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Isolation and cloning of human VEGF promoter region in PGL3 Basic Vector

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Abstract- Worldwide mortality and morbidity from infectious diseases is being replaced by non infectious chronic diseases, such as cancer, obesity, type II diabetes, cardiovascular diseases, neurodegenerative diseases and aging which may involve inflammation. Vascular endothelial growth factor (VEGF) as a potent pro-inflammatory cytokine is elevated in many human diseases, or animal models of human disease, which are mentioned above. Compounds derived from botanic sources, such as polyphenolic compounds express anti-inflammatory activity by modulation of pro-inflammatory gene expression. We hypothesized this effect may related to control regulation of VEGF gene promoter. In this study, the VEGF promoter was isolated using nested PCR to define the transcription factors binding sites. The human VEGF promoter region was cloned in DH5 alpha Ecoli for future investigation.

Index Terms- Inflammation, VEGF (Vascular Endothelial Growth Factor), Polyphenols, NFκB.

I. Introduction

Inflammation is a biological response of vascular tissues to harmful stimuli, such as pathogens, damaged cells, or irritants. Although in the absence of inflammation, wounds and infections would never heal, chronic inflammation can also lead to host potential diseases, such as high fever, atherosclerosis and rheumatoid arthritis [4-8]. The inflammatory response is a complex self-limiting process regulated to prevent damage to the host [2]. Angiogenesis is the key process involved in normal development and wound repair and is motivated by different kinds of growth factors for instance vascular endothelial growth factor (VEGF) which is crucial angiogenic growth factor through increasing vascular leak of both proteins and particulates or permeability [3-5]. VEGF main function is to produce new blood vessels throughout embryonic development. In adult human they are responsible in recovering vascular injuries, by creating new vessels to evade blocked vessels. It is proposed that VEGF levels increase before and/or during the angiogenic process. This changes in VEGF level is observed in many human diseases which are characterized by inflammation and vascular leak, for instance cerebral ischemia, tumor ascites, trauma, early diabetic retinopathy, preeclampsia, ovarian hyper stimulation syndrome, and status epilepticus [7-1].

Some compounds extracted from botanic sources, such as phenolic compounds, have shown anti-inflammatory activities *in vitro* and *in vivo*. They act directly or indirectly through cyclooxygenase, lipoxygenase, nitric oxide synthases and several cytokines, mainly by acting through nuclear factor-kappa B and mitogen-activated protein kinase signaling [2]. Since NFκB coordinate the induction of a numerous genes [3], the inhibition of NFκB as a transcription factor is a useful strategy for treatment of inflammatory disorders. It is suggested that the anti-inflammatory effects of polyphenols are mediated through the decrease of NFκB gene expression, which in turn leads to angiogenesis prevention [9-6].

Although the function of VEGF is well studied, the mechanism involve in its expression is vague. Hence the aim of this study is to isolate the promoter region that controls the expression of this important gene in order to investigate the factor that may influence its expression.

II. Material and Methods

A. Designing of primer from human VEGF promoter DNA sequence

To isolate the VEGF gene promoter region, both forward and reverse primers were designed for human VEGF promoter structure using only 3480 bps from the transcription binding site of the overall sequence of the gene by using the primer 3 Input. In addition the sequences for the whole human VEGF gene and its promoter were obtained in EMBL-EBI database (Accession number AF095785). The primer specificity was checked using bioinformatics tools in order to avoid from cross reaction. Also primer validation was done through experiments. Moreover, nested primer sequences were designed for the introduction of restriction enzyme cutting site (Table.1).

Table.1: Primer sequences for isolation of human VEGF promoter regions

	Forward primer	Reverse primer	Species
Normal PCR	5´GAGGAACTTGCGGTGTTAGC 3´	5´GCGTCTTCGAG-AGTGAGGAC 3´	Human
Nested PCR	5´AAAAACGCGTCTTCGAGAGT 3´ *Mlu I* cutting site	5´AAAAAGATCTCATGGTGGAGGTA 3´ *Bgl II* cutting site	Human

B. DNA extraction from human blood

The blood sample from human was assembled in EDTA tubes; then DNA isolation was carried out using Wizard Genomic DNA Extraction Kit Promega. The DNA quality and purity were analyzed using nanodrop ND1000 and agarose gel electrophoresis.

C.PCR amplification

VEGF gene promoter was amplified by polymerase chain reaction (PCR) and gel purified for further use as DNA sample for nested PCR.

D. Nested PCR amplification VEGF with two restriction sites

Nested polymerase chain reaction was carried out for some reasons: first, as an alteration of polymerase chain reaction in order to decline the contamination in products caused by the amplification of unanticipated primer binding sites; second, to produce different sized segments of the postulated promoter regions (thereby removing regions thought to be important in the regulation of transcription); third, to allow cloning into reporter gene because the primers were designed with two different restriction sites; Mlu I for the upstream and Bgl II for the downstream. Then agarose gel electrophoresis as well as gel extraction and PCR product clean up carried out to prepare the sample in order to be digested for ligation.

E. Isolation and purification of PGL3 Basic vector from E.coli DH5α

The transformation technique was used to amplify the plasmid; then the *E.coli* DH5α containing plasmid (PGL3) was spread onto LB agar containing ampicillin and incubated at 37ºC overnight; after that plasmid was cultured overnight in LB broth comprising ampicillin at 37 ºC with shaking in order to grow up for both amplifying and storage as glycerol stocks for future use. The plasmids were isolated and purified for digestion process before ligation with T4 ligase.

F. Double digestion of VEGF and PGL3 basic vector, using Mlu I and Bgl II

The gel purified nested PCR products which were believed to be VEGF promoters (the insert) and also PGL3 Basic vector were digested by using restriction enzymes Mlu I and Bgl II.

G. Ligation of VEGF into PGL3 Basic

The digested PGL3 Basic vector was treated with Calf Intestinal Alkaline Phosphatase (CIAP), before ligation with the insert. After treatment, the vector was purified by phenol: chloroform extraction followed by ethanol precipitation. The digested insert (the VEGF promoter) was then ligated with digested and CIAP treated PGL3 Basic vector.

H. Transformation of Insert-PGL3 (Plasmid-DNA) construct into host cell

The constructed recombinant plasmid was transformed into E.coli strain DH5α competent cells; then, they were streaked onto LB media plates comprising ampicillin and incubated at 37ºC overnight, for screening.

III. Result and Discussion

A. Polymerase Chain Reaction and Nested PCR

The bands observed in the 3000-3500 bp (3 kb) from the upstream of transcriptional binding site were assumed as the promoter regulatory sequences same as predicted amplicon by the designed primer. The product from this PCR was taken to undergo a second run with the second set of primers containing the restriction cutting sites (Fig.1).

Since the nested PCR is a very specific PCR amplification, its product has little contamination from unwanted products of primer dimmers, hairpins, and alternative primer target sequences. The nested PCR products were observed approximately in 1500 bp as anticipated amplicon by designed primers including restriction cutting sites (*Mlu I* for upstream and *Bgl II* for downstream) (Fig.2).

1)

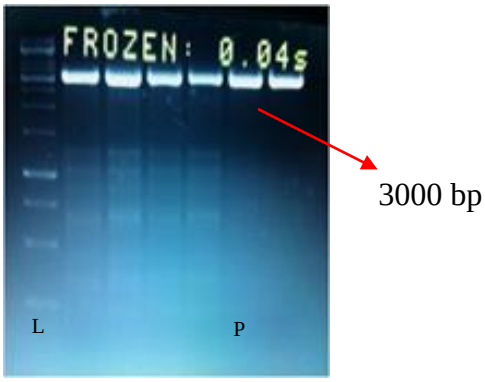

Fig.1: Gel electrophoresis of PCR product;
L-1kb DNA ladder, P-PCR product

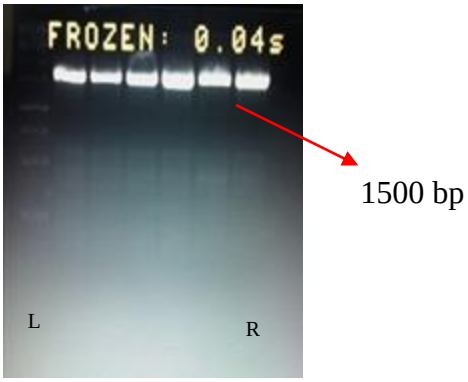

Fig.2: Gel electrophoresis of Nested PCR products;
L- 1kb DNA ladder, R- Replicate of nested PCR product

B DNA sequencing of human VEGF promoter and Similarity search

PCR products were purified and sent with forward and reverse primers to a Laboratory for DNA sequencing and the result was conducting with capillary electrophoresis method applied biosystem genetic analyzers; also the full sequence of VEGF promoter obtained from Bioedit software was BLASTn as query sequence for similarity; the result showed 98% similarity to homo species chromosome 6 (ref|NW_923073.1|). Further alignment of the sequencing was carried out for both forward and reverse primer to verify high similarity with the human VEGF promoter sequence where the primers were designed. In addition the potential transcription factors with binding sites in human VEGF promoter region were identified using TFSEARCH site; http://www.cbrc.jp/research/db/TFSEARCH.html. According to these discovered sites, the crucial part of promoter region required for promoter regulation activities were anticipated.

C Isolation and purification of PGL3 Basic Vector from E.coli DH5α

Since it was shown in Fig.3, there are transformed colonies in the plate comprising ampicillin (antibiotic) which demonstrates only transformed cultures can survive on this media as they posse the plasmid carrying the antibiotic resistance gene.

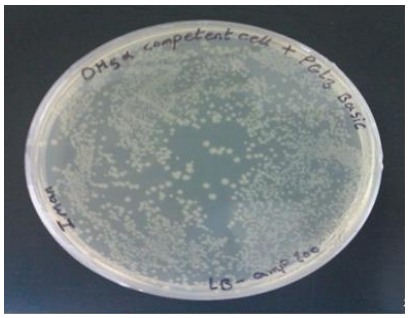

Fig.3: Transformed colonies containing PGL3 Basic Vector

D. Digestion of the vector and insert

Digested insert believe to be VEGF promoter was observed approximately at 1500 bp, while the digested vector (PGL3) was detected at 5000 bp as it expected (Fig.4).

Fig.4: Gel electrophoresis of digested insert and digested CIAP treated vector. L- 1 kb DNA ladder,
DI- Digested insert, TV- Treated digested PGL3 basic vector with CIAP

E. Transformation of VEGF promoter constructs into E.coli DH5 alpha

These two controls were done in order to ensure the digestion on vector (PGL3) was done properly; Transformed circular vector and digested vector (positive and negative controls), which indicated the vector before digestion has its origin form (circular) that is carrying ampicillin (antibiotic) resistance gene, while the linear vector (digested), because it lost its resistance to antibiotics was not able to grow in media comprising ampicillin (Fig.5).

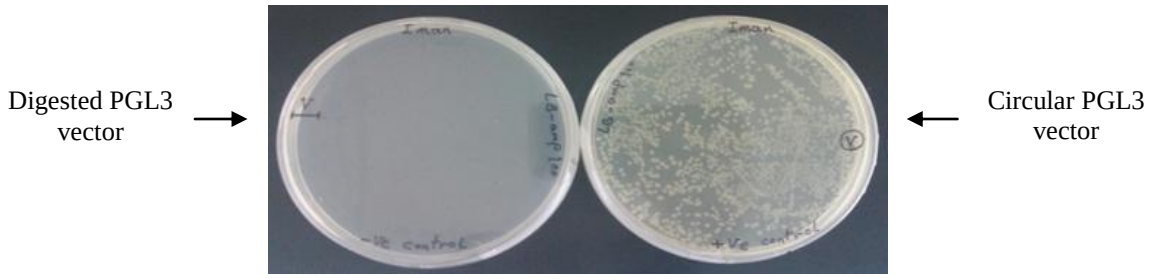

Fig.5: Transformed circular vector and digested vector (positive and negative controls)

Bacteria transformation is a reaction that host organism (E.coli DH5 alpha) takes in plasmid (PGL3 Basic with VEGF promoter gene) and expresses the foreign gene. The presentation of the plasmid including antibiotic resistance (ampicillin) into a bacterial strain was needed to verify the transformation. If the susceptible bacteria merge the foreign DNA, they will have resistance to the ampicillin. As Fig.6, shows, only transformed colonies can persist in this plates comprising ampicillin since they posse the plasmid carrying the ampicillin resistance gene. These transformed colonies may include the recombinant plasmid.

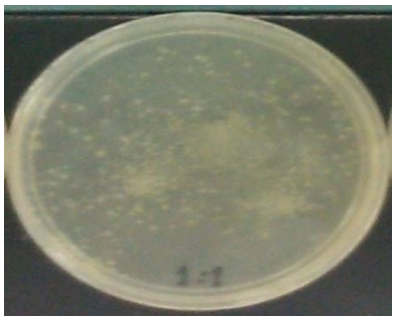

Fig.6: Transformed colonies containing possible recombinant plasmid

Finally the colony PCR was done in order to verify the accuracy of transformation which was supposed to get the same band like what was gotten for VEGF promoter (approximately1500 bp).

IV. CONCLUSION

In conclusion, the promoter region of human VEGF was successfully isolated from human blood. Based on the sequencing result and bioinformatic tools, several overlapping consensus sequence on the human VEGF promoter believe to be the main regulator for VEGF expression were identified. The human VEGF promoter region was cloned in *E.coli* DH5 alpha. In the future, promoter reporter assays can be conducted in order to investigate the effect of polyphenols on the human VEGF promoter. This will provide information on mechanism involve in inflammatory diseases.

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REFERENCES

[1] A. S. R. Maharaj, and P. A. D'Amore. Roles for VEGF in the adult. Microvascular Research. (2007), 74: pp. 100–113.

[2] C. Santangelo, R. Varì, B. Scazzocchio, R. D. Benedetto, C. Filesi, and R. Masella. Polyphenols, intracellular signalling and inflammation. (2007), 4: pp.394-405.

[3] M. Karin, and Y. Ben-Neriah. Phosphorylation meets ubiquitination: the control of NF-[kappa] B activity. Annu Rev Immunol. (2000), 18: 621-63.

[4] M. M. Mueller. Inflammation in epithelial skin tumours: Old stories and new ideas. Im Neuenheimer Feld. Heidelberg, Germany. (2006), 280: 735-7447.

[5] M. Ushio-Fukai. Redox signaling in angiogenesis: role of NADPH oxidase. Cardiovasc. Res. (2006), 71:pp. 226–235.

[6] P. Xia, J. R. Gamble, K. A. Rye, L. Wang, C. S. Hii, P. Cockerill, Y. Khew-Goodall, A. G. Bert, P. J. Barter, and M. A. Vadas. Tumor necrosis factor-alpha induces adhesion molecule expression through the sphingosine kinase pathway. Proc. Natl. Acad. Sci. U. S. A. (1998), 95: pp.14196–14201.

[7] S. D. Croll, T.Wei, R. M. Ransohoff, L. J. Kasselman, G. D. Yancopoulos, N. Cai, J. Kintner, Q. Zhang, M. Karin, Y. Yamamoto, and Q.M. Wang. The IKK NF-kappa B system: a treasure trove for drug development. Nat Rev Drug Discov. (2004), 3:pp. 17-26.

[8] Tischer, R. Mitchel, T. Hartman, M. Silva, D. Gospodarowicz, J.C. Fiddes, and J.A. Abrahamll. The Human Gene for Vascular Endothelial Growth Factor ,Multiple Protein Forms Are Encoded Through Alternative Exon Splicing. (1991), 25: pp.11947-11954.

[9] Y. Osawa, H. Uchinami, J. Bielawski, R. F. Schwabe, Y. A. Hannun, and D. A. Brenner. Roles for C16-ceramide and sphingosine 1-phosphate in regulating hepatocyte apoptosis in response to tumor necrosis factor-alpha. J. Biol. Chem. (2005), 280:pp. 27879–27887.

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