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Research Article

PURIFICATION OF VEGF AND EVALUATION OF ITS ROLE AS PROGNOSTIC TISSUE BIOMARKER IN DMH INDUCED COLORECTAL CANCER RATS TREA FED WITH COATED AND UNCOATED PRE AND PROBIOTICS

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ABSTRACT

Vascular Endothelial Growth Factor (VEGF) or Vascular Permeability Factor (VPF) is an angiogenic cytokine expressed by many human and animal tumors. It is important as prognostic tissue biomarker due to high expression level in tumors. Synbiotic feed additive is a combination of prebiotic (fructo oligosaccharides) and probiotic (Lactobacillus casei strain-17) and is said to have therapeutic role in prevention and treatment of colorectal cancer (CRC) when administered during initiation and post initiation periods. The present study was aimed to analyze the role of VEGF as a prognostic tissue biomarker in 1, 2 dimethyl hydrazine (DMH) induced CRC in female Sprague Dawley (SD) rats treated with coated and uncoated symbiotic. Four treatment groups were used (sham, CRC control, CRC fed with coated and uncoated synbiotic). VEGF was purified from colorectal tissue extracts by heparin sepharose column chromatography (HSCC) at 1.2M NaCl-TBS elution. The eluents of all the treatment groups were analyzed by 12% SDS PAGE under non reducing condition. Two prominent protein bands having molecular weights of 37kDa and 34 kDa were identified in CRC samples but not in control. They were assumed to be VEGF isoforms based on elution characteristics on HSCC and high expression in CRC. The expression level of these two proteins was assessed based on the relative staining intensity on SDS PAGE gels stainability decreased slightly in CRC treated with uncoated symbiotic while there was significant reduction in their expression level in CRC treated with coated synbiotic. The two purified proteins in the present study are hypothesized to be VEGF isoforms which may be used as good prognostic tissue biomarkers in monitoring CRCprophylaxis and therapy.

Key Words: *VEGF, synbiotics, CRC, Prognostic Tissue Biomarker*

INTRODUCTION

Angiogenesis has an important role to play in tumor growth and metastases (Folkman, 2002). Angiogenic switch, which occurs due to the disturbance of local balance between proangiogenic factors and antiangiogenic factors, is the main cause of tumor progression (Abdollahi, 2007). Among the several proangiogenic factors identified, vascular endothelial growth factor (VEGF) is identified to be the key mediator of angiogenesis with potential applications as tissue biomarker of cancer and chemotherapeutic target (Pircher, 2011; Lyden, 2001). It is overexpressed in tumours and is also found in body fluids such as serum, urine, and ocular fluids (Tapper *et al.,* 1979; Chodak *et al.*, 1981; Chodak *et al.,* 1988; Nguyen *et al.,* 1994; Yeo *et al.,* 1993). The expression level of VEGF correlates with the disease stage and it can be used as a biomarker in monitoring the response to preventive strategies and treatment of cancer (Pircher *et al.,* 2011). Colorectal cancer (CRC) is the third most prevalent form of cancer in men and

second in women for which diet and gut microflora are the major risk factors (Fotiadis, 2008; Mohandas, 2011). It implies that incidence of CRC can be prevented. The use of synbiotics (a combination of prebiotics and probiotics) in the prevention of initiation or progression of CRC and in the treatment of existing CRC is receiving attention due to their potential beneficial effects and limited or no side effects when compared to synthetic drugs (Fotiadis, 2008). The present study was undertaken to study the role of VEGF expression level as a biomarker in monitoring the response of 1, 2 dimethyl hydrazine (DMH) induced CRC to feeding with coated and uncoated synbiotics in rats.

MATERIALS AND METHODS

Procurement and maintenance of animals: Twenty four Sprague Dawley (SD) female rats aged 2 months were procured from National Institute of Nutrition (NIN), Hyderabad. The animals were fed with standard pellet diet , provided clean drinking water and maintained at 22^0C temperature and 30% relative humidity with 12hr light/dark cycle. Standard pellet diet supplied by NIN was fed along with 20% animal fat (gratis sample from Al-kabeer Exports Pvt.Ltd, Rudraram). SD rats were distributed randomly into four groups of 6 each [G-I sham (control), G II – CRC (cancer control), G III - CRC fed with uncoated symbiotic and G IV - CRC fed with coated symbiotic], permission of the college IAEC was obtained before starting the experiment.

Procurement of probiotics, prebiotics & preparation of uncoated and coated synbiotics: Probiotics (*Lactobacillus casei* strain 17) were procured from NDRI, Karnal. Prebiotics (fructo oligosaccharides) were purchased from Xena products, Hyderabad. Uncoated synbiotic was prepared by dissolving 100g of prebiotic and 100mg of freeze dried probiotic (10^{14}CFU/gm) in 5L of distilled water. Coated synbiotic beads were prepared by adding the mixture of 1% sodium alginate, 100 g prebiotic and 100 mg probiotic and spraying into 0.1M $CaCl_2$ solution accompanied by continuous stirring on a magnetic stirrer.

Induction of CRC: The carcinogen, viz., 1, 2 dimethyl hydrazine (DMH) at the rate of 30 mg per kg body weight was injected S/C for 6 weeks to induce cancer into the three treatment groups (G II, G III and G IV) High fat diet (HFD) consisting of 20 % animal fat was offered to G II, G III and G IV. G III and G IV were treated with coated and uncoated synbiotics respectively by incorporating them at the rate of 20mg/kg body weight in the diet.

Collection and processing of samples: At the end of the experimental period, the rats were sacrificed by cervical dislocation. The animals were dissected and colons were collected, cut longitudinally, faecal material was scraped from the inner lining and the tissues were flushed with Kreb's ringer solution. Throughout the dissection process, the colon tissue was flushed with ice cold phosphate buffer saline (PBS, 10mM, pH- 7) to maintain cold chain and to inhibit the activity of proteases. The colon tissues were homogenized in a hand operated homogenizer and centrifuged at 7000 rpm for 20 minutes at 4^0C. The supernatants were collected and preserved at -20^0C until further analysis.

Heparin sepharose column chromatography: VEGF was purified by heparin sepharose column (purchased from Genei, Bangalore) chromatography (HSCC) (Senger et al 1993 and Luo et al 1998). One ml supernatant of colon tissue extract was applied to heparin sepharose column previously equilibrated with 10mM PBS (pH – 7). The column was washed thoroughly with 10mM PBS (pH – 7) to remove the unbound proteins and the bound proteins were eluted with 0.4M – 2M NaCl in 10mM PBS (pH – 7). VEGF was purified by the same procedure in all the groups.

SDS PAGE: The eluents (1.2 M NaCl) of all the groups were analyzed by SDS PAGE using 5 % stacking gel and 1 % resolving gel according to the procedure described by Laemmli (1993). The running buffer was tris-glycine-SDS buffer. The medium range molecular weight markers (97.4kDa to 14.3kDa) were used (Genei, Banglore. Catalogue No. PMWM-'105979).

RESULTS AND DISCUSSION

Analysis of 1.2M NaCl-PBS eluents colorectal tissues of control, CRC induced and CRC treated with coated and uncoated synbiotics by 12% SDS PAGE under non reducing conditions revealed two prominent protein bands having molecular weights of 37 kDa and 34 kDa (Fig. 1 and Fig. 2). These two bands were either faint or absent in control sample but significant in CRC samples (Fig. 1). In rats treated with coated synbiotics during initiation and post initiation periods, there was significant decrease in the intensity of bands when compared to the CRC control, while rats treated uncoated synbiotic revealed very slight decrease in the intensity of bands (Fig. 2).

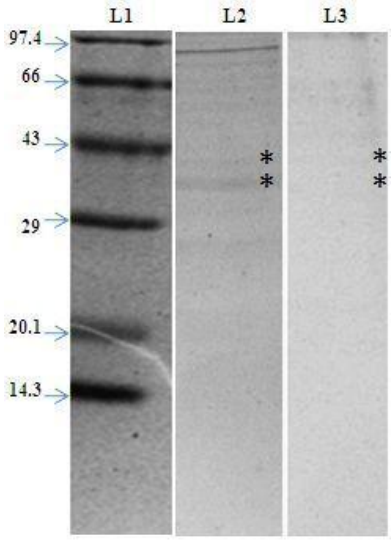

Figure 1: SDS PAGE analysis of heparin sepharose column chromatography eluents of colorectal tissue extracts of CRC (positive) control and healthy (negative) control rats. (L1 – Molecular weight markers, L2 – 1.2M NaCl-PBS eluent of CRC control and L3 - 1.2M NaCl-PBS eluent of control).

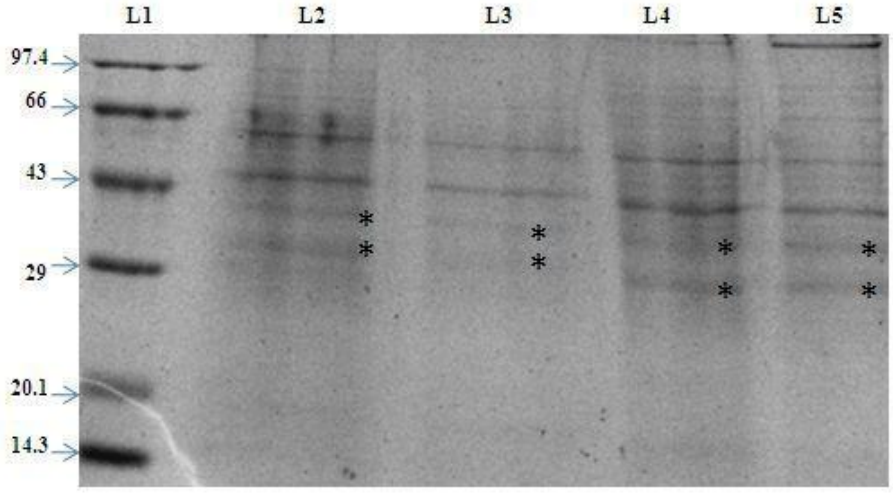

Figure 2: SDS PAGE analysis of heparin sepharose column chromatography eluents of colorectal tissue extracts of CRC (positive) control and coated & uncoated synbiotic treated CRC rats. (L1 – Protein markers, L2 to L4 - 1.2M NaCl-PBS eluents of colorectal tissues of CRC rats (L2 and L4), coated synbiotic treated CRC rats (L3), uncoated synbiotic treated CRC rats (L5).

The differential expression of 37 kDa and 34 kDa protein bands in colorectal tissue of control and CRC rats with high expression level in later indicates that they have a role to play in carcinogenesis. These two bands can be hypothesized to be vascular endothelial growth factors (VEGFs) based on the specific elution characteristic at 1.2M NaCl using heparin sepharose column chromatography and high expression in colorectal tissue of CRC rats. Ferrara and Henzel (1989) purified two proteins from pituitary follicular cell culture conditioned medium using heparin sepharose column chromatography and reverse phase HPLC whose molecular weights were identified as 43kDa and 23kDa by 12% SDS PAGE under non reducing and reducing conditions respectively. These proteins were named as VEGF based on the mitotic effect on vascular endothelial cells. Ghosh and Maity (2004) purified two protein bands of 37kDa and 26kDa by heparin sepharose column chromatography at 1.2M NaCl elution from ascitic fluid of ovarian cancer patients and confirmed them as VEGFs by dot blot, trans-immunoblot and ELISA using polyclonal goat anti-VEGF antibody. The difference in the molecular weights of the two differentially expressed protein bands in the present study is in contrast to the available literature which can be attributed to the presence of different isoforms of VEGF which arise due to alternative mRNA splicing, partial proteolysis and different degrees of N-glycosylation (Connolly, 1989 and Senger, 1990). The molecular weights of VEGF were reported in range from 34 to 42 kDa under non-reducing conditions and 17–24 kDa under reducing conditions (Connolly, 1989 and Senger, 1990). Mineur (2007) reported that VEGF is a disulfide-bonded dimeric glycoprotein with a molecular mass of 34–45 kDa. Salesi *et al.*, (2005) stated that VEGF gene family consists of several members ranging from VEGF A to VEGF E of which VEGF A, commonly referred to as VEGF exhibited most of the angiogenic activity. VEGF had many different isoforms (VEGF 121, 145, 165, 189 and 206) due to alternative mRNA splicing, of which VEGF 165 is the most prominent isoform with 50 to 100 fold potency in endothelial cell growth assays (Tisher *et al.*, 1991 and Keyt et.al., 1996). A very low expression level of the two protein bands (37 kDa and 34 kDa) purified in the present study in CRC rats treated with coated synbiotics in comparison to slightly low expression level in CRC rats treated with uncoated synbiotics and the prominent expression in CRC rats visualized in 12% SDS PAGE gel under non reducing condition emphasized the therapeutic efficacy of coated synbiotics over uncoated synbiotics. It also proves the role of the two purified proteins as prognostic tissue biomarkers in CRC. Studies on the use of coated synbiotics in the prevention and treatment of CRC are scarce and the results of the present study envisaged the advantage of coated synbiotics over uncoated synbiotic in lowering the incidence of CRC when administered during initiation and post initiation period. The two purified proteins (37 kDa and 34 kDa) identified could serve as good prognostic tissue biomarkers in monitoring the response of CRCs to preventive or treatment strategies.

CONCLUSION

Two proteins (37 kDa and 34 kDa) purified by heparin sepharose column chromatography from the colorectal tissues were hypothesized to be VEGF isoforms. Their expression levels varied with progression and regression of CRC elucidating their role as prognostic tissue biomarkers. Coated synbiotics had more therapeutic efficacy in the prevention and treatment of CRC when compared to uncoated synbiotics. Anti angiogenesis could supplement the conventional tumor therapies. Androgen withdrawl, administration of tetracyclines and dopamine have produced antiangiogenesis (Dvorak,2002). The administration of synbiotic could add a new dimension to the existing regimen as an adjuvant therapy. Further studies on the action of synbiotic either on VEGF receptor antagonists/tyrosine kinase inhibitors or other mechanism need to be elucidated. This study was directed only towards preventing the initiation of tumorogenesis as DMH and synbiotics were simultaneously administered. Hence, studies are also desired on the action of synbiotics in clinical cases and assess if they would be of benefit after the progression of tumor.

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REFERENCES

Abdollahi A, Schwager C, Kleeff J, Esposito I, Domhan S, Peschke P, Hauser K, Hahnfeldt P, Hlatky L, Debus J, Peters J M, Friess H, Folkman J, and Huber P E. (2007). Transcriptional network governing the angiogenic switch in human pancreatic cancer. *Proceedings of National Academy of Sciences* **104**(31) 12890–12895.

Chodak G W, Hospelhorn V, Judge S M *et al.* (1988). Increased levels of fibroblast growth factor-like activity in urine from patients with bladder or kidney cancer. *Cancer Research* **48** 2083-88.

Chodak G W, Scheiner C J, Zetter BR. (1981). Urine from patients with transitional transitional- cell carcinoma stimulates migration of capillary endothelial cells. *The New England Journal of Medicine* **305** 869-74.

Connolly D T, Heuvelman D M, Nelson R, *et al.* (1989). Tumor vascular permeability factor stimulates endothelial cell growth and angiogenesis. *Journal of Clinical Investigation* **84** 1470–8.

Dvorak HF(2002). Vascular Permeability Factor/vascular endothelial growth factor: A critical cytokine in tumor angiogenesis and a potential target for diagnostic therapy. *Journal of Clinical Oncology* **20** (21) 4368-4380.

Ferrara N and Henzel W J. (1989). Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells. *Biochemical and Biophysical Research Communication* **161** 851–858.

Folkman J (2002). Role of angiogenesis in tumor growth and metastasis. Seminar on Oncology **29**(6 Suppl 16) 15-8.

Fotiadis C I, Stoidis C N, Spyropoulos B G, Zografos E D (2008). Role of probiotics, prebiotics and synbiotics in chemoprevention for colorectal cancer. *World journal of Gastroenterology* **14**(42) 6453-6457.

Ghosh S and Maity P(2004). Isolation and purification of vascular endothelial growth factor (VEGF) from ascetic fluid of Ovarian cancer patients. *Pathology and Oncology Research* **10**(2) 104-108.

Keyt B, Berleau L and Nguyen H (1996). The carboxyl-terminal domain (111-165) of vascular endothelial growth factor is critical for its mitogenic potency. *Journal of Biology and Chemistry* **272** 7788-7795.

Laemmli UK(1993). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227** 680-685.

Luo JC, Yamaguchi S, Shinkai A et al. (1998). Significant expression of vascular endothelial growth factor in mouse ascitic tumors. *Cancer Research* **58** 2652-2660.

Lyden D, Hattori K, Dias S, Costa C, Blaikie P, Butros L, Chadburn A, Heissig B, Marks W, Witte L (2001). Impaired recruitment of bone-marrow-derived endothelial and hematopoietic precursor cells blocks tumor angiogenesis and growth. *Nature Medicine* **7** 1194–1201.

Mineur P, Colige A C, Deroanne C F, Dubail J, Kesteloot F, Habraken Y, Noël A, Waltenberger S V J, Lapière C M, Nusgens B V, and Lambert C A (2007). Newly identified biologically active and proteolysis-resistant VEGF-A isoform VEGF111 is induced by genotoxic agents. *The Journal of Cell Biology* **179**(6) 1261–1273.

Mohandas K.M. (2011). Colorectal cancer in India: controversies, enigmas and primary prevention. *Indian Journal of Gastroenterology* **30**(1) 3-6.

Nguyen M, Watanabe H, Budson AE, *et al*. (1994). Elevated levels of an angiogenic peptide, basic fibroblast growth factor in the urine of patients with a wide spectrum of cancers. *Journal National Cancer Institute* **86** 356-61.

Pircher A, Hilbe W, Heidegger I, Drevs J, Tichelli A and Medinger M (2011). Biomarkers in tumor angiogenesis and anti-angiogenic therapy. *International Journal of Molecular Science* **12** 7077-7099.

Salesi N, Bossone G, Veltri E, Barbara di cocco, Marolla P, Pacetti U, Larosa G, Muni R and Vecchione A (2005). Clinical experience with bevacizumab in colorectal cancer. *Anticancer Research* **25** 3619-3624.

Senger D R, Connolly D T, Van de Water L, Feder J, Dvorak HF (1990). Purification and NH_2-terminal amino acid sequence of guinea pig tumor-secreted vascular permeability factor. *Cancer Research* **50** 1774–8.

Senger D R, Van de Water L, Brown LF, *et al.* (1993). Vascular permeability factor (VPF, VEGF) in tumor biology. Cancer Metastatic Review **12** 303-324.

Tapper D, Langer R, Bellows A R, Folkman J. (1979). Angiogenesis capacity as a diagnostic marker for human eye tumors. *Surgery* **86** 36-40.

Tischer E, Mitchell R, Hartman T *et al*. (1991): The human gene for vascular endothelial growth factor. Multiple protein forms are encoded through alternative exon splicing. *Journal of Biology and Chemistry* **266**(18) 11947-11954.

Yeo K T, Wang H H, Nagy J A, *et al.* (1993). Vascular permeability factor (vascular endothelial growth factor) in guinea pig and human tumor and inflammatory effusions. *Cancer Research* **53** 2912-18.