

MOLECULAR THERAPEUTIC CANCER PEPTIDES: A CLOSER LOOK AT BOVINE LACTOFERRICIN

ABSTRACT

Bovine lactoferricin (LfcinB) is a cationic peptide with broad spectrum antimicrobial and anticancer activity. It is made up of twenty-five (25) amino acids with a disulphide bridge between two cysteines and thus gives a cyclic structure. The peptide possesses strong cationic charge (+8) which confers the peptide the ability to selectively target the negative charge of cancer cells (-) while sparing healthy cells. It also shows promising activity against many varieties of cancer including colon carcinoma cells, melanoma, fibrosarcoma, monocytic leukemic cells and neuroblastoma. The peptide was reported to be unaffected by multidrug resistance mechanisms seen with conventional chemotherapeutic drugs, and displays a higher specificity for cancer cells versus normal cells in comparison to conventional chemotherapy. Although the exact mechanism of action of LfcinB is not fully understood, subcutaneous administration of LfcinB inhibit lung and liver metastasis of both murine melanomas and lymphomas, confirming the ability of LfcinB to hinder the development and spread of cancer to nearby tissues. The aim of this paper is to review the anti-cancer activity of bovine lactoferricin with its Biochemical and physiological activities within cancer cells.

Keyword: Bovine Lactoferricin; Disulphide Bridge; Apoptosis; Angiogenesis; Cytotoxic Activity

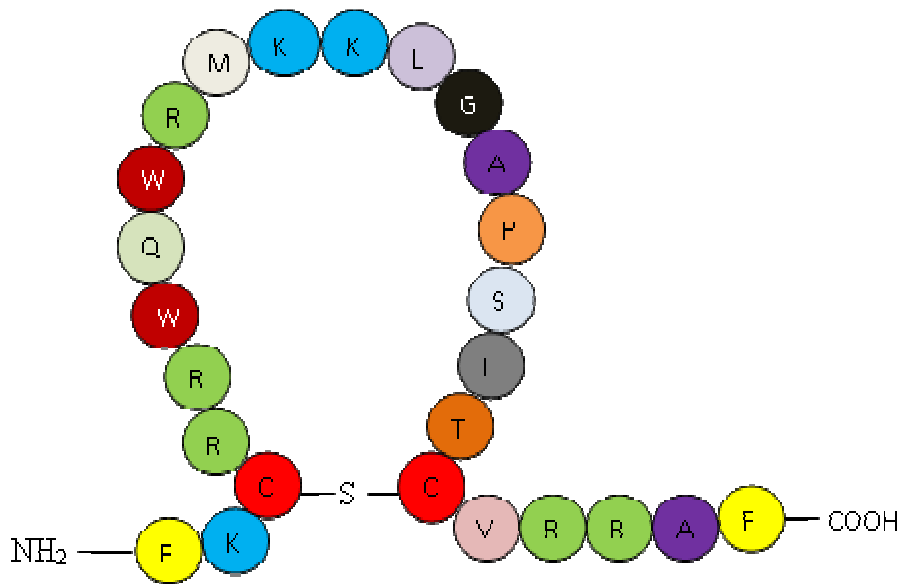
1. INTRODUCTION

Bovine lactoferricin (LfcinB) is a peptide with cationic and amphipathic propriety which was reported to have cytotoxic activity to cancer cells of both human and rodent [1,2]. It is a peptide fragment generated by acid-pepsin hydrolysis of lactoferrin, a peptide obtained from cow's milk [1-3]. It consists of 25 amino acids peptide with a disulfide bridge between two cysteines (Figure 1), thus having a cyclic twisted anti-parallel β -sheet structure [3, 4]. Considerable amount of LfcinB are detected in the human stomach after consumption of bovine lactoferrin [1, 4], proving the natural hydrolysis of LfcinB from the digestion of cow's milk. Due to its well-document antimicrobial activity, many scientists have generated interest in LfcinB and it is now appearing to have antitumor activity. The broad spectrum against microorganisms comprises of activity against bacteria, [5] fungi [6] and viruses [7]. Evidence shows that LfcinB also possesses potent *in vivo* and *invitro* activity against cancer cells [8]. Studies have indicated that subcutaneous administration of LfcinB inhibit lung and liver metastasis of both murine melanomas and lymphomas [8] and it has also been proven to induce apoptosis in human leukemic and carcinoma cell lines, [1,8] all this shows that LfcinB has a broad anticancer activity against many different type of cancers. In addition to its antitumor activities, it is reported to have variety of biological activities, including cells transcriptional activation [9] and regulation of immune response [10, 11]. The peptide may play important role in the protective mechanism against carcinogenic effects due to its cationic property, which may oppose the negative charge of potentially carcinogenic molecules as well as the cancer cell. Research have shown that injections of human

32 lactoferrin reduce the growth of transplanted solid tumors in mice, confirming the inhibitory effect of
33 LfcinB to cancer cells [12].
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35 2. STRUCTURE OF BOVINE LACTOFERRICIN

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38 LfcinB possesses a cyclic structure formed by the disulfide bond that links its two-cysteine residues
39 (Figure 1). In a watery solution, LfcinB assumes an amphipathic beta-sheet conformation in which the
40 residues that are hydrophobic line up on one face of the peptide and the basic residues comprises the
41 opposing face [13]. The primary structure of LfcinB has been described as peptide consisting of 25-
42 residue peptide with an amphipathic, antiparallel β -sheet structure [13]. The 25 AA residue peptide
43 forms looped structure through an intramolecular disulfide bond (Figure 1) [14]. It has a single
44 disulfide bond and no iron-binding capacity, and is active against a broad spectrum of
45 microorganisms, including bacteria [13]. Studies conducted using nuclear magnetic resonance (NMR)
46 spectroscopy reveal that LfcinB exhibit a conformation that is quite different in aqueous solution from
47 that found in the intact protein [13]. The peptide, in low salt solution, loses α -helix structure observed
48 in intact bovine lactoferrin and changes into β -sheet. Adopting the change in structure, the released
49 peptide becomes markedly amphipathic as nearly all the hydrophobic residues lie on one face while
50 the positively charged residues lie on the other [15] The amphipathic property is accounted by β
51 twisted structure that the peptide adopts when it is in aqueous solution. The hydrophobic residues are
52 found in one face while the opposing face consists of the basic positively charged amino acid
53 residues [15]. Molecular dynamic simulation can be used to observe this change in conformation in
54 which LfcinB undergo a transition from an α -helix to a β -sheet hairpin structure in aqueous solution,
55 but the interconversion does not proceed readily in the presence of 250 mM salt [16]. LfcinB contains
56 five arginine and three lysine residues that confer a net charge of +8 (Table 1) on the peptide, as well
57 as two tryptophan and two phenylalanine residues that are both aromatic and hydrophobic [14].
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65 Figure 1: structure of Bovine lactoferricin, all amino acids in the peptide is represented by
66 one letter coded
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3. STRONG CATIONIC CHARGE OF BOVINE LACTOFERRICIN LINKED TO ITS ANTICANCER ACTIVITY

The anticancer activity of LfcinB is attributed to its strong cationic charge +8 which confers the peptide the ability to selectively target the negative charge (-) of the most cancer cells, while healthy normal cells with nil charge (0) remains unaffected due to the high content of zwitterionic phosphatidylcholine located outside the leaflet of the membrane [17]. The net negative charge of most cancer cells of the leaflet of the membrane results due to

The difference that exist in the branch and N-linked glycans of sialic acid which are connected with glycoprotein [18] that crosses the membrane and Over-expression of anionic molecules [19,20-22] such as phosphatidylcholine O-glycosylated mucins [17,21]. The inability of the LfcinB to bind to PC3 carcinoma is probably due to the fact that some cancer cells refract the cytotoxic action of LfcinB due to insufficient negative charge through which electrostatic interaction took place between LfcinB and cancer cells [3]. Derivatives of LfcinB Containing cationic and hydrophobic moieties were reported to have enhanced activity Owing to its high positive charge, structure and amphipathic properties whereas cancer cells cytotoxicity has not been observed with homologue of murine lactoferricin containing glutamic acid [22-24].

4. CYTOTOXIC ACTIVITY OF LFCINB AGAINST CANCER CELLS

Scientists have elucidated the invitro cytotoxic activity of bovine lactoferricin using both human and mice cancer cells such as colon carcinoma cells, melanoma, fibro sarcoma, [25] monocytic leukemic cells [1,26] and neuroblastma, [2] and studies noted that treatment of these cells with LfcinB did not significantly affect the viability of normal human lymphocytes, erythrocytes, endothelial cells and fibroblasts [1,27]. The mechanism of action of LfcinB against neuroblastoma and fibro sarcoma rat cells but not human cells can be attributed to the formation of transmembrane in the plasma membrane through which the peptide is delivered to the component of cytoplasm of cancer cell. The peptide then interacts with the natively charged of the mitochondria, inducing apoptosis mainly via cell injury (necrosis) through cell membrane lytic. When LfcinB is inserted in the membrane, it causes changes in the membrane mimetic systems by fostering the assembling of bicontinues cubic or inverted hexagonal phases [28-32]. Studies have reveal that LfcinB destroy THP-1 human monocytic leukemia cells through the apoptotic pathways activation. Generation of ROS within the cells and activation of $\text{Ca}^{2+}/\text{Mg}^{2+}$ -dependent endonucleases has been linked to the apoptotic-inducing activity of LfcinB to THP-1 human monocytic leukemia cells [25]. In this same experiment, is it noted that THP-1 cells Treated with 100 $\mu\text{g}/\text{mL}$ LfcinB undergo apoptosis after about 10 h with maximal activity, while those cells Treated with bLF did not induce cell death even at a high dose of 500 $\mu\text{g}/\text{mL}$. Fragmented DNA was exhibited by THP-1 cells treated with LfcinB in a dose-dependent manner; a time- and dose-dependent gradually decrease in integrity of the cell membrane which permitted LfcinB to go through the compartment of the cytoplasm. The disruption of cell membrane by LfcinB extracellularly does not depend on internalization of the peptide, but itself may be responsible for the cytotoxic activity of the peptide. LfcinB induce-apoptosis was found to be inhibited by addition of Zn^{2+} , an inhibitor of endonucleases which require $\text{Ca}^{2+}/\text{Mg}^{2+}$ for full activity, however, LfcinB-induced cell death was effectively abolished in THP-1 cells following the addition of antioxidants, such as N-acetylcysteine (NAC) and glutathione (GSH), similarly to that induced by H_2O_2 . [25].

5. THE CASCADES THAT TRIGGERED CANCER CELLS TO APOPTOSIS

The mechanism by which LfcinB destroy cancer cell involved binding of the cancer cell and causes the formation of transmembrane pores allowing the peptide to enter cytoplasmic compartment of the affected cancer cells and co-localized with negatively charged mitochondria [1,2,27]. Mouse fibrosarcoma cells and human neuroblastoma cells subjected to LfcinB die primarily via necrosis caused by a cell membrane lytic effect, [2]. LfcinB has been shown to kills human leukemia and breast carcinoma cells by a process that involves the sequential generation of ROS, loss of mitochondrial transmembrane potential, and activation of the caspase cascade resulting in cell death by apoptosis (Figure 2) [1,8]. Research [1] have further confirmed the ability of LfcinB to induced apoptosis in cancer cells through the generation of ROS and activation of $\text{Ca}^{2+}/\text{Mg}^{2+}$ -dependent

endonucleases. Further, LfcinB-induced apoptosis in human T-leukemia cells was established to be triggered by cascades of events. The events lead to permeabilization of the cell membrane mediated by LfcinB, subsequent crossing of the LfcinB that disrupted cell membrane, colocalization of the LfcinB with mitochondria, release of cytochrome C as a result of depolarization of mitochondria, Interaction of cytochrome c with Apaf-1 and subsequent recruitment and activation of procaspase-9 forming apoptosome which triggered activation of caspases that ultimately lead to cell death through apoptosis (Figure 2). Some studies have suggested that cytotoxicity of the peptide to cancer cells accounted by necrotic or apoptosis depend on the peptide concentration [2, 24]. Even though the action of the peptide decreases with increased serum [8] concentration, intratumoral or systemic administration has been reported to inhibit carcinogenesis and metastasis of a number of tumors in mice [2, 25, and 26]. This effect results due to anionic serum components neutralization rather than proteolytic degradation. In recent times, LfcinB-induced apoptosis in B-lymphoma cells was found to occur via activation of cathepsin B rather than involvement of caspases activation [35]

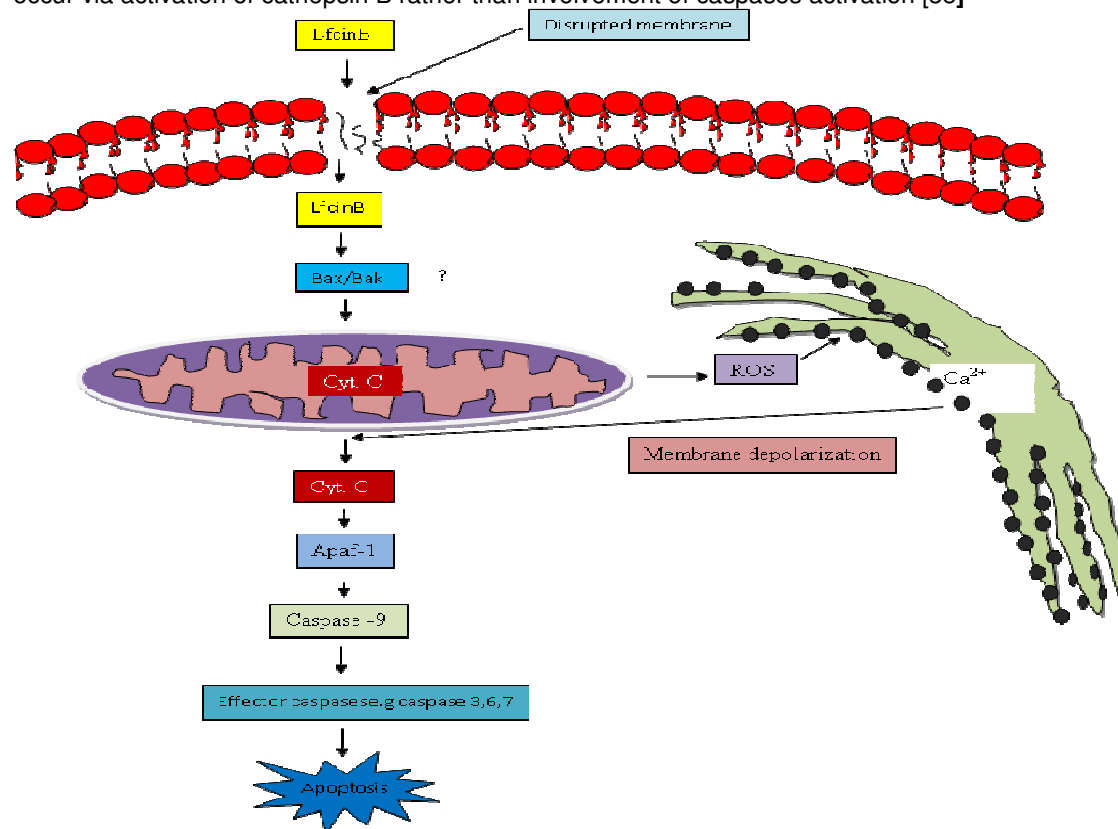


Figure 2: proposed mechanism of action of LfcinB. "In the diagram LfcinB causes membrane lysis and gains entry into the cytoplasm. It co-localizes with the mitochondria and causes the release of cytochrome C. The cytochrome C interacts with Apaf-1 and caspase-9 to form the apoptosome. Caspase-9 then activates caspase 3 which ultimately leads to apoptosis."

6. BOVINE LACTOFERRICIN AND ANGIOGENESIS

Angiogenesis is the process that encompasses the growth, differentiation and migration of endothelial cells within the wall of blood vessels. Research has been directed upon the discovery of peptide-based angiogenic inhibitor as they are least toxic and safe for therapy against abnormal angiogenic associated diseases [36]. Binding of signal molecule are required to their receptors for angiogenesis to occur on endothelial cells. Endothelial growth factors and Signal molecules such like VEGF binds to their receptors and initiate process that lead to growth and differentiation of new blood vessels. Inhibitors of angiogenesis hinder

156 this various process. LfcinB has been shown to repress both basic fibroblast growth factor
157 (bFGF) - and VEGF-driven proliferation and migration of human endothelial cells in vitro, and
158 as well inhibiting with bFGF- and VEGF-induced angiogenesis in mice, it does this by
159 competing with bFGF and VEGF for growth factor receptor-associated heparin sulfate
160 proteoglycans on the endothelial cell surface [37,38]. LfcinB was thought to Hinder with the
161 interaction of signal molecule such as VEGF and bFGF with their receptors on the surface of
162 endothelial cell [37], which result in diminished angiogenesis and decreased endothelial cell
163 proliferation [37]. Even though the precise mechanism of interaction of LfcinB with heparin-
164 like molecules has not been fully understood, it was suggested that the LfcinB demonstrated
165 attraction for heparin-like structures resulting in electrostatic attraction between the positive
166 charge of LfcinB and negative charge of heparin sulfate and heparin (-).The whole anti-
167 angiogenic activity depends on the amino acids sequence (primary structure) of the peptide,
168 in view of the fact that a scrambled peptide composed of the same amino acid residues
169 compete inefficiently with bFGF or VEGF for binding sites on heparin-like endothelial cells.
170 The major drawback of systemic administration of LfcinB as therapy for anti-angiogenesis is
171 the sensitivity of the peptide to enzymatic degradation and inactivation through anionic
172 serum components interactions [37, 38].

173 174 175 176 **7. THERAPAUTICS ASPECT OF BOVINE LACTOFERRICIN**

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178 The use of peptides has gain popularity as therapeutic agents against cancer,
179 cardiovascular diseases and diabetes and it is now growing in a rapid manner as a means of
180 chemotherapeutics to other areas [39]. LfcinB have been proven to be effective anti-
181 carcinogenesis molecule with a unique way of exerting it pharmacological actions. The
182 peptide was reported to bypasses the mechanisms of multidrug resistance frequently
183 encounter with many conventional chemotherapeutic agents [40-45] and shows high
184 specificity to cancer cells than the normal cells in comparison to other chemotherapeutic
185 agents [46, 47]. In addition, invivo studies have established its potential as anticancer agents
186 following primary tumor regression [2, 25] and hindering of metastasis [48, 49]. Intratumoral
187 injection of LTX302, a short peptide obtained from LfcinB which occurs naturally has been
188 shown to cause complete regression of tumors [46-49].

189
190 Active immunization has also emerged as a means of treating cancer and studies uses the
191 component of the immune system - molecules or cells to boost the immune system [50, 51].
192 The concept of fighting cancer through vaccination has also been translated into clinical
193 research and clinical trials in the last decades. Clinical oncologist specifically designed
194 vaccines based on known antigens in order to induced immunity against cancer [50].
195 Vaccines made up of peptides which are obtained from sequences of protein of specific
196 antigens or tumor concerned is used in treating neoplastic cells. In an interesting way, a
197 small peptide derived from LfcinB, LTX302, was also found to evoke immunity hindering
198 carcinogenesis and metastasis [50-52].

199
200 Oncologically, peptides with high affinity for receptors that are over-expressed on tumor cells
201 are used as diagnostic tools and also have broad applicability in clinical research and trials
202 for anticancer therapeutics [53]. Hence, these peptides may emerge as diagnostic tools and
203 as agents of choice for anti-cancer investigation through attachment to chemotherapeutic
204 moieties including cytotoxic agents or radionuclide. The peptides can conveniently be linked
205 directly to halogens (iodine and fluorine) or radionuclide of metals using suitable chelating
206 agents. In view of the fact that these small peptides can be synthesized relatively easy,
207 synthesizing new peptides and enhancing the existing ones will likely be beneficial for
208 clinical investigations [52, 53]. In this scenario, many properties of these small peptides have
209 to be taken into account such as stability of the peptide in body fluids, non- toxic to normal

cells, affinity of the peptide to their receptors and improving its overall pharmacological effects [53].

8. PEPTIDES AS SUITABLE AGENTS FOR DESIGNING EFFECTIVE ANTICANCER AGENTS

In spite of the progress that was recorded in the development of anticancer drugs, challenges continue to rise particularly due to resistance of the cancer chemotherapies and low sensitivity of the commercially available anticancer drugs [53]. Peptides such as LfcinB which are host defense peptides and effectors molecules of innate immunity as well may give rise to a unique way for designing effective anticancer agents. The peptide bypasses the mechanism of drug resistance [54, 55] and provides immunological response [55] against mass of tumors, indicating its suitability for design and development of novel anticancer therapeutics. The anticancer peptide is able to make distinction between cancer and normal cells binding specifically to the negative charge of the component of the plasma membrane (sialic acid, heparan sulfate or phosphatidylserine), which differentiate between neoplastic and non-neoplastic cells. Susceptibility of cancer cells to anti-cancer peptide has been found to be enhanced by increased in the number of microvilli on the cancer cells which also result in increased surface area. Due to their selective target between neoplastic and non-neoplastic cells membrane, host defense peptides have high exceptional penetration power and are taken effectively to both distant metastasis and primary tumors. The chance of resistance occurs less likely due to the fact that this peptide attacked their target rapidly by disrupting plasma membrane integrity [55]. Designing a peptide that is extremely effective having serum stability would lead to a novel approach in cancer therapy. The present cancer situation clearly indicates that better treatment options that selectively target cancer with little or no toxicity to normal tissues are urgently necessary. Novel and cheaper alternatives to current cancer therapeutics, including specific cytotoxic molecules and vaccines that can boost the human immune system to fight deadly forms of cancer are needed these days. The challenge lies in developing the clinical application of therapeutic peptides. Improving delivery to tumors, serum stability, minimizing non-specific toxic effects and discerning pharmacokinetic properties are high among the needs to produce a powerful therapeutic peptide for cancer treatment.

9. DISCUSSION

Bovine lactoferricin has gained a wide range of use in medical and therapeutic applications. A potent cationic anticancer peptide, LfcinB displays its antitumor activity with lysine and arginine accounting for the overall charge of the peptide. It exerts its pharmacological activities against many human and rodent cancer cells including colon carcinoma cells, melanoma, fibrosarcoma, monocytic leukemic cells and neuroblastoma [1,2, 25-27]. Many of the metabolic changes as well as the biochemical changes causes modifications in mechanism of transport, regulation of program cell death and enzymes activities contribute to drug resistance within cancer cells [56].

One peculiar quality about this peptide is that it is unaffected by multidrug resistance mechanisms frequently encountered with many conventional chemotherapeutic agents, and displays a higher specificity for cancer cells versus normal cells in comparison to conventional chemotherapy [46,47]. The peptide rapidly induced apoptosis in several different cancer cell lines indicated by DNA fragmentation assays and phosphatidylserine headgroup inversion detected by Annexin V binding to the surface of cancer cells. Although this peptide did not adversely affect the viability of untransformed human lymphocytes, fibroblasts, or endothelial cells indicating its safety on the normal cells, Production of reactive oxygen species was observed after Treatment of Jurkat T leukemia cells with the peptide which ultimately lead to activation of caspases.

A major limitation of cancer therapeutic efficacy is often systemic non-specific biodistribution which often leads to the reduced bioavailability of drug delivered to target cancer cells and therefore reduce therapeutic index. In this respect, LfcinB bioavailability can be increased to rapidly reach the target tissue without much serum proteases degradation, thus increase its serum stability.

10. CONCLUSION

We come into conclusion that LfcinB possesses strong anticancer activity against many different cancer cells. It exerts its cytotoxic activity causing apoptosis to tumor cells through mitochondria pathway leading to DNA fragmentation without Affecting the Viability of normal Cells. Accordingly, increase in LfcinB activity would lead to designed of better cancer therapeutics.

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480 ABBREVIATIONS

481 AA, amino acids
482 Apaf-1, Apoptotic protease activating factor 1
483 bFGF, Basic fibroblast growth factor
484 bLF, Bovine Lactoferrin
485 GSH, Reduced glutathione
486 LfcrinB, Bovine lactoferricin
487 NAC, N-acetyl cysteine
488 PC 3, Human *prostate cancer* cell line-3
489 ROS, Reactive oxygen species
490 THP-1, Human acute monocytic leukemia *cell* line-1
491 VEGF, Vascular endothelial growth factor