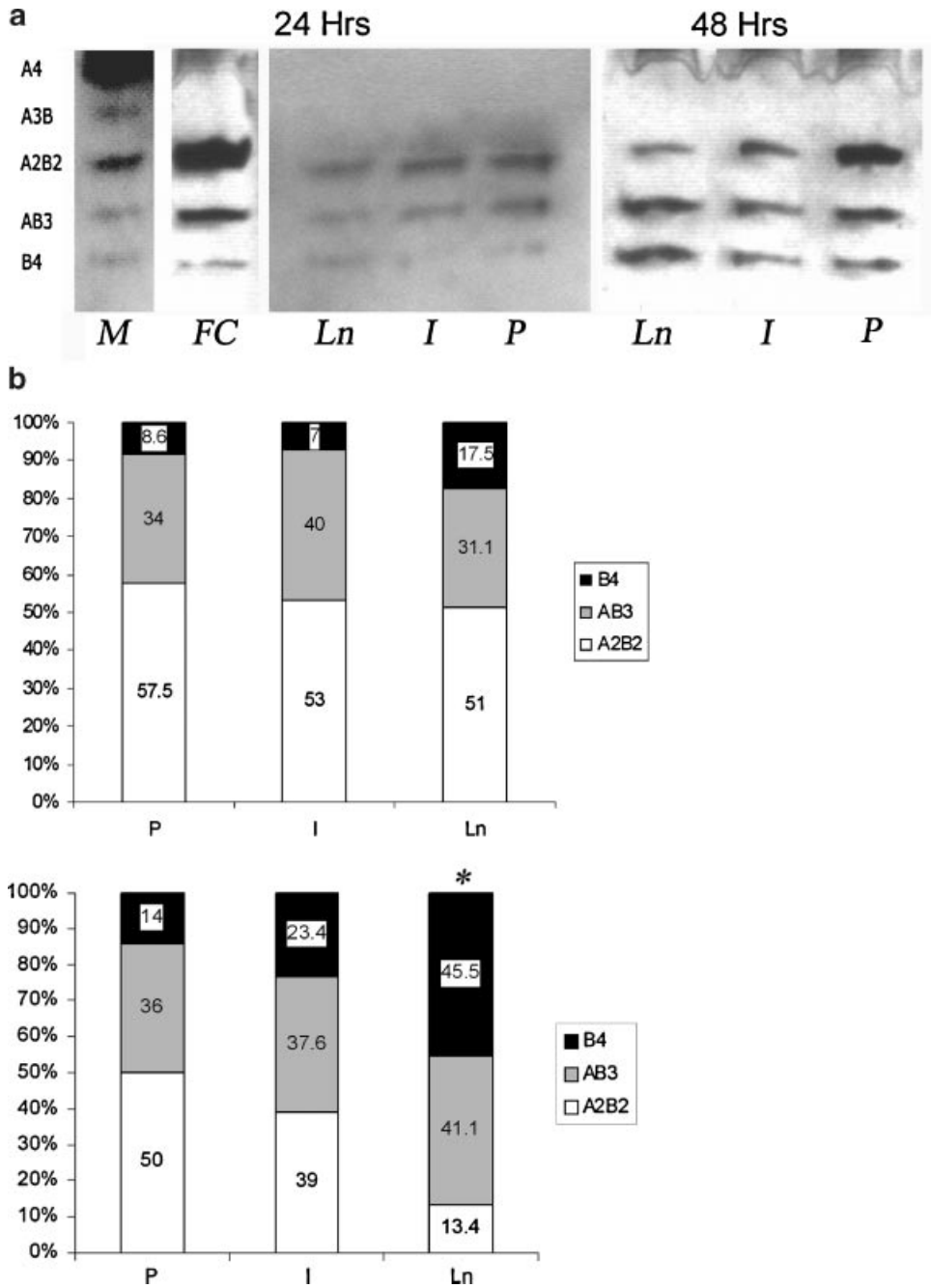


Fig. 8. Modulation of LDH isoenzyme pattern by Ln: HUVECs were maintained in MCDB131 medium on various matrix protein substrata as described in legends to Figure 1 for 24 h (**a**) and 48 h (**b**). At the end of the experiment, cells were harvested, lysed in detergent buffer and the supernatant was used for analyzing the isoenzymes of LDH. Cell lysates were separated on 7.5% native gel and the activity of LDH stained with NBT. The top panel shows the zymogram. M—marker (LDH isolated from muscle); FC—freshly isolated cells; Ln—cells cultured on Ln; I–Col I; and P—polylysine. The intensity of the bands was quantified using BioRad gel doc and the relative distribution of the various isoenzymic forms as percentage of the total is given. The results presented are average of quadruplicate experiments. *Statistically significant compared to control ($P < 0.05$).

et al., 2003], contribution of Ln to the activation of p38 MAPK was studied by analyzing phospho p38 MAPK in cells maintained on Ln and treated with blocking anti-VEGF antibody. p38 MAP kinase activation was about 70% when cells maintained on Ln were treated with blocking antibody against VEGF (Fig. 10). Analysis by HPLC and Western blot of equivalent amounts of VEGF revealed that the level of PAR modified VEGF produced by cells

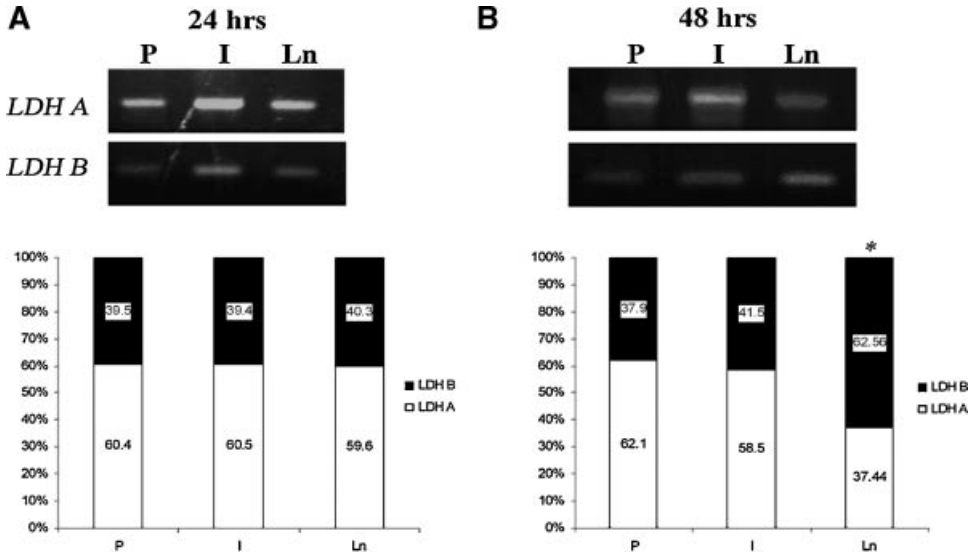


Fig. 9. Differential expression of LDH A and LDH B gene: freshly isolated HUVECs were maintained in culture as described in legends to Figure 1. At the end of the experiment, cells were harvested, total RNA isolated and mRNA levels of LDH A and LDH B were analyzed by semi-quantitative RT-PCR after 24 h (**A**) and 48 h (**B**) as described in methods. The products of PCR were separated on 1.75% agarose gel (**top panel**) and bands visualized by ethidium bromide staining. P—Polylysine, I—Col I, and Ln—Laminin. Relative intensity of bands was quantitated by BioRad Quantity One software and relative proportion of the LDH A and LDH B mRNA is given. The results presented are average of quadruplicate experiments ± SEM. *Statistically significant compared to control ($P < 0.05$).

maintained on Ln was significantly high when treated with inhibitor of p38 MAPK pathway. This appeared to be specific for cells maintained on Ln, as no such effect of SB 202190 was observed in cells maintained on Col I. Immunoblot analysis revealed that treatment of cells with blocking antibody against α6 integrin also caused an increase in the production of PAR modified VEGF by cells maintained on Ln (Fig. 5).

DISCUSSION

Results presented here provide further support to the proangiogenic effect of Ln [Dixelius et al., 2004], the principal glycoprotein of the BM. This was evidenced by the following observations: (a) the levels of E-selectin and CD31, the biochemical markers of angiogenesis were significantly high in cells maintained on Ln substratum as compared to those on Col I and polylysine substrata (b) the expression of VEGF R2, the specific endothelial marker for angiogenesis was significantly high in cells maintained on Ln substratum. CD31 appears to be required for cell elongation, migration as well as for cell–cell association to form the network structures [Yang et al., 1999]. E-selectin plays an important role in the angiogenesis induced by VEGF by promoting cellular interactions [Aoki et al., 2001] and VEGFR-2 is the principal receptor mediating the effects of VEGF [Waltenberger et al., 1996; Zachary, 1998].

VEGF is a key angiogenic factor that can act in an autocrine manner having survival effects on several cell types including ECs [Nor et al., 1999; Gerber et al., 2002; Brusselmans et al., 2005; Byrne et al., 2005]. Dixelius et al. reported that Ln 1 promoted EC differentiation to angiogenic phenotype in Col I gel without affecting the expression of VEGF. Although Ln did not affect the levels of VEGF mRNA and protein, our data indicate that it modulates the activity of VEGF. This was confirmed by CAM assay which showed that the VEGF produced by the cells maintained on Ln exhibited significantly high angiogenic activity. As indicated before, one of the mechanisms that modulate the angiogenic potency of VEGF is PAR, which has been reported to reduce the angiogenic potency of VEGF [Xiong et al., 1998]. This was further confirmed in our laboratory by treating HUVECs with a poly-ADP ribose polymerase (PARP) inhibitor, 3-aminobenzamide; the inhibition of PAR modification of VEGF increased

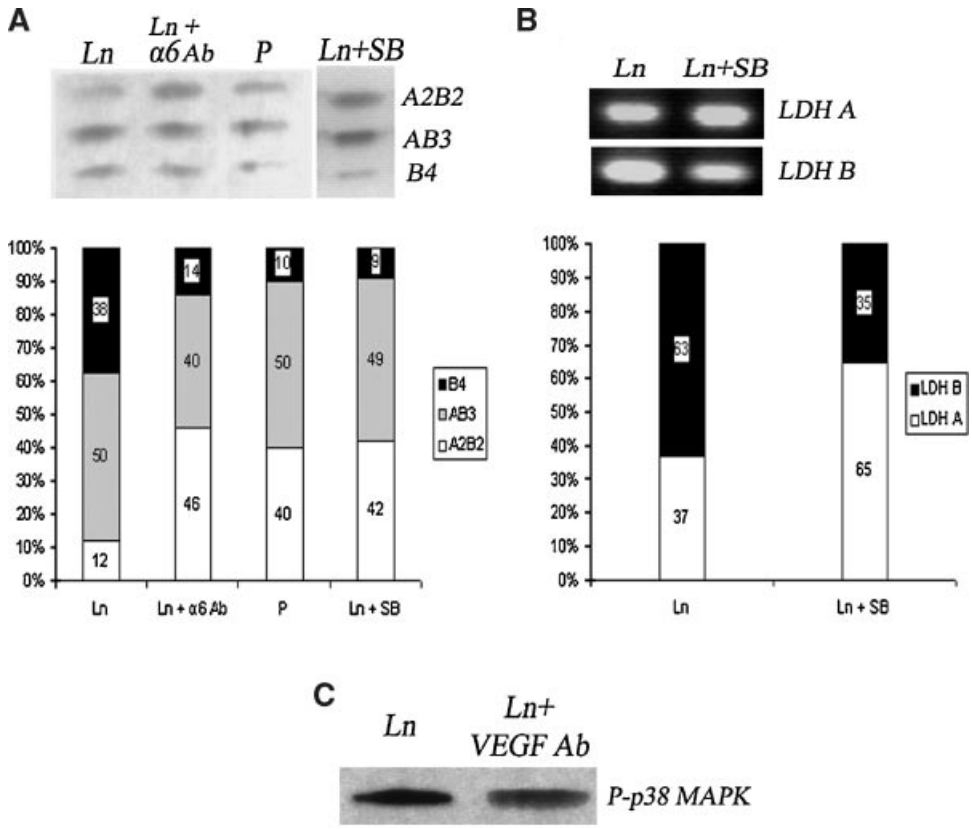


Fig. 10. Ln directed downstream signaling: HUVECs were maintained in MCDB131 medium on Ln matrix substratum as described in legends to Figure 1 for 48 h. **A**: Zymographic analysis of LDH isoenzymes in cells maintained on Ln in presence of blocking antibody against α6 (2 μl/3 ml medium) integrin and inhibitor of p38 MAPK pathway (SB 202190-10 μM). **B**: Total RNA isolated and mRNA levels of LDH A and LDH B were analyzed by semi-quantitative RT-PCR as described in methods. The products of PCR were separated on 1.75% agarose gel and bands visualized by ethidium bromide staining. **C**: HUVECs maintained on Ln substratum were treated with (Ln + VEGF Ab) and without (Ln) VEGF antibody (80 ng/3 ml). Protein equivalent amounts of cell extracts separated on 10% SDS–PAGE and immunoprobed with polyclonal anti-phospho p38 MAPK antibody. The results presented are average of quadruplicate experiments. P—cells maintained on polylysine; Ln—cells maintained on Ln; Ln + α6 Ab—cells maintained on Ln and treated with anti-α6 antibody; Ln + SB—cells maintained on Ln, treated with p38 MAPK inhibitor; Ln + VEGF Ab—cells maintained on Ln and treated with anti-VEGF antibody.

its angiogenic potency significantly in chick CAM (data not shown). Immunoblot and enzymic digestion followed by HPLC revealed that, the PAR modification of VEGF produced by cells maintained on Ln was significantly lower than those maintained on Col I and polylysine substrata suggesting that one of the mechanisms of the proangiogenic effect of Ln is the modulation of angiogenic activity of VEGF by controlling its PAR modification. NAD^+ being one of the substrates for PAR reaction, decrease in NAD^+ in the [NAD^+/NADH] pool in cells maintained on Ln substratum can reduce the rate of PAR modification of VEGF. Modulation of the activity of PARP may also contribute to the differential PAR modification. Matrigel, an Ln rich BM substratum has been shown to induce rapid differentiation of ECs to capillary like network [Grant et al., 1992]. PAR modification of VEGF secreted by HUVECs maintained on matrigel substratum was also significantly reduced and was comparable to that on Ln substratum (data not shown) suggesting that Ln exerts its effects on VEGF in presence of other matrix macromolecules which simultaneously interact with the ECs.

The [NAD^+/NADH] pool in the cytosol is maintained by various dehydrogenases. Of the different dehydrogenases assayed, significant difference in the activity of LDH was observed in cells maintained on Ln substratum when compared to those maintained on Col I and polylysine substrata. The redox state in the cytosol is indicated by the ratios of NADH to

NAD^+ or lactate to pyruvate. The equilibrium of these has been suggested to be maintained by LDH [Connett et al., 1990]. In cells maintained on Ln substratum, there was a shift in favor of the oxidation of lactate to pyruvate, which can shift NAD^+/NADH equilibrium to NADH. Data presented recently showed that supplementing lactate caused a shift in NAD^+/NADH equilibrium in favor of NADH and decreased PAR modification of VEGF, increasing its biological activity [Kumar et al., 2007]. But in the present set of experiments, the cells were not supplemented with lactate and therefore its effect is minimal. Further, as the conversion of pyruvate, which is present in significant amounts in the medium to lactate is slowed down, more of pyruvate might be oxidized shifting the ratio of NAD^+/NADH in favour of NADH.

The change in the rate of oxidation of lactate to pyruvate in cells maintained on Ln substratum appears to be due to a change in the expression of isoenzymes of LDH. In somatic tissues five isoforms of LDH occur by random arrangement of two polypeptide chains to form the tetrameric active enzyme. Analysis of the isoenzymic pattern revealed three major isoenzymic forms in HUVECs which correspond to migration with A2B2, AB3, and B4. Among the three isoforms, A2B2 was found to be the predominant form in freshly isolated cells and in those maintained in culture for 24 h. A shift in the isoenzymic pattern of LDH from A rich form to B rich form was observed in cells maintained on Ln. LDH A rich isoenzyme converts pyruvate to lactate under anaerobic conditions in normal cells. The other isoenzyme, LDH B, kinetically favors the conversion of lactate to pyruvate and is found at high levels in aerobic tissues such as the heart. The B4 isoenzyme has high affinity (low Km) for lactate and a high level of pyruvate inhibits the reduction of pyruvate and ensures lactate oxidation [Li, 1998].

Alteration of LDH isoenzymic pattern has been observed during development and frequently in neoplastic tissues [Goldman et al., 1964; Issbrücker et al., 2003]. Testicular and ovarian germ cell tumors have a predominance of B4 LDH isoenzyme whereas other cancers have different LDH isoenzymic patterns [von Eyben, 2001]. It has also been reported that treatment of leukemic cells with inducers of differentiation resulted in quantitative shift of the isoenzymic pattern [Pantazis et al., 1981]. Interaction of tumor cells with Ln may cause a shift in the expression pattern of LDH isoenzymes to B4 type causing a shift in the rate of oxidation of lactate to pyruvate. The NADH thus formed may not be oxidized unless there is adequate oxygen supply. This results in reduced PAR modification of VEGF causing increase in its biological activity. The intracellular levels of each LDH isoenzyme are dependent on the rate of its synthesis and degradation. The LDH tetramer, and not the monomer, being the degradative unit, the distribution of the LDH isoenzyme is the result of continuous in vivo assembly and disassembly of LDH tetramers combined with the selective degradation of some of them [Nadal-Ginard, 1976].

Ln appears to modulate the expression of genes which code for LDH isoenzymes. In cells maintained on Ln, unlike in cells maintained on other substrata, a downregulation of LDH A gene and an upregulation of LDH B gene were observed. Absence of any effect of high levels of lactate on the distribution of LDH A and LDH B mRNA levels suggests that the shift in the LDH isoenzymic pattern in cells maintained on Ln was not due to a substrate effect. Though LDH A gene expression is reported to be modulated by hypoxia inducing factor and cAMP-mediated pathways [Frith et al., 1995], the regulation of expression of LDH B gene is less understood. The promoter sequence of LDH B gene has a number of transcription factor binding sequences including sites for AP-1, HNF-1, and c-myb, identified by a search for sites by weight matrix library at the Biobase biological database.

Experiments carried out to study the mechanism of action of Ln revealed that Ln caused alteration in LDH isoenzymic pattern through its receptor $\alpha 6\beta 4$ integrin mediated activation of downstream signaling pathway involving p38 MAPK. This was evidenced by the following observations: (a) the effect of Ln on LDH isoenzyme pattern was reversed in cells treated with antibody against $\alpha 6$ integrin, (b) shift in the LDH isoenzyme from the A rich form to the B rich form caused by Ln was reversed in cells treated with SB 202190, an inhibitor of p38 MAPK, (c) immunoblot analysis using specific antibody against phosphorylated p38 MAPK, showed its activation in cells maintained on Ln substratum. Ln interacts with ECs through integrin and generates signals via multiple signal transduction pathways involving various components such as MAPK, phosphatases, focal adhesion kinase (FAK), small GTPases of

the Rho family, and cytoskeletal components [Giancotti and Ruoslahti, 1999]. Blocking of α6 integrin also caused a reversal of the Ln effect on the production of PAR modified VEGF. Interaction of Ln with its integrin receptor leads to activation of FAK followed by association of adaptor protein *src* to phosphorylated FAK, which leads to activation of MAPK. Immunoprecipitation of cell lysate with anti-FAK antibody followed by probing with anti-phospho tyrosine or anti-*src* antibody confirmed activation of FAK and association of *src* with FAK in cells maintained on Ln (data not shown). p38 MAPK has been reported to regulate the activity of the transcription factor AP1 [Zarubin and Han, 2005]. It has been shown that Ln-1 induced a rapid and transient expression of mRNA of c-fos and c-Jun in PC12 cells, and stimulated the DNA binding activity of the complex of these proteins to the AP-1 site [Aznavoorian et al., 1990]. Since the LDH B gene also has an AP-1 binding site in its promoter sequence, the possibility of regulation of the expression of LDH B gene by recruiting p38-AP-1 pathway cannot be excluded.

p38 MAPK pathway constitutes an important component of the signaling network which transduces the migratory signals generated by VEGF suggesting that it may play an important role in regulating angiogenesis [Gerber et al., 2002]. p38 MAPK has also been reported to play an important role in placental angiogenesis. p38 mutant placentas display lack of vascularization of the labyrinth layer as well as increased

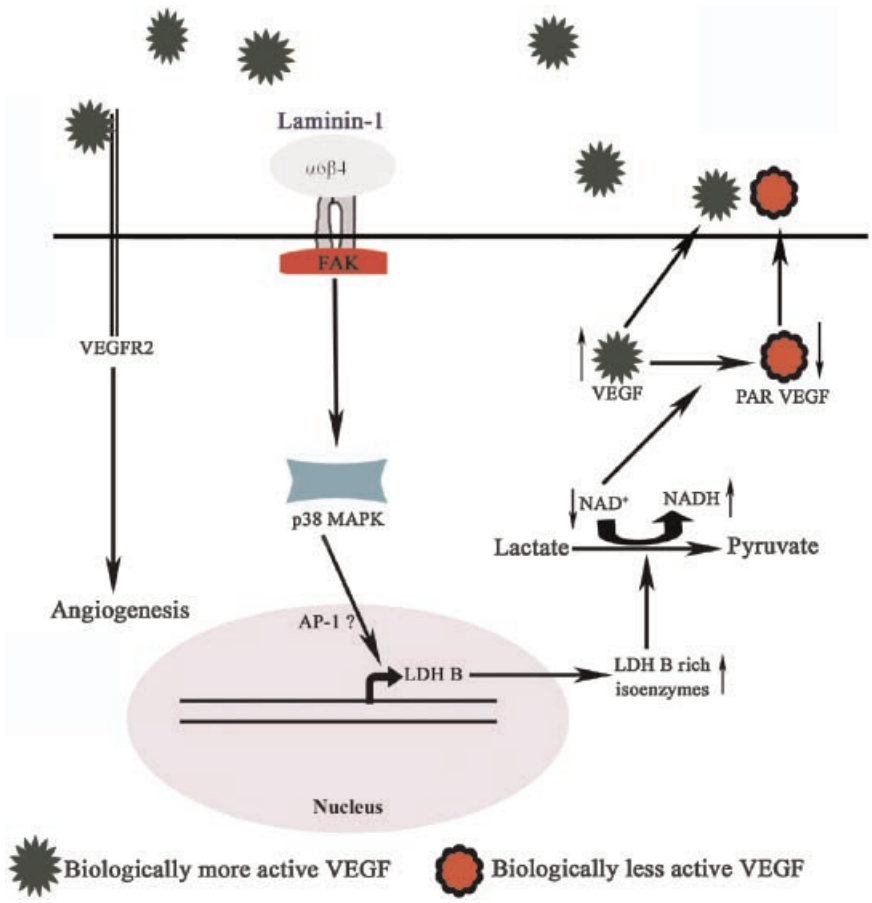


Fig. 11. Scheme for Ln mediated modulation of VEGF activity: Ln-1 engages α6β4 integrin, causing activation of FAK and downstream signaling leading to activation of p38MAPK which (probably through recruitment of AP-1 transcription factor) causes an upregulation of LDHB gene. LDH B rich isoforms more efficiently catalyze the oxidation of lactate to pyruvate causing reduction of NAD^+ to NADH. Decrease in levels of NAD^+ in the NAD^+/NADH pool decreases the PAR of VEGF thereby increasing unmodified, biologically more active form of VEGF. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

rates of apoptosis, consistent with a defect in placental angiogenesis. Furthermore, p38 mutants display abnormal angiogenesis in the embryo proper as well as in the visceral yolk sac [Mudgett et al., 2000]. The blocking of p38 MAPK pathway, apart from reversing the Ln dependent reduction in the production of PAR modified VEGF, also caused inhibition of angiogenesis related EC activities such as production of E-selectin, CD31, and expression of VEGF R2 (data not shown). Apart from modulation of the biological activity of VEGF, the proangiogenic effect of Ln may also involve integrin mediated p38 MAPK signaling. Even though induction of VEGF by ECM components, such as collagen types I and IV or Ln has not been observed [Sheta et al., 2000], the possibility of cross talk between integrin and VEGF R2 signaling cannot be excluded.

In summary, it appears that Ln can affect the aerobic metabolism of ECs by modulating the expression of LDH isoenzymes from the A rich forms to B rich forms, which more efficiently catalyzes the conversion of lactate to pyruvate and the reduction of NAD^+ to NADH. Decrease in the ratio of NAD^+/NADH reduces the rate of PAR of VEGF which renders it biologically more active (Fig. 11). The effect of Ln appears to be mediated through $\alpha 6$ integrin dependent downstream signaling involving p38 MAPK pathway.

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