CHAPTER 4

ANGIOGENIC NATURE OF FIBRONECTIN
4.1. Introduction

The results presented in the previous chapter showed that FN promotes EC survival and suppressed apoptosis. As discussed before, FN is known to play a critical role in vascular development (Risau and Lemmon, 1988; Risau, 1991). It can promote EC adhesion, growth and migration, which are characteristic of angiogenesis (Kim et al., 2000; Wijelath et al., 2002; Ilic et al., 1998; Hynes, 1990). Further, both FN and the α,β₁ integrin receptor are reported to be up-regulated in the vascular ECM following treatment with angiogenic factors in vivo (Kim et al., 2000). It is not known whether FN can influence phenotype of ECs. As described before, VEGF is a key angiogenic factor produced by different types of cells including ECs. Although FN is known to influence activity of a number of different types of cells, it is not known whether FN can influence EC function, particularly the production of VEGF that can cause an autocrine effect on ECs. The present study was therefore designed to analyze the angiogenic response of endothelial cells to FN particularly, the modulation of production and biological activity of VEGF in cells maintained on FN.

4.2. Methods

The angiogenic nature of FN was analysed using three model systems viz. in vivo chick chorioallantoic membrane (CAM) assay, organ culture of rat aortic ring and in vitro culture of HUVECs. The angiogenic markers E-selectin and CD 31 were analyzed by ELISA. Levels of mRNAs of VEGF, VEGFR2, FGF2 and FGFR1 were analysed by RT-PCR. VEGF protein was quantitated by western blotting and ELISA and FGF2 protein by ELISA. Biological activity of VEGF was examined by CAM assay. To analyze the PAR modification of VEGF, co-immunoprecipitation followed by western blotting was employed using anti-PAR antibody. The PAR moiety associated with VEGF was analysed using HPLC. The level of NAD⁺ in HUVECs was quantitated flourimetrically. The details of the methods used in these experiments have been given in chapter 2.
4.3. Results

4.3.1. Angiogenic nature of fibronectin

4.3.1.1. In vivo CAM assay: - The angiogenic effect of FN was assessed by CAM assay (Figure 4.1.). The neovascularisation and the level of hemoglobin as a measure of vessel density were significantly high in CAMs treated with FN when compared to control and Col I suggesting its angiogenic nature.

![Image of CAM assay](image)

**Figure: 4.1. Angiogenic nature of FN - CAM assay:** - 10μg of different matrix proteins, Col I (I) and Fibronectin (FN) were applied on to the CAMs of 4 days old chick embryos. CAMs treated with PBS served as control (C). After 8 days from treatment, the vessel density in the CAM were photographed and quantitated in terms of hemoglobin. The top panel shows representative photographs of vascularised CAMs and the level of hemoglobin is given below. The results presented are the average of quadruplicate experiments ± SEM.

*Statistically significant compared to C (p<0.05)*

4.3.1.2. Rat aortic ring assay: - The angiogenic effect of FN was also tested in organ culture model of angiogenesis, rat aortic ring assay (Figure: 4.2.). The endothelial sprout density was significantly high in FN treated aortic rings as compared to Col I and control, confirming the pro-angiogenic nature of FN.
Angiogenic nature of FN

Figure: 4.2. Angiogenic effect of FN - Aortic ring assay:- Rat aortic ring explants in culture were treated with various matrix proteins (10μg) and the morphological changes were photographed under a microscope [4x] after 48 hrs of rings in culture. The arrow indicates the endothelial sprout. Col I (I), Fibronectin (FN) and control (C). The photographs are of a representative experiment.

4.3.1.3. In vitro culture of HUVECs:- The angiogenic nature of FN was further tested using in vitro culture of HUVECs (Figure: 4.3.). Cells were maintained in culture on FN, Col I or polylysine coated substrata and monitored for morphological changes at regular intervals. The morphological analysis of HUVECs in culture showed grouping and capillary-like network formation in cells maintained on FN at a faster rate as compared to Col I and polylysine. After 48 hours of culture on FN, the cells started grouping and established cell-cell contacts and by 72 hours, they started forming network-like structures, whereas the cells were still spherical in shape on Col I as well as polylysine.

Figure: 4.3. Angiogenic nature of FN – HUVECs in culture:- HUVECs were maintained in culture on polylysine (P), Col I (I) and fibronectin (FN) for 48 hours (upper panel) and 72 hours (lower panel) and the morphological changes were photographed microscopically [40x]. Arrows indicate network-like structures.
4.3.2. Changes in biochemical markers of angiogenesis - Effect of FN

To study the effect of FN on biochemical markers of angiogenesis, HUVECs were maintained in culture on FN, Col I and polylysine and the marker proteins relevant to angiogenesis were analyzed by ELISA (Figure: 4.4.). The level of von Willebrand factor (vWF), an EC-specific protein, was similar in cells maintained on polylysine and Col I whereas that in cells maintained on FN was significantly high. Angiogenic marker activities such as the production of E-selectin and CD31 were significantly high in cells maintained on FN after 48 hours of culture when compared to those on Col I or polylysine.

4.3.3. VEGF and VEGFR2 expression by HUVECs maintained on FN

In order to study whether the pro-angiogenic nature of FN was due to a change in the production of VEGF, the levels of VEGF mRNA and protein were quantitated. The mRNA level of VEGF was found to be significantly high in cells maintained on FN (Figure: 4.5.). The mRNA expression of VEGFR2 was also significantly high in cells maintained on FN when compared to that in cells maintained on Col I or polylysine.
Figure: 4.5. RT-PCR analysis of VEGF and VEGFR2 expression in cells maintained on different substrata: Isolated HUVECs were maintained in culture on fibronectin (FN), Col I (I) and polylysine (P) coated plates for 48 hrs in MCDB 131 medium in a carbon dioxide incubator. At the end of the experiment, cells were harvested, total RNA isolated and mRNA levels of VEGF and VEGFR2 were analyzed by RT-PCR as described in methods. The products of PCR were separated on 1.75% agarose gel and bands visualized by ethidium bromide staining (upper panel). The gel given is of a representative experiment. Relative intensity of VEGF (A) and VEGFR2 (B) bands were quantitated and normalized with the intensity of the band for internal control (GAPDH). The results presented are the average of quadruplicate experiments ± SEM. *Statistically significant compared to P (p<0.05)

The level of VEGF secreted by cells maintained on different substrata was analyzed by ELISA and immunoblot analysis (Figure: 4.6.). The amount of VEGF
produced by cells maintained on FN was significantly high as compared to Col I and polylysine substrata. Immunoblot analysis of protein equivalent volumes of media with monoclonal anti-VEGF antibody revealed two bands of ~50 kDa and ~20 kDa. The quantitation of intensity of both bands showed similar result as ELISA with maximum band intensity in cells maintained on FN.

**Figure: 4.6. Protein levels of VEGF in cells maintained on different substrata:** VEGF secreted into the medium of HUVECs maintained in MCDB131 medium on polylysine (P), Col I (I) and fibronectin (FN) was estimated by ELISA (B). The top panel A shows the intensities of bands of VEGF after immunoblot analysis. A representative blot is given as inset in A. The results presented are the average of quadruplicate experiments ± SEM. *Statistically significant compared to P (p<0.05)
4.3.4. FGF2 and FGFR1 expression in HUVECs maintained on FN

The mRNA expression of another angiogenic growth factor, FGF2 and its receptor in ECs, FGFR1 were analysed by RT-PCR (Figure: 4.7). The results showed that in contrast to VEGF, the mRNA expression of FGF2 was less in cells maintained on FN as compared to polylysine. The mRNA level of FGFR1 in cells maintained on FN was found to be not significantly different from that on polylysine. The mRNA expression of both FGF2 and FGFR1 were very low in cells maintained on Col I.

![RT-PCR analysis of FGF2 and FGFR1 expression in cells maintained on different substrata](image)

**Figure: 4.7. RT-PCR analysis of FGF2 and FGFR1 expression in cells maintained on different substrata:** Isolated HUVECs were maintained in culture on fibronectin (FN), Col I (I) and polylysine (P) coated plates for 48 hrs in MCDB131 medium in a carbon dioxide incubator. At the end of the experiment, cells were harvested, total RNA isolated and mRNA levels of FGF2 and FGFR1 were analyzed by RT-PCR as described in methods. The products of PCR were separated on 1.75% agarose gel and bands visualized by ethidium bromide staining (inset). The gel given is of a representative experiment. Relative intensity of FGF2 and FGFR1 bands were quantitated and normalized with the intensity of the band for internal control (GAPDH). The results presented are the average of quadruplicate experiments ±SEM. *Statistically significant compared to P (p<0.05)
Analysis of the protein level of FGF2 showed no significant difference between FN and Col I whereas in contrast to the FGF2 mRNA, the protein level of FGF2 was low in cells maintained on non-matrix substratum, polylysine (Figure: 4.8.).

Figure: 4.8. Protein levels of FGF2 in cells maintained on different substrata:- FGF2 secreted into the medium of HUVECs maintained in MCDB131 medium on polylysine (P), Col I (I) and fibronectin (FN) was estimated by ELISA. The results presented are the average of quadruplicate experiments ± SEM.
*Statistically significant compared to P (p< 0.05)

4.3.5. Angiogenic potency of VEGF from cells maintained on FN

As VEGF was found to be the major angiogenic factor in cells maintained on FN, the angiogenic potency of affinity isolated VEGF on heparin-sepharose from the media was tested in CAM assay using equivalent amounts of VEGF as determined by ELISA. VEGF isolated from cells maintained on FN had significantly higher angiogenic potency as evidenced by the higher vessel density and hemoglobin level as compared to VEGF isolated from cells maintained on Col I and polylysine substrata (Figure: 4.9.). The heparin binding fraction from which VEGF was removed by immunoprecipitation showed vascularization and hemoglobin levels similar to the vehicle control indicating that VEGF is the major heparin-binding growth factor.
Figure: 4.9. Angiogenic potency of affinity isolated VEGF - Effect of FN: -
HUVECs were maintained in MCDB131 medium on polylysine (P), Col I (I) and fibronectin (FN). 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. Vehicle control (VC). VEGF was removed from heparin isolated fractions by immunoprecipitation and the extract was given to CAM (VEGF-). The top panel shows photographs of vessel densities and below is given the levels of hemoglobin, less the vehicle control in each group. The results presented are the average of quadruplicate experiments ± SEM.
*Statistically significant compared to P (p<0.05)

4.3.6. Post-translational modification of VEGF - Effect of FN

As one of the factors affecting the angiogenic potency of VEGF is its post-translational PAR modification, the PAR modification of VEGF was analysed.
Equivalent amounts of VEGF immunoprecipitated from the media of cells maintained in culture on different matrix proteins, was probed with anti-PAR antibody (Figure: 4.10.). Two bands corresponding to ~50kDa and ~20kDa were identified. The intensity of both 50kDa and 20kDa bands were significantly low in cells maintained on FN as compared to cells on other substrata, suggesting that poly ADP ribosylation of the VEGF produced by cells maintained on FN was significantly low when compared to cells maintained on other substrata.

**Figure: 4.10. PAR modification of VEGF in cells maintained on different substrata:** HUVECs were maintained in MCDB131 medium on polylysine (P), Col I (I) and fibronectin (FN) for 48 hours. Equivalent amounts of VEGF, immunoprecipitated from media, were separated by SDS PAGE, transferred to NC membrane and probed with anti PAR antibody. The intensity of the bands, ~50 kDa and ~ 20 kDa was quantified using BioRad gel doc and the total intensity plotted. The results presented are the average of quadruplicate experiments ± SEM.

*Statistically significant compared to P (p<0.05)

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 (Figure: 4.11). The amount of PR-AMP was significantly low in VEGF isolated from cells maintained on FN substratum as compared to that from cells maintained on Col I and polylysine. The PAR associated with VEGF was found to be high in cells on Col I as was found in the immunoblot analysis.
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**Figure: 4.11. PAR associated with VEGF produced by cells maintained on different substrata:** HUVECs were maintained in MCDB131 medium on polylysine (P), Col I (I) and fibronectin (FN) for 48 hours. 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 minutes each as described in the methods. 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 methods. The values presented are the mean of duplicate experiments.

*Statistically significant compared to P (p<0.05)

4.3.7. Level of NAD⁺ in HUVECs cultured on various matrix substrata

The level of NAD⁺, the principal substrate involved in poly ADP ribosylation reaction, was estimated in cells maintained on different matrix substrata (Figure: 4.12.). The level of NAD⁺ in cells maintained on FN was significantly less than that in cells maintained on Col I or polylysine substrata, suggesting a shift in the NAD⁺/NADH pool towards NADH in cells maintained on FN.

**Figure: 4.12. NAD⁺ levels in cells maintained on FN substratum:** HUVECs were maintained in MCDB131 medium on polylysine (P), Col I (I) and fibronectin (FN) for 48 hours and the level of NAD⁺ in the cells were estimated fluorimetrically. The values given as nanomoles of NAD⁺ are the average of quadruplicate experiments ± SEM. *Statistically significant compared to P (p<0.05)
4.3.8. p38 MAPK inhibition reversed the pro-angiogenic effect of FN

As p38 MAPK was found to influence the survival signals from FN, the effect of p38 MAPK inhibition on angiogenesis was also analyzed. It was found that inhibition of p38 MAPK with SB202190 caused a significant reduction in both EC marker (vWF) and angiogenic markers, E-selectin and CD31 (Figure: 4.13.). Further, the protein levels of the growth factors, VEGF and FGF2 were also found to decrease significantly in cells treated with SB202190 (Figure: 4.14.). But, the PAR modification of VEGF remained unaltered on treatment with p38 MAPK inhibitor as evidenced in both immunoblot and HPLC analysis of VEGF associated PAR (Figure: 4.15.).

Figure: 4.13. Changes in angiogenic markers on inhibition of p38 MAPK:
Isolated HUVECs were maintained in culture on fibronectin and polylysine coated plates for 24 hrs with (P+SB, FN+SB) or without (P, FN) SB202190 (10 µM). At the end of the experiment, cells were harvested and the levels of vWF (A) and E-selectin (C) in medium and CD31 (B) in cell layer were determined by ELISA. The values given are the average of quadruplicate experiments ± SEM.
*Statistically significant compared to FN (p<0.05)
Figure: 4.14. Changes in protein levels of VEGF and FGF2 on inhibition of p38 MAPK: - VEGF (A) and FGF2 (B) secreted into the medium of HUVECs maintained in MCDB131 medium on polylysine and fibronectin in the presence (P+SB, FN+SB) and absence (P, FN) of SB202190 (10 µM) was estimated by ELISA. The results presented are the average of quadruplicate experiments ± SEM.
*Statistically significant compared to respective controls (p<0.05)

Figure: 4.15. PAR modification of VEGF on p38 MAPK inhibition: - A) HUVECs were maintained in MCDB131 medium on polylysine and fibronectin for 24 hours in the presence (P+SB or Pi, FN+SB or Fn) and absence (P, FN) of SB202190. VEGF, immunoprecipitated from media, were separated by SDS PAGE, transferred to NC membrane and probed with anti PAR antibody (inset in A). The intensity of the bands, ~50 kDa and ~20 kDa, were quantified using BioRad gel doc and the total intensity plotted. The results presented are the average of quadruplicate experiments ± SEM. B) PAR associated with VEGF produced by cells - HUVECs were maintained in culture as described above for 24 hours. 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 minutes each and the amount of PR-AMP was quantitated by HP LC as described in methods. The values presented are the average of duplicate experiments.
4.4. Discussion

Angiogenesis requires the interaction of ECs with both angiogenic growth factors and ECM components (Eliceri and Cheresh, 2001; Hynes et al., 1999; Yancopoulos et al., 2000). Both FN and its receptor, $\alpha_5\beta_3$ integrin are poorly expressed in quiescent endothelium but strongly expressed in proliferating endothelium suggesting their important roles in angiogenesis (Kim et al., 2000). Results presented in this chapter confirmed the pro-angiogenic nature of FN. This was evidenced by the following observations: (a) higher vessel density and significantly high level of hemoglobin as a measure of vascular density in CAMs treated with FN (b) significantly high vascular sprout density observed in aortic ring explants in the presence of FN (c) establishment of cell-cell contacts in HUVECs and significantly high levels of the biochemical markers of angiogenesis, E-selectin and CD 31 (PECAM-1) in HUVECs maintained in culture on FN substratum. E-selectin appears to play an important role in the angiogenesis induced by VEGF by promoting cellular interactions (Aoki et al., 2001) whereas CD31 is required for cell elongation, migration and cell-cell association to form the network structures in angiogenesis (Yang et al., 1999). Moreover, PECAM-1 homophilic adhesion rescues ECs from serum deprivation-induced apoptosis (Bird et al., 1999). The angiogenic nature of FN was found to be regulated by p38 MAPK signaling as there was a significant decrease in the angiogenic marker levels on its inhibition indicating that in addition to survival signals, p38 MAPK also elicits a downstream angiogenic signaling in cells maintained on FN. Ligation of $\alpha_5\beta_3$ integrin on ECs to FN in interstitial matrix provides a possible mechanism for the activation of NF-$\kappa$B that leads to transcription of a number of proteins involved in angiogenesis including E-selectin and VCAM-1. The possible role of NF-$\kappa$B activation in HUVECs maintained on FN was analyzed and discussed in chapter 5.

VEGF is an endothelial cell-specific mitogen that promotes the proliferation, survival and migration of ECs, induces vascular permeability and formation of capillary tubes. VEGF is produced by many cell types including epidermal keratinocytes (Ferrara and Davis-Smyth, 1997), macrophages (Xiong et al., 1998) tumor cells (Kondo et al., 1993), cardiomyocytes (Shifren et al., 1994), monocytes (Connolly et al., 1989) and smooth muscle cells (Kuzuya et. al., 1995), which was thought to stimulate ECs in a paracrine manner. However, now it has been established that VEGF can also act in an autocrine manner in a number of cell types including ECs,
embryonic stem cells and hematopoietic stem cells (Gerber et al., 2002; Brusselmans et al., 2005; Nor et al., 1999; Byrne et al., 2005). The results of our investigations also revealed the autocrine action of VEGF produced by ECs maintained on FN probably through VEGFR2, the principal receptor mediating the effects of VEGF (Waltenberger et al., 1994; Zachary, 1998). The expression of VEGF and VEGFR2, the specific endothelial angiogenic marker was significantly high in cells maintained on FN substratum. On p38 MAPK inhibition VEGF production was found to decrease significantly again confirming the role of this intracellular signaling molecule in angiogenesis.

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, 2001). One of the major post-translational modifications reported to reduce the biological activity of VEGF is poly ADP ribosylation (PAR) (Xiong et al., 1998). Further, result from our laboratory has also shown that PAR modification of VEGF reduces its angiogenic activity and cells maintained on the basement membrane protein, laminin produced VEGF with less PAR modification and high biological activity (Kumar et al., 2007). Poly ADP-ribosylation of proteins plays diverse roles by altering the protein function by the covalent modification and introducing steric hindrance effects. The biological potency of equivalent amounts of VEGF isolated from media of cells maintained on FN to induce neovascularisation in CAM model was significantly different from that isolated from cells maintained on polylysine or Col I indicating that the angiogenic potency of VEGF secreted by cells on FN is greater. So the possibility of difference in PAR modification of VEGF resulting in higher biological activity was analysed. Both immunoblot analysis and enzymatic digestion of VEGF followed by HPLC revealed that the PAR modification of VEGF produced by cells maintained on FN was significantly lower than those maintained on Col I and polylysine substrata suggesting that the modulation of angiogenic potency of VEGF by regulating its PAR modification may be one of the possible reasons for the pro-angiogenic effect of FN.

Eventhough the production of VEGF was lowered by more than 50% on treatment with p38 MAPK inhibitor in ECs maintained on FN, there was no significant difference in the PAR modification of the growth factor in cells treated with SB202190 and the untreated controls as evidenced by both western blot analysis and HPLC. This
suggests that on inhibition of p38 MAPK, there is a significant reduction in the translation of VEGF in cells maintained on FN with very minimal effect on its post-translational modification.

Poly ADP-ribosylation is catalyzed by the family of poly (ADP-ribose) polymerases (PARPs) (Amé et al., 2004). PARPs catalyze the polymerization of ADP-ribose units from donor NAD$^+$ molecules on target proteins, resulting in the attachment of linear or branched polymers. PAR is a branched polymer of repeating ADP-ribose units, which are linked via glycosidic ribose–ribose 1' → 2' bonds. PAR is heterogeneous with respect to length (as many as 200 ADP-ribose units in vitro) and extent of branching (approximately one branch per 20–50 ADP-ribose units) (D'Amours et al., 1999). The enzymatic activity of PARPs requires a ready supply of the substrate molecule, NAD$, which is hydrolyzed to produce ADP-ribose units for the PARylation of protein targets. Unlike cellular redox reactions that use NAD$^+$ as a cofactor without a net loss of pyridine nucleotide, PARPs cleave the glycosidic bond between nicotinamide and the ADP-ribose moiety of NAD$. Consequently, the resynthesis of NAD$^+$ is essential for maintaining PARP functions. In fact, the regulated (and perhaps localized) availability of NAD$^+$ may represent a key point of control for PARPs. In order to examine the mechanism that affects the PAR modification of VEGF in cells maintained on FN, the levels of NAD$, the substrate for poly ADP ribosylation was analyzed. The levels of NAD$^+$ was significantly low in cells maintained on FN matrix substratum when compared to that of cells maintained on Col I and polylysine substrata, suggesting a shift in the [NAD$^+$/NADH] pool towards NADH causing a reduction in the PAR modification of VEGF. Thus it appears that FN causes changes in the metabolic activity of the cells maintained on it, contributing to the shift in the [NAD$^+$/NADH] pool and thus less PAR modification of VEGF. However, the regulation of activity of PARP cannot be excluded.

The level of FGF2 protein, another angiogenic growth factor required for the proliferation of ECs was found to be significantly high in cells maintained on FN when compared to polylysine, but the amount of FGF2 was low as compared to VEGF. Moreover, removal of VEGF by immunoprecipitation, from the heparin isolated fractions and subsequent CAM assay revealed that VEGF is the major angiogenic growth factor produced by HUVECs in culture. FGF2 exerts angiogenic activity in vivo and is reported to induce angiogenic phenotype in cultured ECs both in a paracrine
and autocrine manner. The inhibition of p38 MAPK caused a reduction in the FGF2 protein levels though to a less extent compared to VEGF.

![Diagram](image)

**Figure: 4.16. Scheme for mechanism of angiogenic action of FN:** FN interacts with αβι integrin and recruits FAK-Src and activates p38 MAPK dependent downstream signaling. p38 MAPK through the regulation of transcription factors activate the transcription of VEGF and VEGFR2. Further, the production of PAR modified VEGF was found to be low in cells maintained on FN by shifting the NAD+/NADH equilibrium towards NADH.

As discussed before, FN interacts with the cells through αβι integrin and mediates downstream signaling through FAK/Src (Chapter 3). Results presented in this chapter indicate that FN can modulate the production of angiogenic markers and growth factors in an αβι integrin-p38 MAPK dependent pathway regulating the angiogenic phenotype of ECs (Figure: 4.16). Further, FN can also regulate the post-
translational PAR modification of VEGF thereby affecting its biological activity through alterations in cellular NAD$^+$ levels. The nature of the transcription factors involved in regulating VEGF/VEGFR2 expression is discussed in detail later. These results thus indicate that FN is pro-angiogenic in nature and one of the mechanisms responsible for the pro-angiogenic effect of FN is the modulation of the expression and angiogenic activity of VEGF.