Introduction

From the words angio (blood vessels) and genesis (creation), angiogenesis is the creation of new blood vessels. The formation of new blood vessels out of pre-existing capillaries, or angiogenesis, is a sequence of events that is of key importance in a broad array of physiologic and pathologic processes. Normal tissue growth, such as in embryonic development, wound healing, and the menstrual cycle, is characterized by dependence on new vessel formation for the supply of oxygen and nutrients as well as removal of waste products. Also, a large number of different and nonrelated diseases is associated with formation of new vasculature. Among these pathologies are diseases, such as tissue damage after reperfusion of ischemic tissue or cardiac failure, where angiogenesis is low and should be enhanced to improve disease conditions (***Carmeliet et al., 1999; Ferrara and Alitalo, 1999).***

In several diseases, excessive angiogenesis is part of the pathology. These diseases include cancer (both solid and hematological tumors), cardiovascular diseases (atherosclerosis), chronic inflammation (rheumatoid arthritis, Crohn's disease), diabetes (diabetic retinopathy), psoriasis, endometriosis, and adiposity. These diseases may benefit from therapeutic inhibition of angiogenesis ***(Folkman, 1995; Hanahan and Folkman, 1996).***

The blood vessels in the body have long been considered to only function as a transport compartment of the blood. Nowadays, it is appreciated that the vasculature is one of the main organs in the body, extending more than 900 m^2 and playing a major role in maintaining the body's integrity in various ways ***(Rajotte et al., 1998).***

Blood vessels consist of endothelial cells that are in direct contact with the blood, and subendothelially located pericytes, smooth muscle cells, fibroblasts, basement membrane (BM), and extracellular matrix (ECM). Depending on the location in the body, the organ microenvironment, the cellular constituents, BM, and ECM of the vasculature differ in phenotype, composition, and function ***(Rajotte et al., 1998).***

The endothelial cells from a monolayer in every single blood vessel in the circulation and are actively involved in several regulatory processes in the body (Figure. 1).

(1) Beside being metabolically active and selectively permeable for small solutes , peptides and proteins , (2) the endothelial cells regulate blood coagulation . when their integrity is maintained endothelial cells exert anticoagulative properties via the synthesis of thrombomodulin, tissue factor (TF) pathway inhibitor and tissue-type plasminogen activator (t-PA). ***(Verstraete,1995).***

(3)Another important feature of endothelial cells is their ability to direct cells of the immune system to specific sites in the body ***(Carlos and Harlan 1994).***

(4) Last, endothelial are actively involved in vascular remodeling during, for example ,ovulation, wound healing, tumour growth, and diabetic retinopathy .***(Rajotte et al 1998).***

The body control of angiogenesis occurs in the healthy body for healing wounds and for restoring blood flow to tissues after injury or insult. In females, angiogenesis also occurs during the monthly reproductive cycle (to rebuild the lining of uterus, to mature the egg during ovulation) and during pregnancy (to build the placenta, the circulation between mother and fetus), The healthy body controls angiogenesis through a series of "on" and "off" switches:

- The main "on" switches are known as angiogenesis-stimulating growth factors e.g. angiopoietin-1, fibroblast growth factors: acidic (aFGF) and basic (bFGF), hepatocyte growth factor (HGF), interleukin-8 (IL-8),placental growth factor (PGF), tumor necrotic factor (TNF), transforming growth factor-alpha (TGF-alpha), and beta (TGF-beta), vascular endothelial growth factor (VEGF) .etc .

- The main "off switches" are known as angiogenesis inhibitors e.g. endostatin, human chorionic gonadotropin (hCG), interferon alpha / beta / gamma, vasostatin, placental ribonuclease inhibitor, plasminogen activator inhibitor ...etc .

When angiogenic growth factors are produced in excess of angiogenesis inhibitors, the balance is directed toward blood vessel growth. When inhibitors are present in excess of stimulators, angiogenesis is stopped. The normal, healthy body maintains a perfect balance of angiogenesis modulators. In general, angiogenesis is "turned off" by the production of more inhibitors than stimulators ***(Risau,1997)*** .

In vasculogenesis during embryonic development, new endothelial cells differentiate from stem cells. In contrast, in angiogenesis new blood vessels mainly emerge from pre-existing ones ***(Risau, 1997)*** .

In adult life, physiologic stimuli during wound healing and the reproductive cycle in women lead to angiogenesis, whereas vasculogenesis is absent. Pathologic conditions such as tumor growth, rheumatoid arthritis, and diabetic retinopathy are characterized by abundant angiogenesis. The active vascular remodeling phase in tumors, e.g., is reflected by the fact that tumor endothelial cells proliferate 20 to 2000 times faster than normal tissue endothelium in the adult ***(Denekamp, 1984)*** .

Angiogenesis is rapidly initiated in response to hypoxic or ischemic conditions. Vascular relaxation, for example, mediated by nitric oxide (NO) is a prerequisite for endothelial cells to enter the angiogenic cascade. Likely, morphologic changes of the endothelial cells lead to a decrease in confluency status to make them susceptible to mitogens (***Folkman, 1997***) .

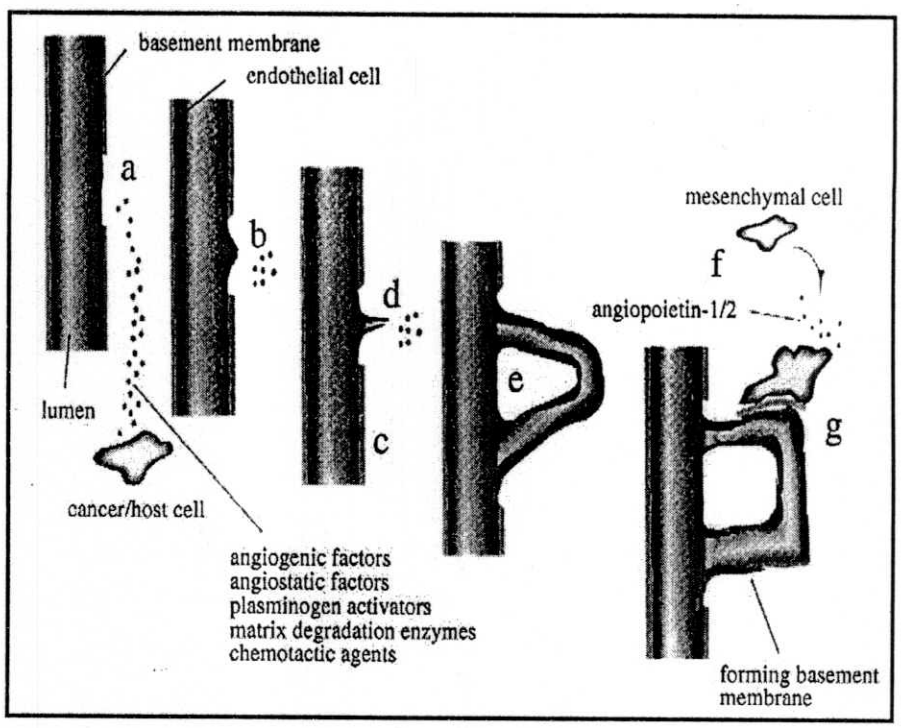
The process of angiogenesis, either under physiologic or pathologic conditions which: (figure 2).

1. **Endothelial cell activation** is the first process to take place. Cytokines from various sources are released in response to hypoxia or ischemia. It is suggested that vascular endothelial growth factor (VEGF) is a major player in angiogenesis initiation based on its ability to induce vasodilatation via endothelial NO production and its endothelial cell permeability increasing effect ***(Ziche et al., 1997).*** This allows plasma proteins to enter the tissue to form a fibrin-rich provisional network ***(Dvorak, 1986).***

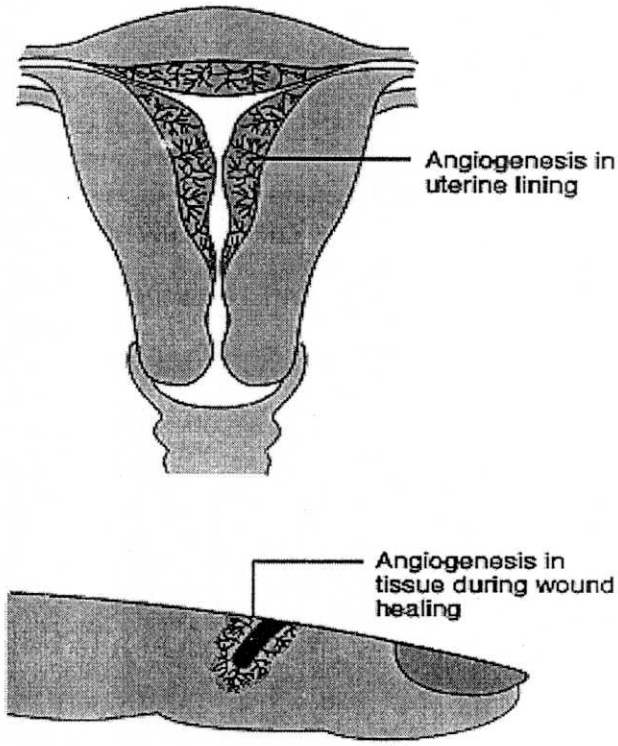
The observation that VEGF production is under control of hypoxia inducible factor (HIF) strengthens the suggestion of an early involvement of VEGF in the angiogenic response. Moreover, VEGF receptor (VEGFR) expression is up-regulated under hypoxic or ischemic conditions as well ***(Forsythe et al., 1996).***

VEGF is abundantly produced by hypoxic tumor cells, macrophages and other cells of the immune system ***(Brown et al., 1997).***

Besides affecting vasodilatation and vascular permeability, VEGF can induce the expression of proteases and receptors important in cellular invasion and tissue remodeling and is able to prevent endothelial cell apoptosis ***(Ferrara and Keyt, 1997; Gupta et al., 1999).***



The angiogenic process



Physiological angiogenesis

Figure: 2

After proper activation of the endothelial cells, endothelial penetration into new areas of the body is achieved by degradation of the BM by matrix metalloproteinases (MMPs). These extracellular endopeptidases are secreted as zymogens that become activated in the ECM compartment and subsequently selectively degrade components of the ECM ***(Stetler Stevenson, 1999).***

These (MMPs) are produced by a variety of cells, including epithelial cells, fibroblasts, inflammatory cells, and endothelial cells. ***(Gomez et al., 1997; Valente et al., 1998).***

2. **Endothelial Cell Migration and Proliferation**. Plasminogen activators urokinase- plasminogen activator u-PA and tissue type – plasminogen activator t-PA convert the plasma protein plasminogen to plasmin. Plasmin has a broad trypsin-like specificity and degrades, e.g., fibronectin, laminin, and the protein core of proteoglycans. In addition, plasmin activates certain metalloproteinases. Plasmin is believed to be the most important protease for the mobilization of fibroblast growth factor-2 (FGF-2 or basic FGF) from the ECM pool.

FGF members are directly acting proangiogenic molecules. FGF-2 consists of, in two modifications, an 18-kDa low-molecular weight form and a 22- to 24-kDa high-molecular weight form. During angiogenesis, low-molecular weight FGF-2 binding to endothelium induces FGF receptor (FGF-R) down-regulation, increased motility, proliferation and proteinase activity, and modulates integrin levels. High-molecular weight FGF-2 may act on endothelial cell proliferation after nuclear translocation in the endothelial cells ***(Gleizes et al., 1995;Klein et al., 1997).***

Recently, it was shown that a secreted FGF-2-binding protein could bind FGF-2 that is normally inactive due to strong adherence to heparin sulfate proteoglycans in the ECM. The displaced FGF-2 molecules were thus released to mediate biological function ,angiogenesis seems exquisitely sensitive to small changes in factors such as VEGF and FGF-2 that drive the angiogenic process. This may have important therapeutic implications in treating angiogenesis-driven disorders ***(Czubayko et al., 1997).***

Besides its effect on angiogenesis initiation, VEGF also affects endothelial cell proliferation. ***(Czubayko et al., 1997).***

Integrins are transmembrane proteins composed of an α and β subunit in over 20 different heterodimeric combinations. They bind to ECM proteins or cell surface ligands through short peptide sequences. Combinations of different integrins on cell surfaces allow cells to recognize and respond to a variety of different ECM proteins ***(Varner, 1997).***

3. **Maturation of the Neovasculature.** Endothelial cell interaction with ECM and mesenchymal cells is a prerequisite to form a stable vasculature. Therefore, after endothelial cell proliferation and maturation, and the formation of endothelial tube structures, surrounding vessel layers composed of mural cells (pericytes in small vessels and smooth muscle cells in large vessels), need to be recruited. Endothelial cells may accomplish this via the synthesis and secretion of platelet-derived growth factor (PDGF), a mitogen and chemoattractant for a variety of mesenchymal cells. Subsequent differentiation of the mural precursor cells into pericytes and smooth muscle cells is believed to be a cell-cell contact-dependent process.

On endothelial cell-mural cell contact, a latent form of transforming growth factor (TGF-β,) produced by both endothelium and mural cells, is activated in a plasmin-mediated process. Activated TGF-β can induce changes in myofibroblasts and pericytes, which may contribute to the formation of a quiescent vessel, ECM production, and maintenance of growth control. The coinciding investment of growing capillaries by pericytes with the deposition of BM and cessation of vessel growth during wound healing also indicates vessel growth regulation by pericytes ***(Hirschi and D'Amore, 1997).***

FGF-1 is also implicated in endothelial cell differentiation leading to vascular tube formation. Besides inducing plasminogen activator and endothelial cell proliferation and migration, FGF-1 receptor signaling resulted in endothelial tube formation ***(Kanda et al., 1996) .***

4. **Other Mechanisms Implicated in Angiogenesis Control**. Although the roles of several factors during angiogenesis have been discussed here separately, it is important to note that the activity of an angiogenesis-regulating cytokine depends on the presence and concentration of other factors or cytokines in the environment of the responding endothelium ***(Pepper et al., 1998) .***

For example, exogenous factors such as hormones can affect conditions leading to angiogenesis ***(Schiffenbauer et al., 1997).***

Isoforms of VEGF that bind to ECM-associated heparin sulfate proteoglycans can release ECM-stored FGF-2 in a bioactive form ***(Jonca et al., 1997).***

and angiopoietins potentiate the effects of VEGF ***(Asahara et al., 1998).***

Although their relative role in angiogenesis is not yet fully elucidated, it is now well appreciated that cells of the immune system such as monocytes/macrophages, lymphocytes, and mast cells can affect pro- and antiangiogenic balances ***(Sunderkotter et al., 1996; Blair et al., 1997).***

T- lymphocytes were able to activate endothelial expression of various metalloproteinases As a consequence, increased tube formation in a three-dimensional gel was observed ***(Mach et al., 1999).***

Recently, **Keshet** and **coworkers** identified the importance of the presence of periendothelial cells in the microenvironment as a control mechanism of angiogenesis. Loss of VEGF by androgen ablation therapy lead to selective apoptosis of endothelial cells in vessels devoid of periendothelial cells. Based on this observation, it is now hypothesized that VEGF is required to maintain cell anchorage to a provisional ECM until periendothelial cells facilitate a more permanent mode of adhesion ***(Benjamin et al., 1999).***

Besides the already mentioned proangiogenic factors, VEGF, FGF-1, and FGF-2, many others have now been identified in various settings of physiologic and pathologic angiogenesis. Among them are TGF-α and TGF-β, granulocyte macrophage-colony-stimulating factor, epidermal growth factor, interleukin-1 (IL-1), scatter factor, platelet-activating factor, IL-8, and substance P ***(Bouck et al., 1996; Yoshida et al., 1997).***

Their effects can be either directly or indirectly on the endothelium via activation of surrounding cells to produce other factors with proangiogenic activity or modulation of receptors/receptor activities ***(Yoshida et al., 1997; Giraudo et al., 1998).***

Therapeutic angiogenesis is an experimental treatment that medically promotes the creation of new blood vessels, Much attention has been payed to therapeutic strategies that are able to stop the angiogenic cascade in tumor growth (and more recently, in chronic inflammatory situations such as rheumatoid arthritis (There are, however, various diseases affecting millions of people every year that would benefit from the induction of angiogenesis, so-called therapeutic angiogenesis ***(Takeshita et al., 1994).***

The disease conditions that may benefit from therapeutic angiogenesis encompass ischemic diseases such as ischemic coronary artery disease, critical limb ischemia with various etiologies. In these diseases, functional blood flow is partially lost in an organ or limb. For coronary artery disease, the leading cause of morbidity and mortality in Western countries, the therapeutic options (reducing the risk factors, restoration of the blood flow by angioplasty, or coronary bypass grafting), are insufficient. In critical limb ischemia, estimated to develop in 500 to 1000 individuals per million per year. ***(Baumgartner et al., 1998).*** A specific form of vascular occlusive disease that leads to critical limb ischemia, is thromboangitis obliterans, or Burger's disease. The disease affects arteries of young smokers and is characterized by the onset of distal extremity ischemic symptoms, leading to ulceration and gangrene ***(Isner et al., 1998).***

Gastro duodenal ulcers, also caused by local insufficient perfusion, have been subject of angiogenesis stimulation therapies ***(Wolfe et al., 1995).***

It has recently been suggested that for congestive heart failure, possibly a result of myocardial ischemia, stimulation of angiogenesis may

also become a therapeutic option ***(Carmeliet et al., 1999; Isner and Losordo, 1999).***

The treatment of arterial occlusions by balloon angioplasty is frequently associated with delinquent re-endothelialization and smooth muscle cell proliferation. One therapeutic option to reduce subsequent intimal thickening is the induction of apoptosis in infiltrating immune cells ***(Sata et al., 1998).*** Therapeutic angiogenesis to facilitate endothelial cell regeneration in this specific pathology has been proposed as well ***(Callow et al., 1994; Asahara et al., 1996).***

In the case of organ transplantation, surgical procedures decrease vessel integrity and function of the transplanted organ ***(Taub et al., 1998).*** Transplantation of encapsulated pancreatic islets as a treatment modality for type I diabetes, for example, may be more successful when prevascularized solid supports are used or solid supports are pretreated with proangiogenic factors ***(De Vos et al., 1997).***

Ischemic diseases from different etiologies may improve when treated with agents that induce neovascularization. Although a vast number of proangiogenic factors are available to date mostly VEGF and FGF-2 have been explored for this purpose. More recently, the proangiogenic protein angiopoietin-1 (Ang-1), has been applied in therapeutic angiogenesis strategies as well ***(De Vos et al., 1997)*** .

Angiogenesis is driven by numerous mediators produced by numerous cells under a variety of conditions. These mediators are either soluble, ECM or membrane bound growth factors, or components of the ECM itself. Of the soluble factors, one of the best studied and the most

potent proangiogenic factor is VEGF, discovered in the early eighties by Dvorak and colleagues ***(Senger et al., 1983).***

VEGF (also known as VEGF-A) isoforms VEGF-121, -145, -165, -183, -189, and -205 are a result of alternative splicing from a single VEGF gene located on chromosome 6 ***(Mattei et al., 1996)..***

Together with VEGF-B, -C, and -D, they belong to the VEGF/PDGF super family. Recently, a viral VEGF family member, designated VEGF-E, was described ***(Meyer et al., 1999).***

The two VEGF-specific tyrosine kinase receptors, VEGFR-1 (Flt-1) and VEGFR-2 (Flk-1), are expressed on vascular endothelium, and to a lesser extent on monocytes/macrophages and certain tumor cell types. VEGFR-3 (Flt-4), which binds VEGF-C and VEGF-D, is mainly expressed on lymphatic endothelium ***(Kaipainen et al., 1995).***

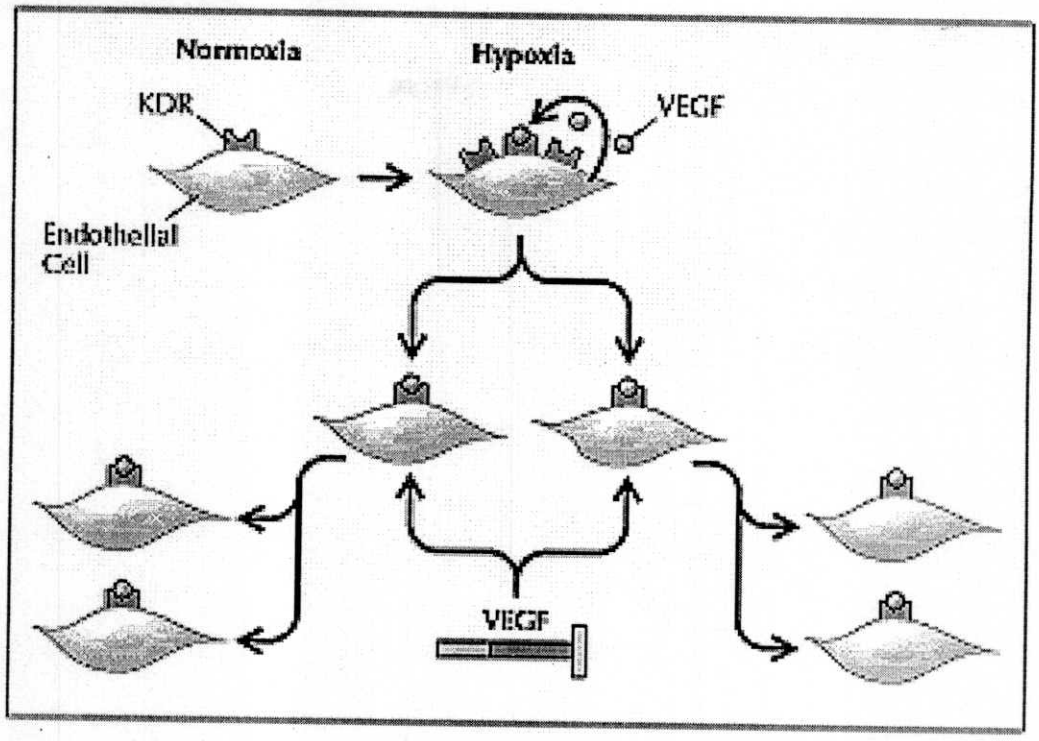
Interaction of VEGF with VEGFR-2 is a critical requirement to induce the full spectrum of VEGF biologic responses. Intracellular signal transduction pathways in endothelial cells through VEGFR-2 dimerization lead to permeability enhancement, cellular proliferation, and migration, ***(Abedi and Zachary, 1997; Kroll and Waltenberger, 1997; Wheeler Jones et al., 1997; Ziche et al., 1997; Gerber et al., 1998; Hood and Granger, 1998; Wellner et al., 1999; Doanes et al., 1999; Shen et al., 1999; Yu and Sato, 1999).***

Actually, VEGF and other endothelial cell mitogens should not promote angiogenesis indiscriminately but rather limit it to sites of wound healing and tissue ischemia, where vascular growth may be beneficial. Studies in animal models using fibroblast growth factor (FGF) or VEGF have shown that administration of angiogenic cytokines produces

neovascularization in the region of ischemia. For example, when recombinant VEGF was injected into the normal or ischemic limb in rabbits studied, angiogenesis was observed only in the ischemic limb, Analysis of ^{165}VEGF-binding indicated no substantial increase in VEGFR-2 affinity in normoxic or hypoxic endothelial cells. Instead, increased binding during hypoxia was associated with a 13-fold increase in the number of VEGFR-2 receptors. VEGF site-specificity is thus a function of VEGFR-2 expression by endothelial cells. Factors secreted from hypoxic myocytes in ischemic tissues upregulate VEGF expression on adjacent endothelial cells, which then attract circulating VEGF into the ischemic tissue and amplify its effects . ***(Baumgartner et al., 1998).*** figure (3) .

It is well established that the immune system plays an important role in the regulation of angiogenesis. Multiple studies indicate that leukocytes can induce vascular proliferation ***(Sidky and Auerbach, 1975; Polverini et al., 1977; Camussi et al., 1997).*** and specific leukocyte-derived cytokines have been identified to induce angiogenesis ***(Koch et al., 1992; Hashimoto et al., 1994;Richardson et al., 1994; Vanhee et al., 1994; Freeman et al., 1995).***

Next to the regulation of angiogenesis by leukocytes, angiogenic processes can have a major impact on cells of the immune system and on the development of an immune response as well. Normal endothelial cells contribute to the recruitment of immune cells to the site of inflammation by the expression of adhesion molecules. Different families of adhesion



Bioactivity of vascular endothelial growth factor (VEGF) appears to derive from an autocrine mechanism that upregulates expression of VEGF and specific receptors in response to hypoxia. Experimentally, hypoxic endothelial cells increase both VEGF secretion and the number of VEGF receptors on endothelial cells. Thus, any VEGF molecule secreted- or administered- into an ischemic territory would have amplified effects. Endothelial cells stimulated to proliferate in response to VEGF may then serve as additional sources of the cytokine's synthesis, thus amplifying the angiogenic effect of the initial VEGF dose. (from Baumgartner et al., 1998).

Figure : 3

Molecules play a role in this process. The most important families identified at present are: "1" the Ig superfamily of Ig related molecules, such as intracellular adhesion molecule – 1(ICAM-1), vascular cell adhesion molecule-1(VCAM-1), and CD31; "2". The selectins, molecules that initiate the adhesion cascade by mediating leukocyte rolling through recognition of carbohydrate epitopes; and 3) a group consisting of among others, CD34 an L-selectin binding glycoprotein that is expressed on hematopoietic progenitor cells and on the luminal side of vascular endothelial cells ***(Kuzu et al., 1992)*** . and CD44, the lymphocyte homing receptor that is expressed on activated endothelial cells ***(Griffioen et al., 1997)*** .

Expression of endothelial adhesion molecules is controlled by cytokines such as tumor necrotic factor (TNF), inter lukin-1 (IL-1), and interferon(IFN). These cytokines facilitate leukocyte adhesion to endothelial cells and extravasation into tissues by inducing an enhanced expression of ICAM-1, VCAM-1, and E-selectin among others ***(Carlos and Harlan, 1994).***

A link between leukocyte-endothelium adhesion and angiogenesis seems to be present as suggested by the observation that endothelial markers originally identified to play a role in leukocyte recruitment appear to be involved in neovascularization as well ***(Ferrara, 1995).***

Immunohistochemistry

Is a technique for identifying cellular or tissue constituents (antigens) by means of antigen- antibody "Ag-Ab" interactions, the site of Ab . binding being identified either by direct labeling of Ab. or by use of 2ry labeling method ,an antigenic protein ,carbohydrate or lipid molecule bears one or more antibody binding sites ,these are highly specific topographical regions composed of small numbers of amino acids or monosaccharide units and are known as antigenic determinant groups or epitopes ,while antibodies belong to a class of serum proteins known as immunoglobulins, they are formed in the humoral immune system by plasma cell which is the end cell of B-lymphocyte transformation after recognition of foreign antigen, there are 5 types of antibodies found in the blood of higher vertebrates :IgA,IgD,IgE,IgG,IgM.

IgG is the commonest and the most frequently used antibody for Immunohistochemistry , the IgG molecule is composed of two pairs of light and heavy polypeptide chains linked by disulfide bonds to form Y-shaped structure , the terminal regions of each arm vary in amino acid sequence and are known as (variable domains) , this variability in amino acid provides specificity for particular epitope and enables the antibody to bind specifically to the antigen against which it was raised , the amino acid chains of the variable domain of an antibody form a cavity which is geometrically and chemically complementary to a single type of Ag-epitope as described by ***Capra and Edmundson 1977*** .

The analogy of a lock (antibody) and key (antigen) has been used, the associated Ab. and Ag. Are held together by a combination of hydrogen bonds, electrostatic forces and **Van der Waals** forces. ***(Bancroft and gamble 2002).***

Enzymes are the most widely used labels in Immunohistochemistry and incubation with a chromogen using a standard histochemical method produces a stable, colored reaction end- product suitable for light microscope, horse radish peroxidase is the most widely used enzyme, and in

Recombinant Human Vascular Endothelial Growth Factor 165 (rhVEGF165)

Recombinant Human VEGF produced in E.Coli is a double, non-glycosylated, polypeptide chain containing 165 amino acids and having a molecular mass of 38231 Dalton, The sequence of the first five N-terminal amino acids was determined and was found to be Ala-Pro-Met-Ala-Glu, which agrees with the sequence of native human VEGF.

Inform of Sterile Filtered White lyophilized (freeze-dried) powder And it is very soluble in water and most aqueous buffers below and above the isoelectric point (1mg/ml) .

Lyophilized rhVEGF165 although stable at room temperature, should be stored desiccated below 0°C. Reconstituted rhVEGF165 is best stored refrigerated at 4°C.

Aim of the work

This work was performed to study the effect of aging on angiogenesis in response to regional ischemia in experimental animal models and to study the effect of administration of one of angiogenic growth factors on angiogenesis.