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Effect of glycated opticin on angiogenesis: mechanism & relevance to Proliferative Diabetic Retinopathy

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A Thesis submitted in partial fulfilment of the requirements of the Manchester Metropolitan University for the degree of
Doctor of Philosophy

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Abstract

Diabetic retinopathy is one of the major microvascular complications of diabetes and considered as the common cause of blindness worldwide. Various studies have indicated that increased AGE formation during diabetes might play an essential role in the development and progression of retinopathy. Preretinal neovascularisation is responsible for the onset of diabetic retinopathy and ultimately blindness. CollagenIV of the vitreous humour provides an essential substrate for this process.

The blindness is normally associated with pathologies including non proliferative diabetic retinopathy (NPDR), retinopathy of prematurity (ROP), and retinal vein occlusion. It is characterised by the angiogenic growth of neovessels from the preexisting inner retinal vasculature into the vitreous humour. Visual loss is caused by vitreous haemorrhage and/or tractional retinal detachment induced by the contraction of fibrous tissue associated with these new blood vessels which are immature, with a low pericyte and smooth muscle cell covering, and often abnormally shaped.

Opticin is an extracellular matrix glycoprotein and a member of the small leucine– rich repeat protein/proteoglycan family. It is highly expressed in the eye throughout life and localises to the vitreous humour. It has been previously shown to be a potent endogenous inhibitor of angiogenesis, although the signalling mechanisms responsible for its action have not been determined. Here, it is demonstrated that opticin significantly inhibited migration and tube-like structure formation of bovine aortic endothelial cells (BAEC) and human retinal microvessel endothelial cells (HREC) in matrigel, induced by FGF-2. Regarding cellular signalling, opticin also inhibited FGF-2-induced phosphorylation of MEK1/2, p38 and JNK2, confirmed by Western blotting, which could explain its anti-angiogenic properties. We also observed increasing in Akt expression. Protein glycation is also a feature of chronic diabetes and can result in modified cellular function. Here we show that exposure of opticin to high methyalglyoxal produces glycation that inhibited its ability to reduce endothelial cell migration and tube-formation, and reduced its ability to inhibit cell signalling through ERK/MEK and JNK. This may be one mechanism through which the angiogenic switch is altered in pro-angiogenic proliferative retinopathy.

Here it was observed that when opticin became glycated, it was less able to bind to collagen, the collagen, thereby inhibiting α2β1 integrin binding to collagen affecting the angiogenesis inhibiting properties of opticin.

AGEs are localized in retinal vessels and neuroglia of diabetic patients where they exert a range of deleterious effects on cell function. *In vivo* and *in vitro* studies suggest that elevated AGE level occurring in diabetes may be an important factor in retinopathy initiation and progression. Our result shows the distributions of the opticin mostly in front of the eye with a small amount in retina and vitreous. Furthermore, the diabetic mouse has more AGEs than the non-diabetic mouse, and the increased co-localization of AGEs with opticin was very clear in diabetic mouse compared with non-diabetic mouse.

We can conclude the opticin become glycated if exposed to high sugar level and this reduces its ability to bind to collagen and impair angiogenic signalling. The accumulation of AGEs in retina may play a causative role in the development of corneal epithelial disorders of diabetic patients. Glycation of opticin could one of mechanisms allowing venel growth into the vitreous during PDR.

Declaration

I declare that this work has not been accepted for any degree before and is not currently being submitted in candidature for any degree other than the degree of Doctor of Philosophy of the Manchester Metropolitan University.

Ahmeda I. Benjama

Dedication

This study is dedicated to those who sacrificed their lives during the struggle for freedom in my country, Libya. Also to my wonderful and loving family for their untiring support and endless encouragement especially through the difficult times.

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Abbrreviations

Ω (AMO)	Acetol/acetone monooxygenase.
Ω (AGEs)	Advanced Glycation End-Products.
Ω (AMD)	Age-related macular degeneration..
Ω (α-SMA	ALpha smooth muscle actin.
Ω (Ang-1)	Angiopoietin-1.
Ω (Ang2)	Angiopoietins -2.
Ω (ACE)	Angiotensin changing enzyme.
Ω (BDR)	Background Diabetic Retinopathy.
Ω (BM).	Basement membrane.
Ω (bFGF)	Basic fibroblast growth factor.
Ω (bms)	Bone marrows.
Ω (CVD)	Cardiovascular disease.
Ω (cm)	Centimetres.
Ω (DM)	Diabetes mellitus.
Ω (DR)	Diabetic retinopathy.
Ω (DAG)	Diacylglycerol.
Ω (DHAP)	Dihydroxyacetone phosphate
Ω (esRAGE)	Endogenous secretory RAGE.
Ω (enos)	Endothelial nitric oxide synthase.
Ω (EGF)	Epidermal growth factor.
Ω (EC)	Epithelium cell.
Ω (ECM)	Extracellular matrix.
Ω (FACIT)	Fibril associated collagens with interrupted triple helices.
Ω (GSH)	Glutathione.
Ω (hba_{1c})	Glycated haemoglobin.
Ω GOPT	Glycated opticin
Ω (G-3-P)	Glyceraldehyde 3-phosphate.
Ω (GAPDH)	Glyceraldehyde-3-phosphate.
Ω (Gly)	Glycine.
Ω (GAG)	Glycosamino glycan.
Ω (HSPG)	Heparan sulphate proteoglycans.

Ω (Tie-2)	Homology domains-2.
Ω (H_2O_2)	Hydrogen peroxide.
Ω (OH^-)	Hydroxyl ion.
Ω (HIF-1)	Hypoxia inducible factor1.
Ω (HIF-2)	Hypoxia inducible factor-2.
Ω (Ig)	Immunoglobulin.
Ω (IGCAMs)	Immunoglobulin superfamily-related cell adhesion molecules.
Ω (IGF-I)	Insulin-like growth factor-I.
Ω (IL-8)	Interleukin-8.
Ω (ICAM-1)	Intracellular adhesion molecule-1.
Ω (KGF/FGF-7)	Keratinocyte growth factor.
Ω (kDa)	Kilo Dalton.
Ω (mRNA)	messager Ribonucleic acid.
Ω (ΔM)	Mass diffrence
Ω (MMP)	M et alloprotease.
Ω (MG)	Methylglyoxal.
Ω (μm)	Micrometers.
Ω (MCs)	Mural cells.
Ω (NO)	Nitric oxide.
Ω (PDGFR-β)	PDGF receptor-β.
Ω (PIGF)	Placental growth factor.
Ω (PM)	Plasma membrane.
Ω (PA)	Plasminogen activator protease system.
Ω (PECAM-1)	Platelet/EC adhesion molecule-1.
Ω (PDGF)	Platelet-derived growth factor.
Ω (PDGF-B)	Platelet-derived growth factor-B.
Ω (PRELP).	Praline, arginine-rich end leucine-rich repeat protein.
Ω (PDR)	Proliferative diabetic retinopathy.
Ω (PKC)	Protein kinase C.
Ω (PTKS)	Protein Tyrosine Kinases.
Ω (PM)	Pyridoxamine.
Ω (RCS)	Reactive carbonyl species.
Ω (RTKs)	Receptor tyrosine kinases.

Ω (RBCs)	Red Blood Cells.
Ω (RAS)	Renin-Angiotensin System.
Ω (SSAO)	Semicarbazide-sensitive amine oxidase.
Ω (LRR)	Small leucine rich repeat.
Ω (SLRP)	Small leucine-rich proteoglycans.
Ω (SMCs)	Solitary smooth muscle cells.
Ω (SHR)	Spontaneously hypertensive rat.
Ω (O_2^-)	Superoxide anion.
Ω (RAS)	The rennin-angiotensin system.
Ω (VCAM-1)	The vascular cell adhesion molecule-1.
Ω (TGF-β)	Transforming growth factor-β.
Ω (1DDM)	Type1 diabetes mellitus.
Ω (NIDDM)	Type2 diabetes mellitus.
Ω (VE)	Vascular endothelium.
Ω (VE-Cadherin)	Vascular endothelial cadherin.
Ω (VEGF)	Vascular endothelial growth factor.
Ω (VSMC)	Vascular SMC.
Ω (vWF)	Von Willebrand factor, factor VIII-related antigen.
Ω (WKY)	Wistar-Kyoto.
Ω (WHO)	World Health Organisation.

Chapter 1: Introduction

1 Introduction

1.1 Ocular angiogenesis

Ocular angiogenesis is one of the most common complications in many pathological conditions affecting different structures in the eye, for instance, the cornea, retina and choroid. Corneal angiogenesis, 'corneal neovascularization' (Faraj *et al.*, 2011) is related to the second most common cause of blindness worldwide, trachoma, (Whitcher *et al.*, 2001) and to the most common cause of corneal blindness in the industrialized countries, herpetic keratitis (Liesegang *et al.*, 1989). The corneal neovascularization rate in USA is 4%, affecting 1.4 million people (Lee *et al.*, 1998; Chang *et al.,* 2001) inducing visual damage through edema, lipid deposition and scarring. Furthermore, corneal vascularity introduces circulating immune cells, decreasing the immune privilege and subsequently grafts survival probability after transplantation (Niederkorn, 2003; Cursiefenet *e*t *al.,* 2004; Chongand Dana, 2008; Hos *et al*., 2008). Diabetic retinopathy (DR) effects the posterior part of the eye. It is the leading cause of blindness in Americans of working age and the third leading cause of blindness in the US (Morrello, 2007). In DR, retinal hypoxia, caused by a subsequent breakdown of capillaries, induces the release of a number of vasoactive factors such as VEGF and IGF, which promote angiogenesis, tissue remodelling and consequently visual impairment. Age related macular degeneration (AMD) is a major cause of blindness and visual impairment in older adults (>50 years).There are two types of AMD - "wet" or neovascular and "dry" or atrophic. There is no cure for AMD, but new treatments are available for the wet form of the disease is a multi-factorial disease that progresses from damage of the retinal pigment epithelium. In the exudative form (10-15 % of cases), abnormal angiogenesis causes choroidal neovascularization under or above the pigment epithelium, inducing severe visual impairment in untreated cases (Qazi *et al.,* 2009). Retinopathy of prematurity (ROP) or Terry syndrome, previously known as retrolental fibroplasias (RLF) is a blinding eye disease of premature infants (Terry, 1942), The causes of ROP are not completely understood , 55% of babies who are premature develop ROP. The retinal blood vessels in ROP are not completely developed, with peripheral retinal avascularity. It results in disorganized growth of retinal blood vessels, which may lead to scarring and

retinal detachment. When such infants are brought out of the hyperoxic incubator into normoxia, relative retinal hypoxia will occur and consequently widespread hypoxia-induced retinal angiogenesis. (Jager *et al*., 2008)

1.2 Angiogenesis

Judah Folkman first used term of angiogenesis to describe new blood vessel growth. In 1787, John Hunter discovered that interrupting the blood flow in stag antlers resulted in newly formed blood vessels to carry the existent blood flow, and that gunshot wounds only healed if there was an adequate blood supply (Greenberg and Jin, 2004). Angiogenesis is a multi-step process that is crucial in developmental, physiological and pathological processes and involves the formation of new blood vessels from pre-existing vasculature. This is distinct from vasculogenesis, which refers to the differentiation of angioblasts (precursors of endothelial cells) into blood islands that fuse to form a primitive cardiovascular system or vascularise endodermal organs (Biancone *et al*., 1997). Angiogenesis is two types sporting and intrsussotion.

The blood vessel wall surface is made up of endothelial cells, found in a monolayer lining the lumen and covered by mural cells; smooth muscular tissue cells for macro-vessels (vein and artery) and pericytes for micro-vessels (capillary). In pathological conditions, the dysregulation of pro- and antiangiogenic factors can increase vascularisation and may lead to conditions such as tumour development and arthritis, or inadequate vascularisation in the context of ischemia and myocardial infarction (Hall and Ran, 2010). Promoted by inflammatory mediators or hypoxia-inducible factors, the procedure of angiogenesis takes place in numerous steps, with the key players being endothelial and mural cells and the destruction of the micro-environment extracellular matrix (e.g. cellar membrane layer, bordering cells) (Hickey and Simon, 2006; Presta *et al*., 2009). This results in cell membrane destruction causing the migration and sprouting of Epithelium cell (EC) in to the extracellular space towards angiogenic stimulations by VEGF. In the sprouting, ECs elongate and align to develop a lumen, and ultimately develop in to tubular structures that anatomise with neighbouring vessels enabling blood circulation (Folkman, 1995; Risau, 1995; Risau, 1997). Vascular remodelling and

regeneration takes place through paracrine signals. The endothelial cells proliferate and migrate, to increase the length of the neo-vessel, and then differentiate into a sprout, which undertakes stabilisation with the recruitment of mural cells permitting vessels growth with the deposition of extracellular matrix proteins. In adults, ECs are quiescent unless activated during tissue repair, endometrial modifications throughout menstruation and body regeneration (Stephen *et al.*, 1996; Feldman *et al.*, 2002). Angiogenic disease is caused by either the extreme development of blood vessels (e.g. cancers cells, diabetic retinopathy and skin psoriasis) or insufficient capillary formation (e.g. chronic wounding and ischaemic heart disease) (Carmeliet, 2005; Tai *et al.,* 2006).

Angiogenesis may be divided into four stages (Chuan-Yuan *et al*., 2000): (I) activation of the endothelial cells leading to the localized degradation of the basal membrane of the parent vessel and of the extra-cellular surrounding matrix; (II) oriented migration of endothelial cells in the extracellular matrix; (III) proliferation of endothelial cells; (IV) differentiation of these cells with organization into tubular structures with a new basal lamina. Through these stages, the new capillaries form a new vascular network. It is well established that angiogenesis plays a crucial function in atherosclerotic plaque development and in tumour growth, with a boost in the build-up of immature capillary that are badly spent by mural cells (Slevin *et al*., 2009). The weakness of these vascular structures facilitates the infiltration of red blood cells and inflammatory cells, which lead to plaque build-up weakness with hemorrhagic danger of stroke and/or tumour spread (Slevin *et al*., 2009; Izquierdo *et al*., 2009). Tumor vascularization occurs via a number of potential mechanisms. Endothelial progenitor cells (EPCs), which can reside in the vascular wall or migrate from bone marrow in response to chemoattractants from the tumor cell, can differentiate into ECs and contribute to vessel formation. Vascular mimicry can also occur, whereby tumor cells can act as replacement cells for ECs. Another possibility is that chromosomal abnormalities in putative cancer stems cells allow tumor cells to differentiate into ECs. Other mechanisms by which tumor cells can obtain a blood flow include vessel cooption, whereby the tumor cell arises near to (or migrates toward) a preexisting blood vessel, or the process of intussusception, whereby a preformed vessel splits into two daughter vessels by the insertion of a tissue pillar.(Jonathan *et al*., 2013).

1.3 Vasculogenesis:

1.3.1 Vascular development and growth

The cardiovascular system consists of the heart and blood, and blood vessels, that is the very first body organ to develop during embryogenesis. The vascular system appears in the human embryo during the thirth week of development and the heart begins to beat after roughly 23 embryonic days (Van Leeuwen *et al*., 1999). The vascular system is made up of a network of blood vessels composed of the endothelium; a monolayer of EC lining the lumen of the vessel, vascular smooth muscle cells that surround the EC and a basement membrane covering the vascular tube (Gerhardt and Betsholtz, 2003). Larger arteries and veins have a thick layer of vascular smooth muscle cells to stabilize the vessels and to enable vessel contraction. In contrast, the smallest vessels (capillaries) are partially covered by solitary smooth muscle cells (SMCs), called pericytes that promote vessel stabilization (Casley-Smith, 1987; Gerhardt and Betsholtz, 2003) (Figure1.1).

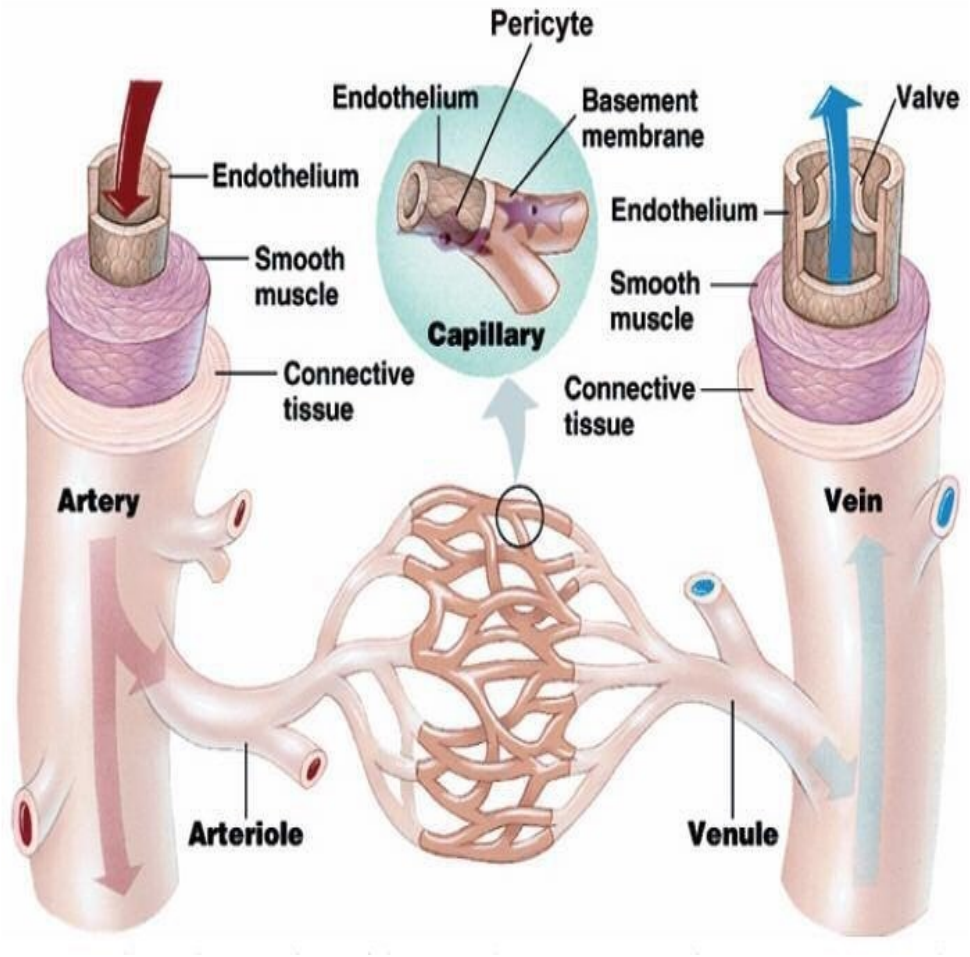


Figure 1 .1 Schematic overview of the vascular system

The arteries carry oxygenated blood to the organs of the body. Oxygen and nutrient exchange takes place in the smallest vessels, the capillaries. Deoxygenated blood is carried back to the heart via the veins to be re-circulated to the lungs where it becomes oxygenated. (Take from; http://www.infibeam.com/Books/info/Randall-Phillis/Biology-ofCancer/0805348670.html).

1.3.2 Structure of the blood vessels

Blood flow proceeds from the upstream arteries to the downstream veins through the capillaries. However, its spatial organisation is more complicated, since it is a three-dimensional ramified structure covering a wide range of diameter scales from centimetres (cm) at heart level to tens of micrometers (μm) at the capillary compartment (microcirculation). A single, continuous monolayer of ECs lined of the vessels arranged in a mosaic pattern around a central lumen in which the blood can flow. In some tissues, such as the liver, spleen and basement membrane

(BM), the ECs are discontinuous, whereas in the kidneys, joints and intestines, the ECs are fenestrated (Risau and Flamme, 1995). The EC layer controls the passage of nutrients and other materials between the blood stream and around the tissue and is attached to the luminal side of a basement membrane. The ECs are separated from the surrounding connective tissue by the basement membrane that is composed of laminins, collagen IV, perlecan and other matrix components (Plank and Sleeman, 2003). The basement membrane separates ECs and SMCs in smaller vessels, but in larger vessels, basement membrane is separated with a layer of mesenchymal cells and extracellular matrix (ECM) (Gerhardt and Betsholtz, 2003; Armulik *et al.,* 2005). Although ECs share common characteristics, they also display considerable heterogeneity. Differences have been observed in their morphology, function and gene expression profile. Morphological diversity includes changes in size, shape and thickness. For instance, ECs on micro-vessels are flattened and elongated while the ECs of large vessels are thicker and polygonal (Thorin and Shreeve, 1998).

ECs can be characterised by specific cell surface marker proteins. Hematopoietic precursor cells or mature blood cells (Garlanda and Dejana, 1997) also express many of these proteins. For example, platelet/EC adhesion molecule-1 (PECAM1), also referred to as CD31, a glycoprotein of the Ig super-family, is expressed on the surface of some hematopoietic precursor cells, circulating platelets, subsets of leukocytes and in the intercellular junctions of ECs (Baumann *et al*., 2004). Vascular endothelial cadherin (VE-Cadherin) is an EC specific adhesion molecule expressed by most EC populations in large and small vessels, arteries and veins. It is localised at cell-cell junctions of EC. VE-Cadherin differs from other EC markers in that it is not expressed on blood cells or HSCs (Breier *et al*., 1996).

Specialised cells, the pericytes (PCs), are located at the abluminal side of the basement membrane. PCs distributed tightly on capillaries and small vessel, while large vessels coated by smooth muscle cells (SMCs) (Risau and Flamme, 1995; Risau, 1997), and matrix (Figure 1.2). Cellular markers used to identify peri-ECs include α smooth muscle actin (α-SMA), NG2 chondroitin sulphate proteoglycan,

PDGF receptor-β (PDGFR-β) and desmin (Bergers and Song, 2005). α-SMA is an isoform of the cytoskeletal protein actin family normally restricted to SMCs, It is suggested as a marker of differentiated PCs. α-SMA expression has been detected in the peri-ECs of most intermediate sized vessels, but not in capillaries.

Therefore, not all PCs can be identified solely based on α-SMA. In contrast, desmin, another contractile filament, as well as NG2 chondroitin sulfate proteoglycan, PDGFR-β, and a tyrosine kinase receptor, are also expressed in the immature peri-ECs of developing micro-vasculature (Ozerdem *et al*., 2001; McDonald and Choyke, 2003; Bergers and Song, 2005).

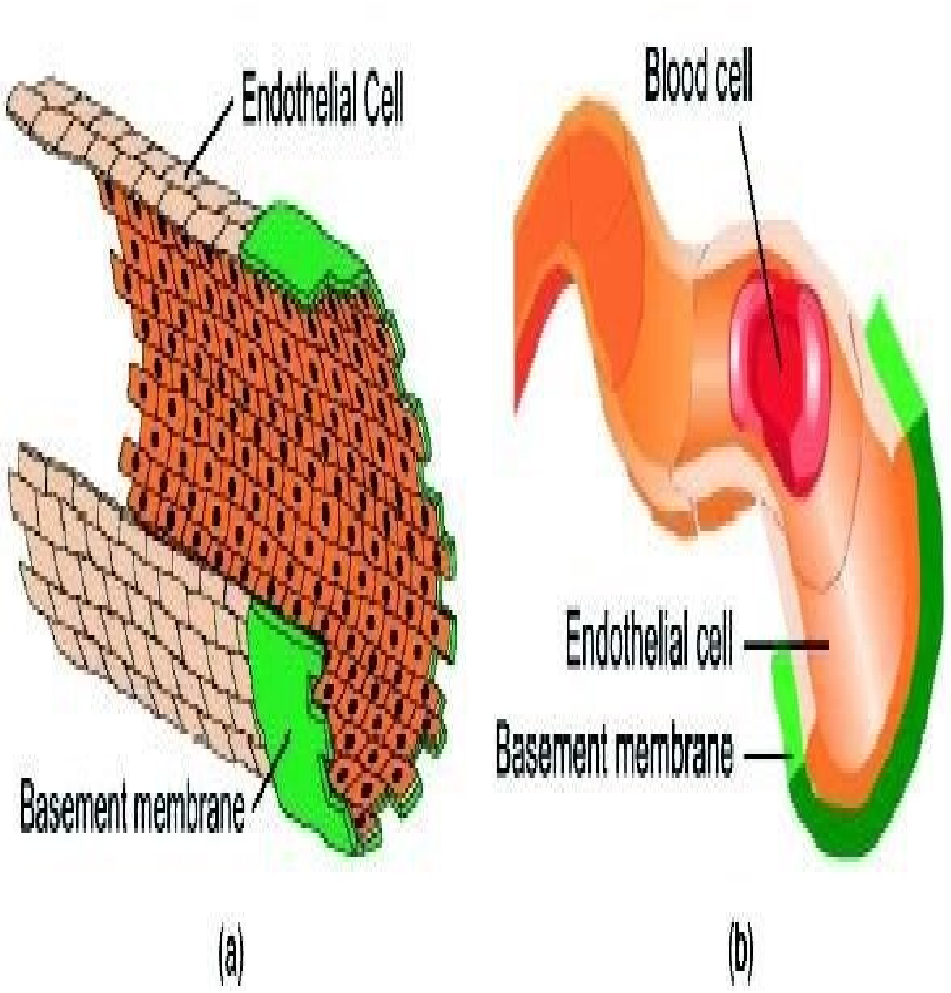


Figure 1 .2 Blood vessel morphology
A blood vessel consists of an EC monolayer, arranged in a mosaic model. a) Large vessel. b) Capillary, (adapted from Jekunen and Kairemo, 2003).

1.3.3 Function and structure of the lymphatic vasculature:

The lymphatic system consists of lymphatic vessels and lymphoid organs, which include lymph nodes, thymus, tonsils, spleen and Peyer's patches. It is a one-way system; collecting proteins and fluid from body tissues and transporting them back into the subclavian veins of the blood vasculature (Karkkainen and Alitalo, 2002; Nguyen *et al*., 2001). A major part of the transportation of the fat absorbed by the gut is performed by lymphatics. Foreign particles, such as bacteria and

viruses, are transported to the phagocytes in the lymph nodes, as part of the host defense (Alitalo and Carmeliet, 2002; Karkkainen and Alitalo, 2002). The lumen of the lymphatic vessels is lined with a discontinuous layer of EC, sitting on an irregular layer of the basement membrane. The lymphatic EC have a valve-like arrangement that permits influx of the tissue fluid into vessels, but blocks reverse transport, and are anchored by filaments into surrounding tissue (Figure 1.3). In addition, initial lymph sacs can arise from primitive precursors (lymphangioblasts), independent of the veins, and, subsequently, from lymphatic vessels that connect to the veins (Alitalo and Carmeliet, 2002; Nagy *et al*., 2002)

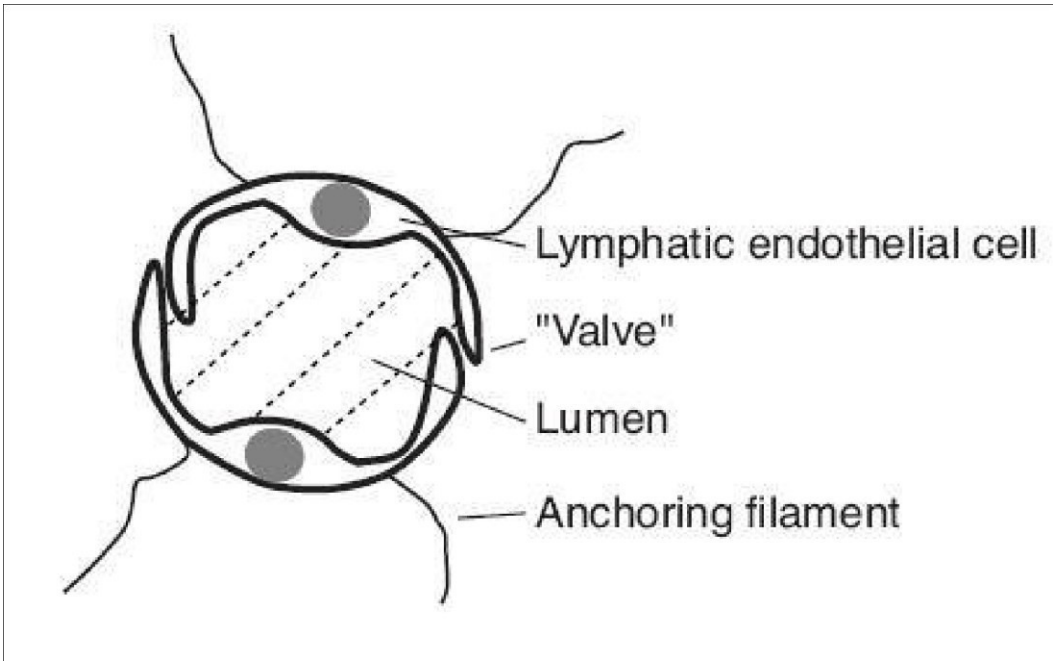


Figure 1 -3 Schematic drawing of a lymphatic vessel
(Taken from Nguyen *et al*., 2001)

1.3.4 Types of Angiogenesis:

Angiogenesis is only switched on under specific conditions. (Figure 1.4). Physiological angiogenesis is activated during embryogenesis and in the female reproductive system, for example. Pathological vascular remodelling is found in tumour growth, atherosclerosis and diabetes, as prime examples. There are thought to be major differences between the blood vessels associated with tumours and those associated with physiological angiogenesis with the former

being described as more leaky and less well organised compared to the normal vasculature (Liekens *et al*., 2001).

1.3.4.1 Sprouting angiogenesis:

There are three described mechanisms of angiogenic growth, the main difference being the mode of stimulation for the production of new vessels. The classical form of angiogenesis is sprouting angiogenesis, which occurs when a stimulus from the abluminal side of the vessel, for example muscle overload (extirpation), initiates the release of cytokines and growth factors from skeletal muscle. Proteolytic degradation of the ECM occurs following an increase in the permeability or integrity of the basement membrane (BM), a barrier made of structural proteins that provide support to the capillaries. Disruption of the BM allows the growth and migration of the EC into new vessel architecture (Haas *et al*., 2000). The BM breakdown is mediated by matrix mettaloproteinases (MMPs) activated by vascular endothelial growth factor VEGF (Folkman, 1971).

1.3.4.2 Splitting angiogenesis:

Splitting angiogenesis can also occur due to a luminal-triggered stimulus, for example, an increase in blood flow, increasing shear stress and up regulated nitric oxide (NO) production. There are two forms of splitting angiogenesis: intussusception occurs when a single capillary splits into two capillaries from within by the formation of a longitudinal divide on the luminal side of the capillary (Prior *et al.,* 2004), and involves abluminal activation of mural cells for example fibroblasts. The other form of splitting angiogenesis occurs whereby endothelial cells, as opposed to interstitial cells, send filopodial processes into the capillary lumen, which then join to form a separate branch, and this subsequently propogates down the capillary (Zhou *et al.*, 1998). The main difference between the described mechanisms is that sprouting angiogenesis requires BM and extracellular matrix degradation, allowing migration and proliferation of ECs, and thus the development of the sprouting tube. Work by Egginton *et al*., (2001) supports the notion that BM degradation is not a pre-requisite for splitting capillary growth.

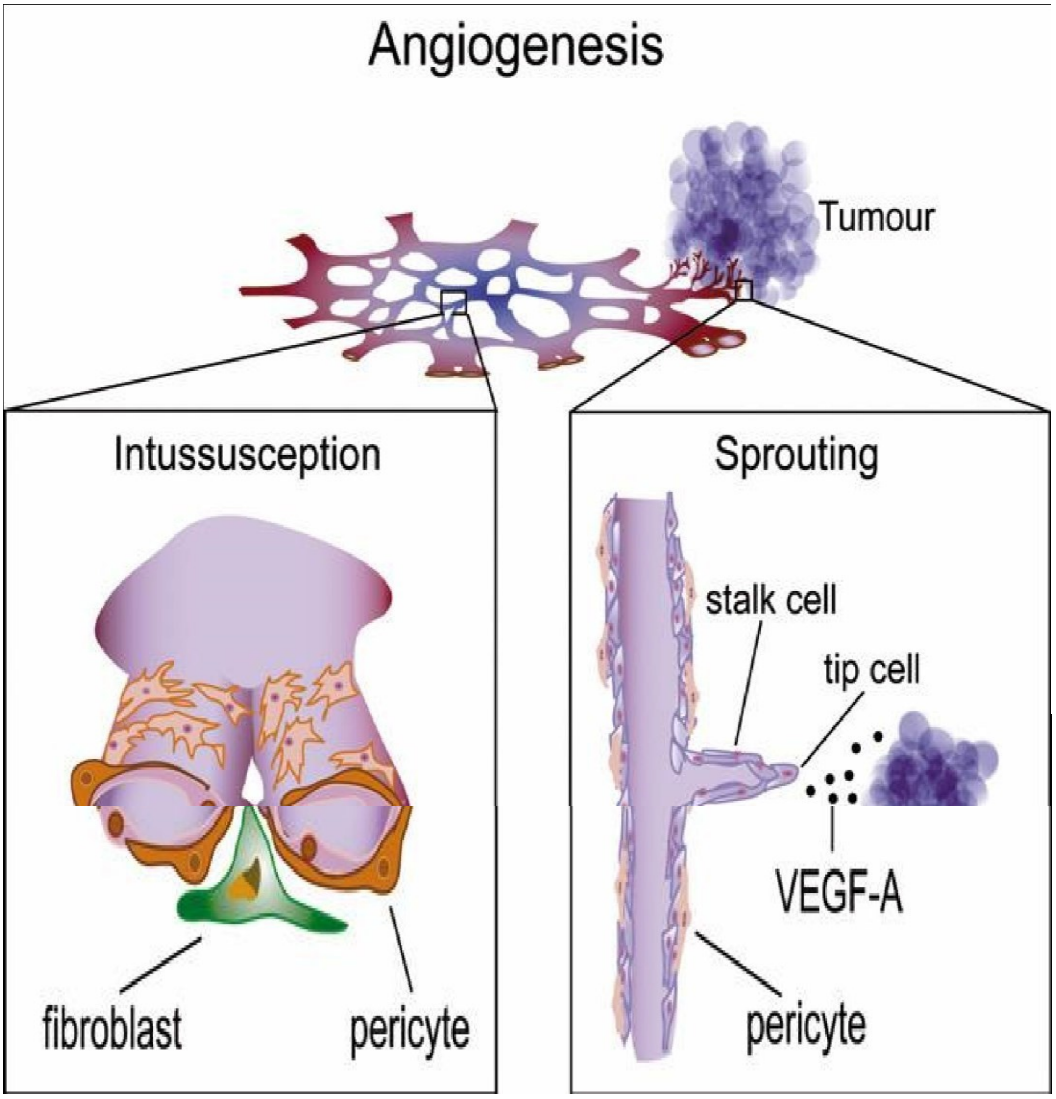


Figure 1 .4 Schematic outlines of angiogenesis by two different mechanisms. Intussusception and sprouting. (Jain, 2003)

1.4 Mechanisms of blood vessel growth:

The sprouting growth of new capillaries is initiated via degradation of the BM. The increased vascular permeability due to a combination of VEGF and the MMPs enables extravasation of plasma proteins that lay down a provisional scaffold for migrating cells (Carmeliet, 2003). This also results in the release of other growth factors involved in stimulating angiogenesis and up-regulating

VEGF mRNA expression, including insulin-like growth factor-1 (IGF-1), transforming growth factor B (TGF-B), platelet-derived- growth-factor (PDGF) and basic fibroblast growth factor (bFGF). ECs and vascular smooth muscle, initiating the establishment of paracrine and autocrine loops for EC activation and proliferation (Dunn *et al*., 2000). The formation of properly patterned and structurally stable microvascular networks requires the coordinated recruitment of pericytes to microvessels (Ponce and Price, 2003) and it has been suggested that pericytes lead capillary sprouts into tissues (Figure 1.5).

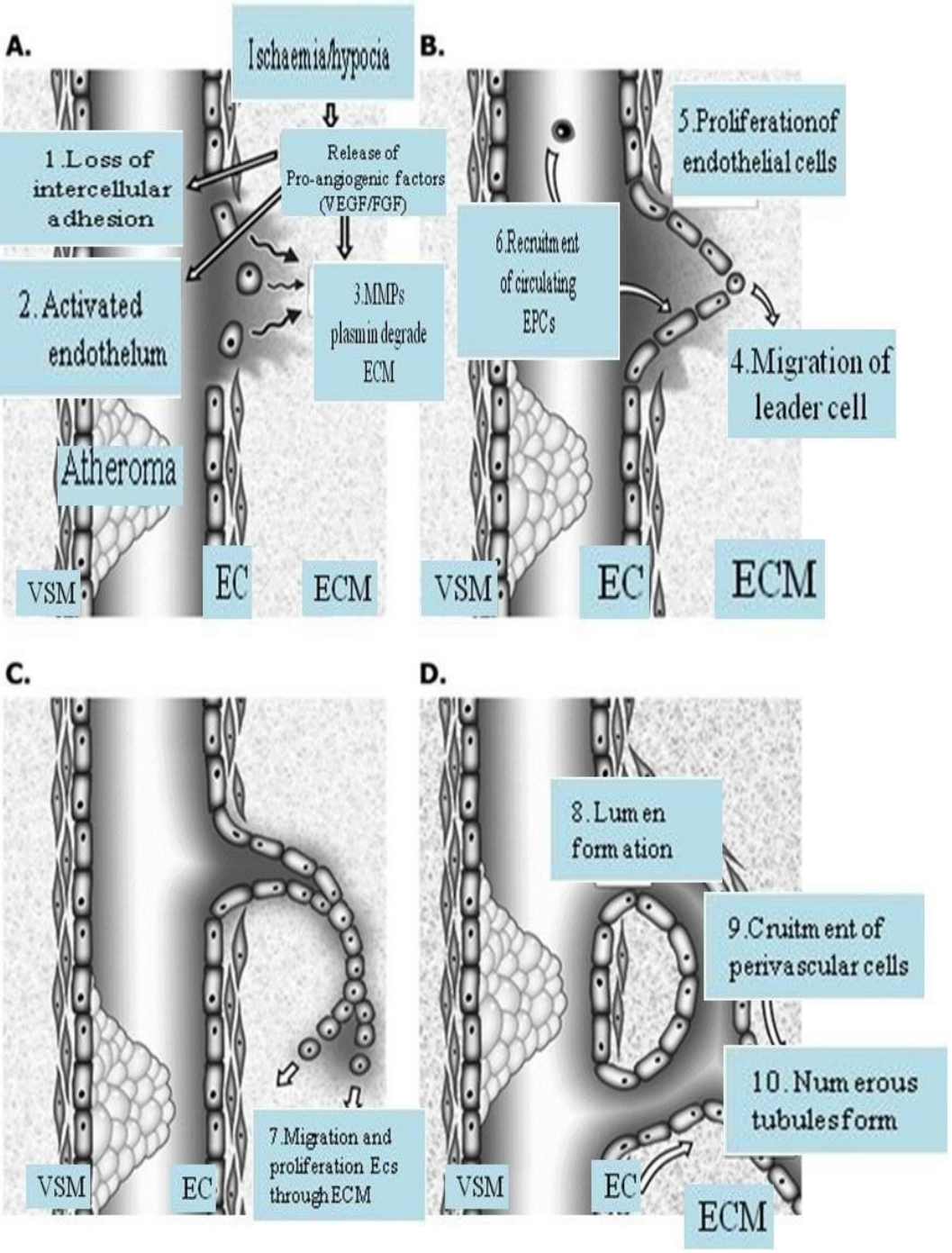


Figure 1 .5 Mechanisms of blood vessel growth

BM degradation, EC migration, differentiation and reformation into tubes that form new capillaries (Taken from Collinson and Donnelly, 2004).

Table 1 .1 Summary of the molecular basis underlying new blood vessel growth (Receptors are indicated in brackets) (Christopher and Peter, 2003**).**

Steps in angiogenesis	Stimulatory factors	Inhibitory factors
Vasodilatation	Nitric oxide synthesis	1.4.1
Increased vascular permeability	VEGF (Flt-1, Kdr)	Ang-1 (Tie-2)
Extravasation of plasma proteins	VEGF	Ang-1 (Tie-2)
Endothelial sprouting	Ang-2 Tie 2 VEGF (Flk-1)	1.4.2
Degradation of extracellular matrix	MMPs & TIMPs (tissue inhibitors) Collagen prolyl-4-hydroxylase	PAI-1
Liberation of growth factors	uPA receptor	Thrombospondin-1 PAI-1
Endothelial cell proliferation & migration	VEGF, Ang 1&2, FGFs, PDGF	1.4.3
Pericyte and smooth muscle recruitment	PDGF	1.4.4
Endothelial assembly and lumen acquisition	VEGF, Ang-1 (Tie-2) Integrins	Thrombospondin
Stablilisation of nascent vessels	PAI-1	1.4.5
Maintenance of differentiation and remodelling	Ang-1 (Tie-2)	Ang-2 (Tie-2)

A number of factors contribute to the dissociation and movement of ECs through the matrix. The Tie-2 receptor is a signalling system involved in vessel maintenance, growth and stabilisation. Tie-2 is a membrane bound receptor tyrosine kinase that binds angiopoietins 1 and 2 (Ang1 and Ang2). To enable ECs to dissociate from each other, competitive inhibition of Tie-2 by Ang2 occurs, initiating the loosening of matrix contacts and support cell interactions, thus allowing access for the above-mentioned growth factors and other angiogenic inducers (Douglas, 1997). Redistribution of intercellular adhesion molecules platelet endothelial cell adhesion molecule (PECAM-1) and vascular endothelial VE-cadherin also contributes to the dissociation of ECs from their neighbouring cells, causing them to undertake mitosis, and, due to loss of contact inhibition, start migrating throughout the matrix. Proteinases also expose new epitopes in ECM proteins that induce migration, along with a matrix of fibronectin and fibrin, and another group of adhesion molecules called the integrins αvβ3 and αvβ5 (Eliceiri and Cheresh, 1999). These all aid in migration the ECs towards their objective sites (Carmeliet, 2003). During the migration of the ECs to distant sites, there is a complex interaction and redundancy between VEGF, bFGF and angiopoietins. Via phosphorylation of Tie2, Ang1 stimulates interactions between pericytes and ECs and induces sprouting of new capillaries (Conway *et al*., 2001), solidifying and stabilising a newly formed blood vessel. Initial breakages in the BM are repaired rapidly as an intact BM was initiated around various EC sprouts (Egginton *et al*., 2001).

1.5 Formation of blood vessels

The formation of new vessels occurs mainly throughout two processes, vasculogenesis and angiogenesis (Conway *et al*., 2001). Vasculogenesis occurs during early embryonic development starting with the formation of hamangioblasts from the mesodermal germ layer (Risau and Flamme, 1995). Blood vessels are formed within an aggregation of multipotent cells derived from the mesoderm called blood islands. These *de novo* blood vessels develop inside the embryo providing support to the heart, the first primitive vascular plexus and in its surrounding membranes as the yolk sac circulation. This primitive network originate from vasculogenesis is formed by angiogenesis, which is a strongly regulated process of amplification of pre-existing vessels by sprouting, bridging,

as well as branching. This angiogenic method occurs in the development of adult tissues (i.e. osteogenesis, menstrual cycle) but moreover in patho-physiological situations such as wound healing along with pathological situations such as atherosclerosis and tumour growth and spread through metastasis (Ozerdem *et al*., 2001;McDonald and Choyke, 2003;Bergers and Song, 2005). Figure 1.6.

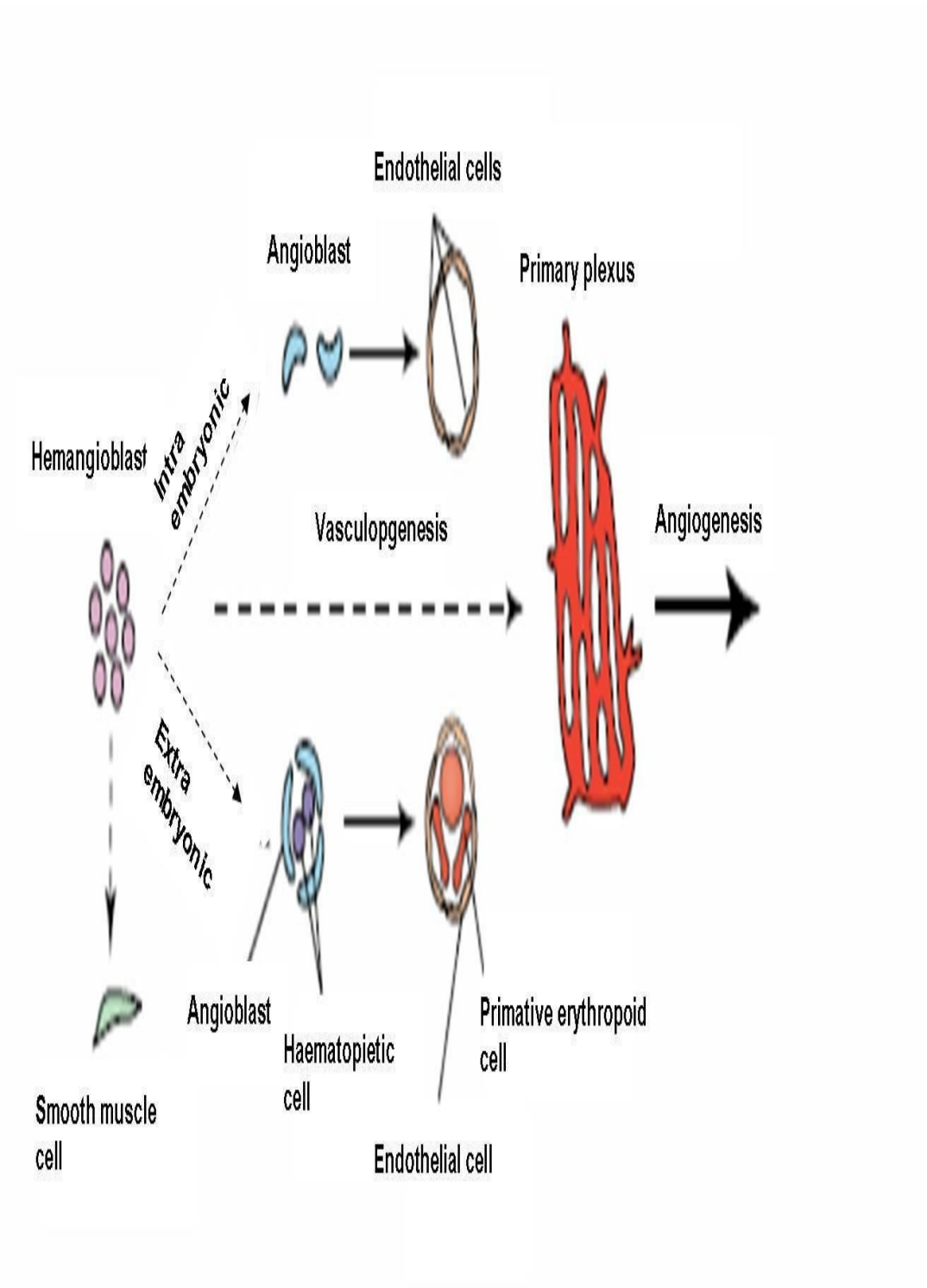


Figure 1 .6 Outline of the vasculogenic process

1.6 Stabilisation of general networks.

Blood vessel development is dependent on the activity of VEGF on its receptor tyrosine kinases, VEGFR-1 / Flt-1 and VEGFR-2 / vascular endothelial cells growth factor receptor 2 antibody (Flk-1), in endothelial cells (Fong *et al*., 1995; Shalaby *et al*., 1995; Carmeliet *et al*., 1996; Ferrara *et al.,* 1996). Deletion VEGFR-2 cause an arrest in vasculogenesis during the angioblasts differentiation state (Shalaby *et al*.,1995), while deletion of VEGFR-1 causes embryonic lethality from edema following initial formation of definitive blood vessels (Fong *et al*., 1995).

The receptor tyrosine kinases (Tie1 and Tie2) are expressed in embryonic angioblasts, vascular endothelium and endocardium, and in adults. Tie1 is expressed in endocardium and lung blood vessels and Tie2 is shown in endocardium and general endothelium (Dumont *et al*., 1994; Korhonen *et al*., 1994). The signalling through angiopoiens and Tie1 and Tie2 essential in the stabilisation of immature vascular networks. The importance of the Tie receptor system was outlined with genetic studies carried out with Tie1 deficient mice where vascular integrity is compromised leading to embryonic lethality. Tie2 deficient mice showed vascular network formation lacking normal sprouting, branching, and remodelling of vessels also leading to embryonic lethality. Ang-1 in pericytes is involved in vessel creation by promoting endothelial cells to generate the bordering mesenchyme into pericytes or smooth muscular tissue cells (Suri *et al*., 1996). Conversely, mice over-expressing Ang-1 exhibit increased vascularisation (Suri *et al*., 1998). In the absence of VEGF, Ang-2 acts as a antagonist of Ang-1 and destabilises blood vessels, leading to vessels regression, whereas in the existence of VEGF, Ang-2 facilitates growth and branching by blocking Ang-1 signalling (Maisonpierre *et al*., 1997).

1.7 Remodelling of general networks by angiogenesis.

New vessels divisions could grow to a collection of cells in the bordering mesenchyme that generates the angiogenic stimulation. Already existing vessels could split in to specific little daughter vessels by the formation of transendothelial cell bridges or the vessels could branch through

intussusceptions, based after installation of interstitial cells pillars in to the lumen of pre-existing vessels (Figure 1.7) (Djonov *et al*., 2000)

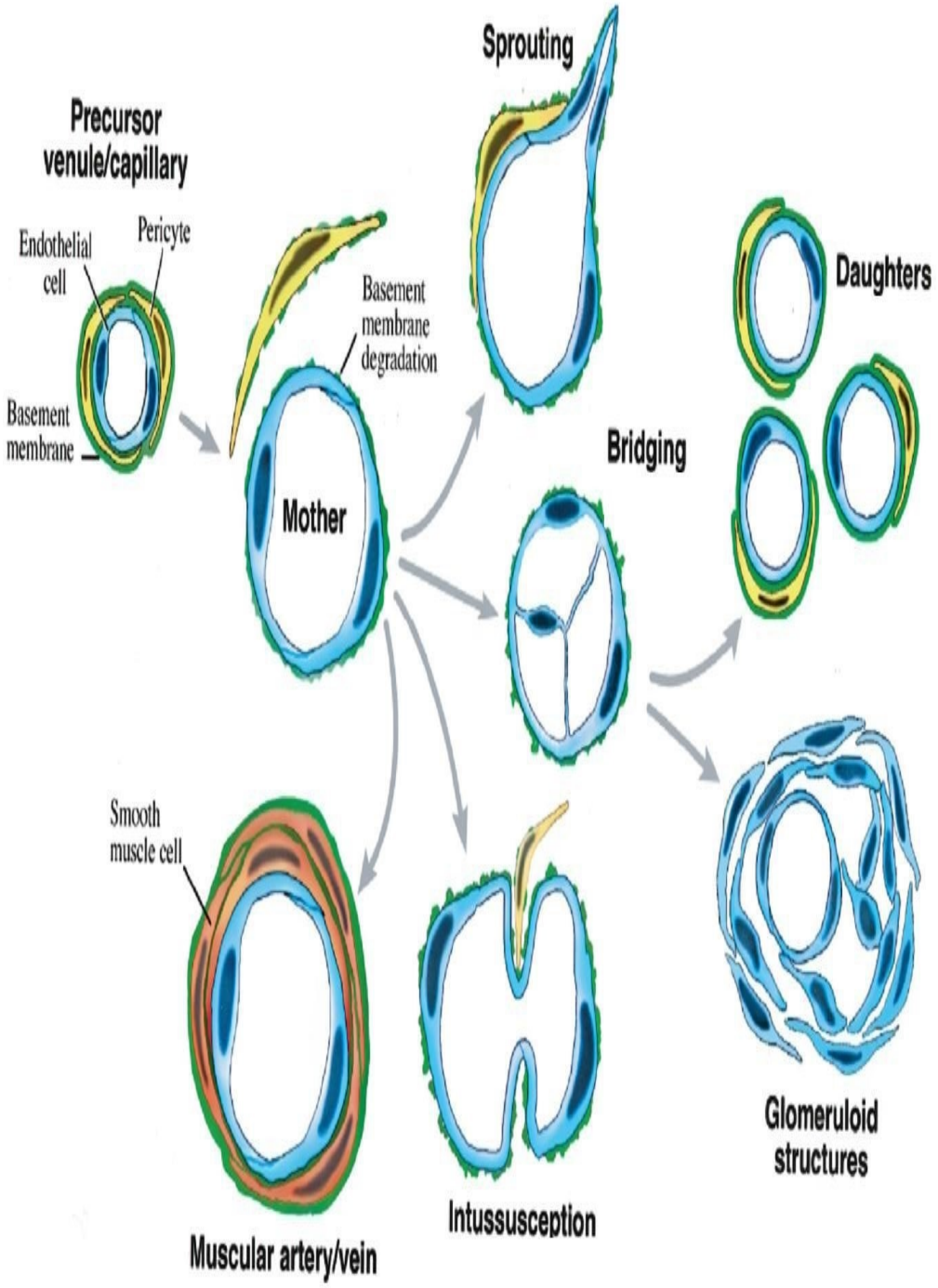


Figure 1 .7 Schematic layouts standing for the progress of the angiogenic response various systems where angiogenesis takes place in the establishing vasculature. (Adapted from Patterson *et al*., 2000).

Angiogenesis features have already been very well characterised, by real-time image resolution in the zebra fish (Lawson and Weinstein, 2002) and by studying the establishing retina (Uemura *et al*., 2006). In growing angiogenesis, ECs that are included in the vessel wall surface become loosened and begin to move after stimulation by development signals, controlled by VEGF (Habeck *et al.,* 2002; Leung *et al.,* 1989; Tischer *et al.,* 1989). Intersegment vessels development in zebra fish, comprises intracellular vacuoles, which link to vacuoles of nearby ECs to develop a continual lumen (Kamei *et al.*, 2006) (Figure 1.8).

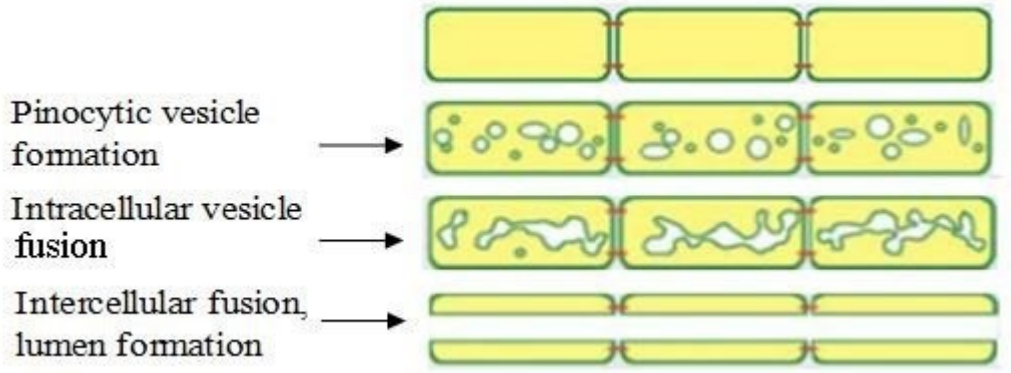


Figure 1 .8 Vacuoles compartments and lumen
Start with pinocytic vesicle, intracellular vesicle formation, intracellular vesicle fusion, and finally intercellular merging of vacuoles compartments and lumen formation (Kamei *et al.,* 2006).

As the tip cell encounters a new sprout or vessel it bunds and circulation is created, for example in the retina, protruding endothelial tip cells generate the majority of PDGF, which in turn entices pericytes by activation of PDGF receptor (Gerhardt *et al*., 2003; Hammes *et al*., 2002). Endothelial cell motion within the ECM is a firmly controlled procedure that requires the polarisation of the molecular components matrix decline to the accelerate side of the relocating cell. The pattern of retinal blood vessels has been revealed to depend on the equilibrium between two features of extracellular VEGF-A. The slope and the stimulation, which is essential for the appropriate support of filopodial expansion from specialized endothelial cells at the tips of the vascular sprouts, and endothelial cell development takes place in the grow stalks in response to

VEGFA stimulation. VEGFR-1 was revealed to promote grow formation by detrimentally regulating the number of VEGF-A signalling to endothelial cells using soluble VEGFR-1 as the primary mediator of the effect (Kearney *et al.,* 2004). Hence, the local presence of VEGF signal is essential to effectively promote morphogenesis and growth (Gerhardt *et al*., 2003; Kearney *et al.,* 2004).

1.8 Angiogenic stimuli

Many factors have the effect of the initiation of angiogenesis, there is a very fine balance between pro- and anti-angiogenic factors, and disruption of this balance provides the driving force for the development of new vessels for example metabolic stress, cytokines, hypoxia, pH, and hypoxlyceamia. Up-regulation of angiogenic inhibitors and/or down-regulation of angiogenic activators may be linked to reduced neovascularization capacity (Figure 1.9).

1.9 The key process of angiogenesis

Endothelial cells that form the inner blood vessel surfaces differentiate in response to angiogenic signalling and also secrete matrix metalloprotease (MMP) which enable migration toward the source of the angiogenic stimuli (Folkman, 1985). The EC processes inside the sprout become vacuolated and subsequently the vacuole will become the extracellular tube, encased by a thin wall membrane on the EC cytoplasm (Folkman, 1985). Once the sprout forms and elongates, EC proliferation occurs, to complete the elongating vessel. The EC processes within the sprout become vacuolated and later the vacuole becomes an extracellular tube, surrounded by thin wall of the EC cytoplasm (Folkman, 1985). The lumen is formed, either through the tubular wall of one cell connecting to the tubular wall of another cell, or the EC within the sprout changes shape, becoming curved to form a lumen (Folkman, 1985).

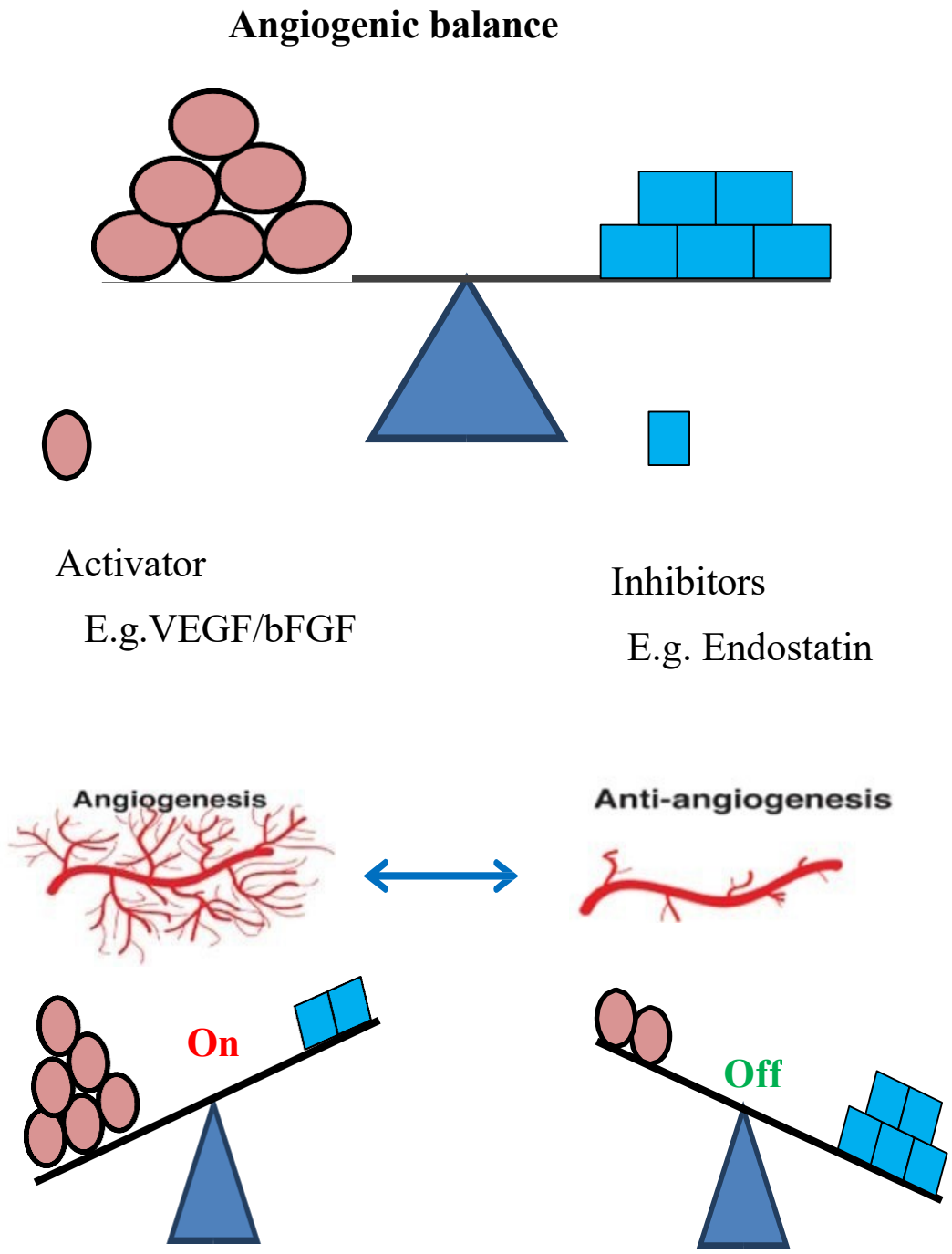


Figure 1 .9 The angiogenic balance between angiogenic activators and angiogenic inhibitors regulate vascular homeostasis. Angiogenesis under physiological and pathological conditions is associated with up-regulation of angiogenic factors and/or down-regulation of angiogenic inhibitors.

Once the growing sprouts are formed by leading migrating cells, they converge and anastomose to form a capillary loop (Folkman, 1985), where in blood, flow may initiate and subsequently the basement membrane is synthesised by ECs (Figure 1.10).

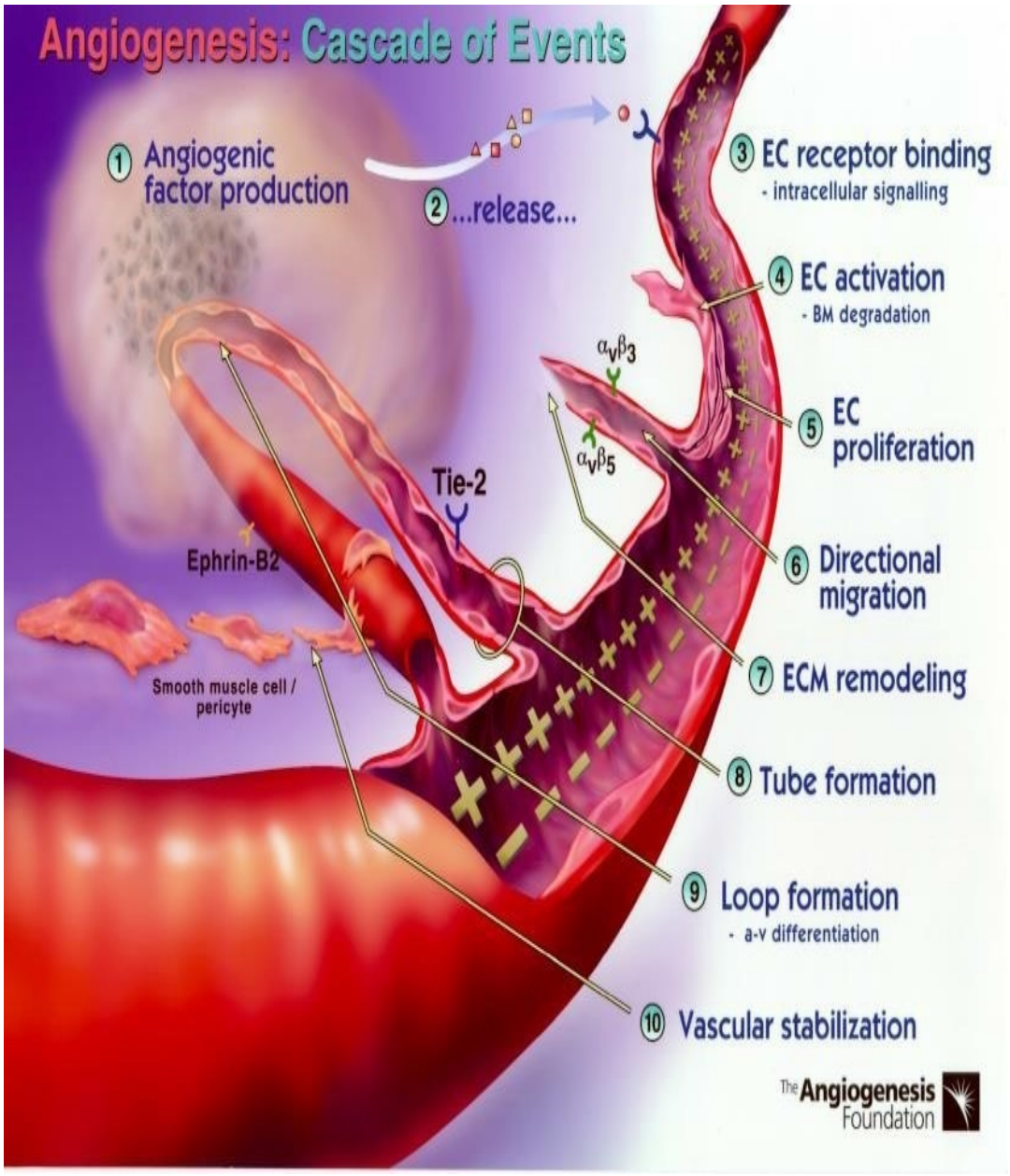


Figure 1.10 Key steps in angiogenesis

The angiogenesis cascade occurs as an orderly series of events. Angiogenic endothelial cells must proliferate, avoid apoptosis, migrate, and produce molecules able to degrade the extracellular matrix and, finally, differentiates into new vascular tubes.

http://www.angio.org/understanding/understanding.html.

1.10 Notch signaling

The Notch signaling pathway is an evolutionarily conserved, intercellular signaling mechanism essential for proper embryonic development in organisms as diverse as insects, nematodes, echinoderms and mammals (Gridley, 2007). Notch receptors initiate a highly conserved signaling pathway that influences cell fate decisions within multiple tissues and regulate the ability of precursor cells to respond to other developmental signals. In mammals, Notch signaling regulates neurogenesis, myogenesis, vasculogenesis, hematopoiesis, skin development, and other aspects of organogenesis. In addition, Notch signaling is involved in other critical cellular processes such as apoptosis, differentiation, migration, and proliferation. Notch proteins are cell surface transmembrane-spanning receptors, which mediate critically important cellular functions through direct cell-cell contact. Interaction between Notch and its proposed ligands initiates a signaling cascade that governs cell fate decisions such as differentiation, proliferation, and apoptosis in numerous tissue types. (Mumm *et al.*, 2000; Radtke *et al.*, 2002). Expression analysis of the Notch signalling pathway in human cancer samples provides encouraging evidence of a role for Notch signalling in human disease. Expression of the Notch ligand Delta-like-4 (DLL-4) is up regulated in human breast cancer but not found in normal breast tissue (Li *et al.,* 2009).

1.11 Angiogenesis in Disease and treatment

The fundamental role of angiogenesis is in embryogenesis/vasculogenesis. Number diseases are connected with dysfunctional capillary development, including diabetic retinopathy, rheumatoid arthritis, skin psoriasis, myocardial anaemia, and coronary artery disease (Folkman *et al*., 1995; Isner, 1999). The formation of new blood vessels is vital for the development of the majority of expanding tumours. Tumor angiogenesis refers to the ability of a tumor to stimulate new blood vessel formation (Hicklin and Ellis, 2005). This critical step in development enables tumor expansion, local invasion, and dissemination through; Delivery of oxygen, nutrients, and survival factors, production of growth factors that benefit tumor cells, and formation of a route for tumor cell egress. As a result, each part of the angiogenesis process is a potential target for new cancer treatments. (Hanahan and Weinberg, 2011).

The tumour is dependent on diffusion for the exchanges of four medicines that target VEGF have been authorized by the government medicine management in the USA to be utilized scientifically.

1. Bevacizumab (Avastin™; a VEGF neutralising antibody) is a monoclonal VEGFA-antibody. It has been approved mainly to treat of metastatic colon cancer, but is broadly used off-label for ocular angiogenesis and has shown promising effects on corneal angiogenesis (Dastjerdi *et al*., 2009; Koening *et al*., 2009; Zaki and Farid, 2010).

2. Pegaptanib, (Macugen™; an oligonucleotide that expecteds VEGF) is the common therapy for most age-related macular deterioration (AMD), by intravitreal injection (Gragoudas *et al*., 2004).

3. Ranibizumab (Lucentis™; an antibody that detects all VEGF isoforms) for treating of damp AMD. (Jain *et al.*, 2006).

4. Aflibercept (VEGFTrap) is a soluble decoy receptor, which binds to all isoforms of VEGFA and placental growth factor (PlGF). A recent study by Oliveira showed a promising effect of VEGFTrap on FGF2-induced corneal neovascularization in a murine model (Oliveira *et al*., 2010).

Several particles having anti-angiogenic properties detected in human cells. They are signified endogenous obstacle, most of them usually are proteolytic pieces of ECM proteins or plasma proteins, shown by endostatin (a piece of collagen XVIII), endorepellin (the C-terminal component of perlecan), angiostatin (a piece of plasminogen) and tumstatin (a piece of the IV alpha3 chain) (Bix and Iozzo, 2005).

1.12 Blood vessels cells

1.12.1 Endothelial cells

In vivo and *in vitro*, endothelial cells is just one cell layer often identified as "cobblestone" in appearance, that could be a criterion for identification, although it is not a solid identification marker (Haudenschild., 1984;Manconi *et al*., 2000).(Figure 1.11). Endothelial cells are the only cells in the body that are exposed to a moving fluid under pressure (blood) and a solid substrate (blood vessel wall) (Haudenschild., 1984; Manconi *et al*., 2000). Within regular circumstances, endothelial tissues keep a quantity epithelioid geometry that really helps to prevent unstable blood circulation within blood vessels and produces an antithrombotic area (Cines *et al.,* 1998).

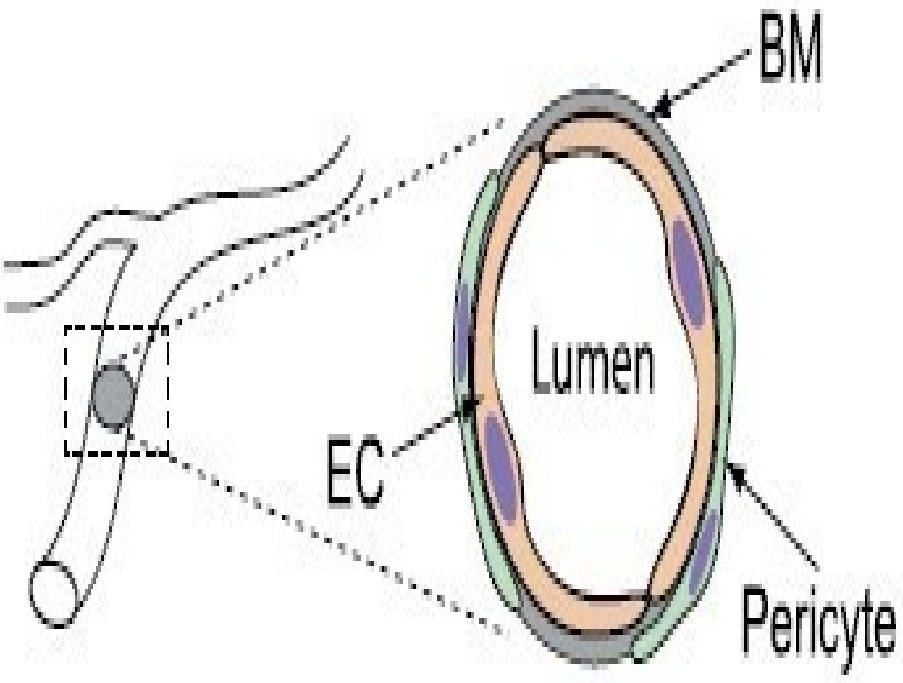


Figure 1.11 Schematic cross section of a capillary. ECs are bounded by pericytes and lean on a cellar membrane layer.

The endothelial cell consists of a variety of frameworks, composed of a round ellipsoid. An organelle that is restricted to endothelial cells is the Weibel-Palade body (Weibel and Palade, 1964), which has been identified in human, pig, rat, and mouse endothelium (Jaffe, 1987). Weibel-Palade bodies are therefore markers for endothelial cells (McNiff *et al*., 1983; Wanger *et al*., 1982), however usually are not present in all endothelial tissues. (Johnson *et al.,* 2002).

There are three considerations widely used to positively identify cultured endothelial cells: 1) contact inhibition resulting in a monolayer of endothelial cells; 2) immunocytochemical staining with vWF (elements VIII associated antibody); and 3) particular uptake of acetylated low-density lipoprotein. Other qualities associated with cultured endothelial cells include CD31+ and CD34+ markers, angiotensin changing enzyme (ACE), endothelial nitric oxide synthase (eNOS) secretion, prostacyclin secretion, cobblestone appearance, and "tube" development in Matrigel (a synthetic extracellular area including laminin and collagen to which endothelial cells could stick) (Manconi *et al*., 2000; Scott *et al*., 1993). Endothelial cells are associated with a number of physiological processes.These includ capillary obstacle feature and leaks in the structure, coagulation, fibrinolysis, blood circulation requirement, and angiogenesis (Ausprunk *et al*., 1977; Mombouli *et al*., 1999; Shimokawa *et al.,* 1999; Taddei *et al.,* 2000). The endothelium is first element to be facing to the blood and its numerous components, including hormones, development elements, different other elements secreted by cells throughout the body, and various other elements presented in to the blood (Mombouli *et al*., 1999).

1.12.2 Endothelial cell differentiation

In the embryo, endothelial cells arise from haemangioblast (Choi *et al*., 1998) present in different organs (Jiang *et al*., 2002) and in the adult, from endothelial parent cells, mesoangioblasts, multipotent mature parent cells or side population cells in the bone marrow (Reyes *et al*., 2002). Endothelial cells interact in between bordering endothelial cells, periendothelial cells/mural cells (such as smooth muscular tissue cells and pericytes) and cells inside the vessels lumen, and dynamically connect with these cells and their bordering extracellular matrix.

Endothelial cells can differentiate to either arterial or venous endothelial cells throughout embryonic development, neonatal retina and even in the heart of an adult (Stalmans *et al*., 2002; Visconti *et al.,* 2002). Growth factor generated endothelial cells have the intrinsic capacity to develop a network of blood vessels (Flamme and Risau, 1992). Many of growth factors and developmental stimuli affect the phenotype of endothelial cell with the signalling of their receptors on the cell membrane consisting of VEGFs, ang-1 and ang-2, TGF- β1 and Ephrin B2. It is very clear that endothelial cells interact with various other endothelial cells and periendothelial cells/mural cells with adherent and void joints, whereas and with strict joints exist in brain and retinal blood vessels. VE-cadherin mediates the adherent endothelial-endothelial cell joints in blood vessels (Lampugnani *et al.,* 1992) and mediates an endothelial cell survival path via its intracellular communications with β-catenin (Carmeliet *et al.,* 1999). The communications and interactions between endothelial cells and the matrix bordering controlled by cell surface integrins.

1.12.3 Mural cells (MCs)

The main function of Peri-ECs, PCs and Vascular SMC (vSMCs), are to stabilise the blood vessels and give physiological support. Without the backing of periECs, vessels become leaky, hyper-dilated, dysfunctional and regressive (Benjamin *et al.,* 1998; Bergers and Song, 2005). Mutal cells subdivided into vSMCs as well as PCs according to their density, morphology, location and the expression of specific protein markers. Human venous malformation characterised by a complete lack of vSMCs around certain veins, is mainly caused by mutations in the tyrosine kinase with immunoglobulin (Ig) and Epidermal growth factor (EGF) homology domains-2 (Tie-2) gene (Vikkula *et al*., 1996). In two independent families, the responsible mutation was found to activate the Tie-2 kinase. Both inactivation (mouse knockout) and constitutive activation of Tie-2 (human venous malformation) results in MCs loss (Hall, 2006). The ligand/receptor pair plateletderived growth factor (PDGF) receptor-β (PDGFRβ) axis forms paracrine endothelium to MC signalling loop. The angiopoietins-Tie receptor axis represents generally a signalling loop of the opposite orientation from MCs to the endothelium (Armulik *et al*., 2005; Hall, 2006).

1.12.4 Pericytes

Pericytes (historically known as Rouget cells) and share a basal membrane with the endothelium. Pericytes are either singly connected with the EC tube or form a single, often discontinuous, cell layer around it. Pericytes morphology and the degree to which they cover the endothelium vary substantially between different tissues. In some tissues, PCs have acquired specialised functions, for example, mesangial cells in kidney glomeruli, as well as peri-sinusoidal fat storing cells (Ito-cells) in the liver (Hall, 2006). Usually, fully developed PCs form intimate, umbrella-like contacts with the ECs in capillaries, pre-capillary arterioles, collecting venules and post-capillary venules. ECs and PCs together synthesise and share a common basement membrane (Mandarino *et al*., 1993). Pericytes are embedded in the basement membrane and are immediately in contact with the ECs through peg-and-socket junctions that extend through a discontinuous basement membrane (Cuevas *et al.*, 1984). These junctions consist of membrane evaginations, which are rich in tight and gap junctions. Pericytes-endothelial adhesion, recognition and signalling are maintained by neural (N)-cadherin adhesion junctions (Gerhardt *et al*., 2000) also with fibronectin-rich dense plaques (Courtoy and Boyles, 1983). Pericytes support vessel stability via physical and chemical signalling with the endothelium and can directly regulate EC-SMC communication (Armulik *et al*., 2005). Moreover, PCs have been suggested to play an essential role throughout angiogenesis, which are recognised at angiogenic sprouts (Morikawa *et al*., 2002) and are a source of VEGF-A(Darland *et al*., 2004). Pericytes also play a role in the regulation of capillary diameter and EC differentiation (Hellstrom *et al.,* 2001). Endothelial cells and PCs share a common progenitor cell in the embryonic mesoderm, the VEGFR-2-positive angioblasts. This progenitor cell can differentiate into ECs upon stimulation by VEGF-A, and into PCs upon stimulation by platelet-derived growth factor-BB (PDGF-BB) (Yamashita *et al.,* 2000).Pericyte contraction could control microvascular blood flow and permeability. At the blood brain barrier, several reports suggest that PCs are able to act as brain phagocytes and can be converting to tissue macrophages (Balabanov *et al.,* 1996; Thomas, 1999). In an ovarian model of angiogenesis, pericytes were the first angiogenic cells to invade the normally avascular Graafian follicle and precede the sprouting vascular tip (Amselgruber *et al*., 1999).

1.12.5 Smooth muscle cells

Vascular SMCs are present in arteries and veins and form multiple concentric layers (Hall, 2006). Here, they create many of the ECM components include collagens, which provide solidity, and elastin, which confers properties of elastic recoil. They also produce proteoglycans, which regulate permeability, viscoelasticity, cell adhesion and migration (Wight, 1989), and regulating short-term vascular tone myogenically. SMCs play a part in vascular remodelling by synthesising and secreting matrix-degradative enzymes that include both plasminogen-dependent as well as plasminogen-independent proteinases (Galis *et al*., 1994), growth factors and other cytokines.

1.13 Extracellular matrix

The extracellular matrix (ECM) is non-cellular meshwork bordering nearly the most of cells in multicellular microorganisms. The ECM is an architectural structure and provides cells with positional and environmental information, however additionally provides specialised components such as cartilage material, ligaments, bone marrows (BMs), bone and teeth. The macromolecules of the ECM usually excreted by neighbouring cells such as fibroblasts. The most rich proteins of the ECM are collagens specified as structurally connected ECM proteins offering mechanical constancy for tissue. A quality of all collagens is the presence of a minimum of one triple-helical domain name consisting of three polypeptides, called α-chains, comprised of a duplicated (Gly-X-Y) amino acid series with glycine (Gly) as every thirth amino acid deposit, and regularly hydroxyl proline and hydroxyl lysine in the X and Y placements (Figure 1.12). This enables α- chains to develop stable triple-helical collagen particles. The triple helix is stiff and in many collagens, it is disrupted by globular particles that make the particle more flexible and functional in a range of macromolecular organic tasks (Bateman *et al*., 1996; Prockop and Kivirikko, 1995; Myllyharju and Kivirikko 2001).

Some collagen types are formed by three identical α-chains, yet some (kinds I, IV, V, VI, VIII, IX and XI) consist of even more, and are coded by different genes (Prockop and Kivirikko, 1995; Bateman *et al*., 1996; Myllyharju and Kivirikko,

2001). The collagens are divided into subgroups based on their structural features, as shown in figure 1.12: fibril-forming collagens (types I-III, V and XI, Figure. 1. 12, A), fibril associated collagens with interrupted triple helices, i.e. the FACIT collagens (types IX, XII, XIV, XVI and XIX, Fig. 1.12, B), hexagonal networkforming collagens (types VIII and X, Fig. 1.12, C), basement membrane collagen (type-IV, Fig. 1.12, D), beaded filament-forming collagen (type VI, Fig. 1.12, E), collagen forming anchoring fibrils of basement membranes (type VII, Fig. 1.12, F), collagens with transmembrane domains (types XIII and XVII, Fig. 1.12, G), and the family of collagen types XV and XVIII, Figure. 1. 12 (Prockop and Kivirikko 1995; Myllyharju and Kivirikko, 2001).

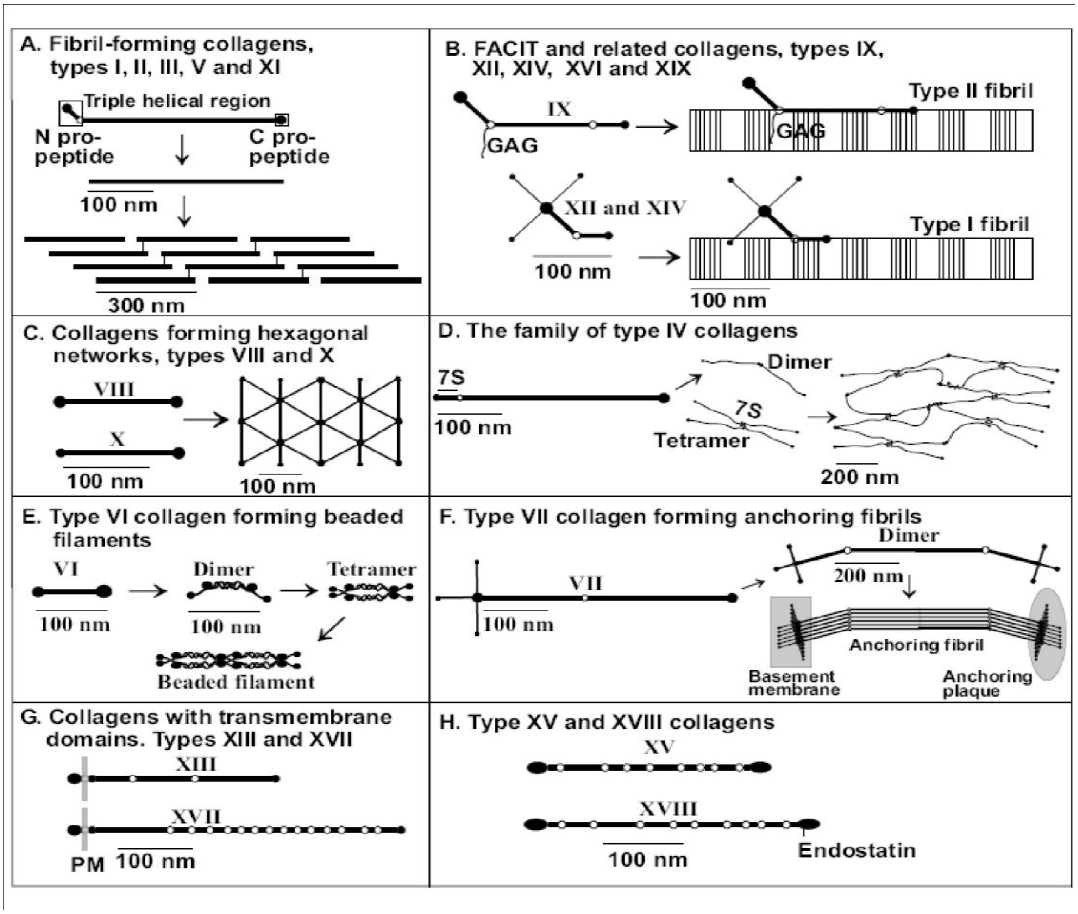


Figure 1 .12 The members of the collagen family of proteins and their molecular assemblies.

The rods show triple helical collagenous domains in each molecule, solid circles N and C terminal non-collagenous domains and open circles interruptions in the collagen triple helix. GAG, glycosaminoglycan; PM, plasma membrane. (Myllyharju and Kivirikko, 2001).

1.14 Basement membrane layers

Basement membranes (BM) or know as basal lamina are thick sheets of extracellular matrices that work as architectural barriers separating epithelial and endothelial cells as well as outer nerve axons, fat deposits, cells and muscular tissues, from the underlying cells stroma. BMs offer structural support, separate tissues into compartments, and regulate cell behaviour (Timpl and Brown, 1994). All cell types produce components of BMs, which include type-IV collagen, laminin, heparan sulfate proteoglycans and nidogen/entactin. Minor components include agrin, SPARC, fibulins, type XV collagen and type XVIII collagen. Fibronectin is present in fetal BMs (Erickson and Couchman, 2000). The

molecular composition of the BM varies among different tissues. The differences are supposed to funding tissue specificity, which is important for defining the specialised functions of epithelial and endothelial cells in different organs. The collagen network/scaffold but another scaffold the laminin scaffold forms independently interacting with the collagen scaffold through nidogen (Aumailley *et al*., 1989; Timpl and Brown, 1994), makes the basic structure of the basement membrane. Additional adhesive glycoproteins, such as tenascin, as well as proteoglycans, adhere to the scaffold and interact with cells in or adjacent to the matrix. Microcellular proteins offer indispens able role in modulating cell-matrix connections. Basement membranes and other ECMs undergo regular dynamic changes in response to a huge number of cellular stimuli, and the altering of the vascular basement membrane is a vital aspect of angiogenesis. After endothelial cells and mural cells migrate to form new blood vessels, the ECM network is not only degraded, but its structure is also reformed. New cryptic epitopes of the ECM proteins show to facilitate the migration of endothelial and mural cells. The basement membrane considered as the main source of angiogenic growth factors such as FGF-2, VEGF, PDGF-B and TGF-β, also angiogenesis inhibitors, such as thrombospondin-1 (TSP-1), canstatin, tumstatin, platelet factor-4 and endostatin. The cells responsbale for production of proteinases are essential for the liberation of these factors from the matrix (Egeblad and Werb, 2002; Kalluri, 2003). ECMbound VEGF can also stimulate endothelial cell adhesion, migration, and survival through integrin ligation. It is very important that the degradation and remodelling of the ECM should occur in a balanced manner, thus, it is a highly regulated process. Deficient breakdown of the ECM will discontinue endothelial and mural cells from leaving their original position, in the opposite site; too much breakdown of the ECM removes critical support and guidance signs for migrating endothelial and mural cells inhibiting the formation of new blood vessels. When endothelial cells and mural cells move to develop brand-new blood vessels, the ECM network is not just abject, yet its make-up is additionally changed and brand-new puzzling epitopes of the ECM proteins are subjected to assist in the migration of endothelial and mural cells. Growth factors stimulation leads to the secretion of ECM absorbing enzymes from endothelial cells, and in some times from the bordering stromal cells and growth cells (Kalluri, 2003).

1.15 Degradation of cellar membrane layers

Proteolyic decline of sub-endothelial BM is crucial for endothelial cell migration into the underlying ECM. Furthermore, proteases stimulate endothelial cell migration by activation of pro-angiogenic growth factors and with their release from the ECM. Proteolytic cleavage of the ECM unmasks cryptic adhesion sites and liberates bioactive destruction merchandise (Pepper *et al*., 1996; Werb *et al*., 1999; Kalluri, 2003). Physiological angiogenesis require a balance in proteolysis. Extreme proteolysis brings about inhibition of angiogenesis because of exploitation of the matrix scaffold that is necessary for endothelial cellular migration and tubular constructions and formation of endogenous angiogenetic inhibitors. Insufficient proteolysis, conversely, prevents migration and tubular morphogenesis because of restricted destruction of ECM and generates propose proangiogenic progress variables (Bergers and Benjamin, 2003). One of the most important protease systems associated with angiogenesis are the plasminogen activators as well as the matrix metalloproteinases. Urokinase-type plasminogen activator (uPA), Urokinase-type plasminogen activator receptor (uPAR), as well as plasminogen activator inhibitor (PAI-1) are not detectable in the quiescent endothelium, but can be detected in the course of effective angiogenesis (Bacharach *et al*., 1992; Larsson *et al.,* 1984). *In vitro*, the particular expression of these proteins can be controlled by angiogenic expansion components for instance FGF-2, as well as VEGF (Mignatti and Rifkin, 1994).

Urokinase-type plasminogen activator has the ability to degrade fibrin, lamanin, fibronectin, and proteoglycans although native collagens are generally resistant to uPA proteolysis. In addition, uPAR as well as PAI-1 are generally upregulated by hypoxia, a known inducer of angiogenesis (Kroon *et al.,* 2000; Uchiyama *et al.*, 2000).

Matrix mettaloproteinases undoubtedly are a large class of MMPs that break down the various protein components of the ECM and BM. Matrix mettaloproteinases participate are implicated in wound healing, angiogenesis, embryogenesis and within pathological conditions including tumour invasion and metastasis. MMPs (Table 1, 2) are divided into four groups; collagenases, gelatinases, stromeolysins and matrilysins, according to their ECM substrate specificity however now are divided into unique structural groups (Egeblad and

Werb, 2002; Stamenkovic, 2003). Matrix mettaloproteinases activity controlled by the activation in transcription, proteolytic activation from the zymogen; they contain a pro-peptide, whose cleavage is required for MMP activation, and by the inhibitory activity of various endogenous MMP inhibitors (Egeblad and Werb, 2002; Stamenkovic, 2003). Many MMPs are stimulated by the proteolytic activity of other MMPs and range of serine proteases. α2- macroglobulin is the main MMP inhibitor inside tissues fluids leading to this clearance of MMP-α2-macroglobulin by scavenger receptors (Sottrup-Jensen and Birkedal-Hansen, 1989). Since cells have receptors, such as integrins for structural ECM components, degradation of ECM components affects cell signalling through most of these receptors. Cleavage of insulin-like growth factor-binding protein and perlecan releases insulin-like growth factor.

Another example is the cleavage of SPARC, a matricellular protein of the ECM, by MMP-3. Produced peptides can regulate EC proliferation and/or migration and angiogenesis (Sage *et al.*, 2003). Moreover, the proteolytic degradation of ECM molecules, recent data has extended the substrate specificity of MMPs to include enzyme inhibitors, cell-bound precursors of cytokines and active cytokines, cell adhesion molecules and growth factor receptors (Monika, 2010).

Table 1 .2 Members of the MMP family.

Subgroup	MMP	Name	Substrate
1. Collagenases	MMP 1	Collagenase-1	Collagen I, II, III, VII, VIII, X, gelatin
	MMP 8	Collagenase-2	Collagen I, II, III, VII, VIII, X, aggrecan, gelatin
	MMP 13	Collagenase-3	Collagen I, II, III, IV, IX, X, XIV, gelatin
2. Gelatinases	MMP 2	Gelatinase A	Gelatin, Col I, II, III, IV, VII, X
	MMP 9	Gelatinase B	Gelatin, Col IV, V

1.16 Integrins and cell-matrix communications

Integrins are found in all multicellular animals, but not in prokaryotes, plants, or fungi. The number of different integrin α and β subunits varies in different species. Mammals have 24 different heterodimeric integrins composed of eight different β subunits and eighteen different α subunits. Though some subunits appear only in a single heterodimer, 12 integrins contain a β1 subunit and five have α V. The occurrence of integrins in all multicellular animals suggests that integrins are essential for the development of multicellular organs and tissues (Hynes, 2002; Kim *et al*., 2011).

Integrins are bidirectionally signalling transmembrane receptors composed of two different subunits, α and β (Hynes, 1992). They integrate the intracellular cytoskeleton, the contractile actin microfilament system and signalling pathways to the network of extracellular matrix (ECM) proteins (Brakebusch and Fässler, 2003; Humphries *et al*., 2004; Kinashi, 2005). In vertebrates, specific integrins

are also involved in cell-cell interactions with other transmembrane proteins of the nearby cells. To facilitate proper function it is important that integrins become active and thus capable of binding their ECM ligands only under specific conditions (Hynes, 2002; Kim and Ginsberg, 2011). Bidirectional signalling and the regulation of integrin activity are mediated via large conformational changes (Hynes, 2004; Mould and Humphries, 2004; Arnaout *et al.,* 2005; Gahmberg *et al.*, 2009; Shattil *et al*., 2010; Springer and Dustin, 2012) (Figure 1.13).

Amongst others, integrins, cadherins, selectins, syndecans and the immunoglobulin superfamily-related cell adhesion molecules (IgCAMs) are suggested as a factor in these situations (Juliano, 2002). Integrins form important family of adhesion molecules of which the main receptors for ECM proteins for example fibronectin, vitronectin, collagen, or even Laminin (Hynes, 1992). These integrin molecules integrate the intracellular cytoskeleton with the ECM-rich extracellular environment by binding to intracellular protein complexes (Critchley, 2000). Signalling molecules, initialized in an adhesion dependent fashion, also recruited into most of these complexes. As a result, ECM proteins, integrins, cytoskeletal proteins along with signalling molecules make into aggregates either side of the cellular membrane. Integrins connect to cell surface receptors, for example, the intracellular adhesion molecule-1 (ICAM-1), the vascular cell adhesion molecule-1 (VCAM-1), some other growth factor receptors such as FGF-2R (Meredith *et al*., 1996) and also bind to co-receptors present on other cells, bacterial polysaccharides, or viral coat proteins (Danen and Sonnenberg, 2003). Integrins perform diverse cellular functions which includes cell adhesion, migration (Hynes, 1992), design, polarity (Schwartz and Ginsberg, 2002), spreading (Lauffenburger and Horwitz, 1996), apoptosis (Assoian and Marcantonio, 1997), survival (Schaller, 2001), angiogenesis (Luscinskas and Lawler, 1994), cell differentiation (Hynes, 1992), homeostasis connected with multicellular microorganisms (Schwartz *et al*., 1995) organogenesis.

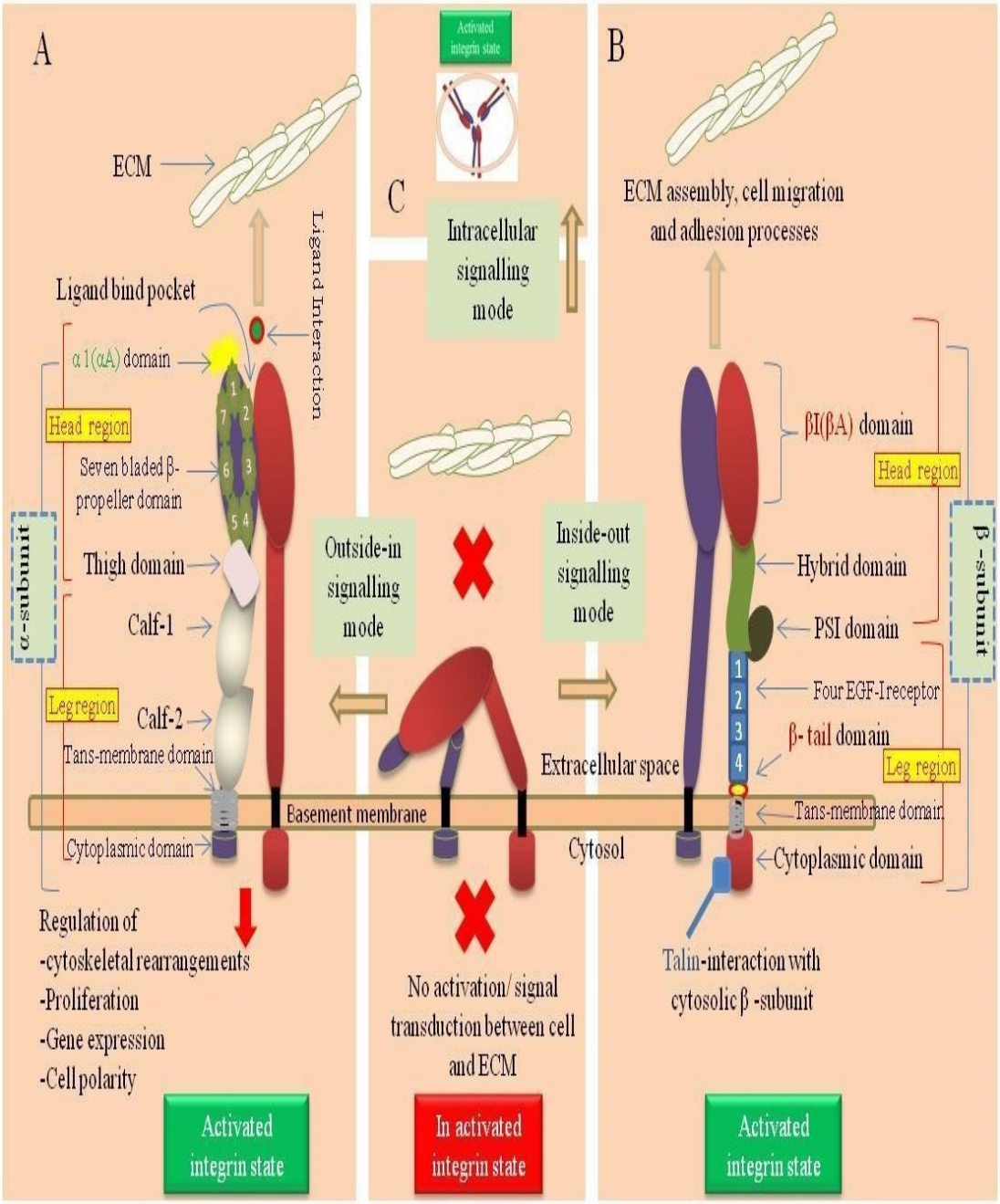


Figure 1.13 Integrin signalling. αβ subunits have head region and leg region α subunit domain head region include; α domain, propeller domain, and thigh domain, leg reigion include: calf1, calf2, trns-membrane and cytoplasmic domain. β sub unit head region include; βI domain, Hybrid domain, and PSI domain, leg region include FGFI receptor, β-tail domain, trans-membrane domain, and cytoblasmic domain.

Integrins are not just involved with typical cellular processes but also pathological disorders such as atherosclerosis, tumorigenesis, metastasis and a number of inflammatory disorders (Schwartz *et al.,* 1995; Simon *et al*., 1997). Integrin cues can be divided into (A) inside-out and (B) outside-in signalling pathways. Based on recent studies, an emerging class of integrin signals can be considered eliciting from (C) intracellularly active integrins that localized to endosome (Luo *et al*., 2007).

Data around the functions of integrins, specificity in cell development has been obtained from integrin-deficient mice. The loss of any kind of integrin, α or, β, leads to severe abnormalities, including lethality in embryonic stage or soon after birth. Particularly, the β1 subunit could bind to one of many distinct α subunits, lack of the β1 integrin subunit ends up with embryonic lethality (Ratnikov I*, et al*. 2005)

Structurally, integrins are usually glycosylated, heterodimeric, transmembrane receptors including non-covalently associated α (120 kDa to 180 kDa) and β subunits (90 to 110 kDa) (Giancotti and Ruoslahti, 1999; Hynes *et al*., 1987).

Nearly 24 different integrins have been reported to be expressed as combinations of the 18 α and 8 β subunits already described and splice variant isoforms of individual subunits produce additional complexity (Burkin and Kaufman, 1999; de Melker and Sonnenberg, 1999). Study survey of the human genome has identified 6 novel α and 1 novel β subunit, however, their cellular expression has not been defined yet (Venter *et al*., 2001). Every single subunit consist of a large extracellular domain (700 to 1100 aa), one particular transmembrane section along with a short intracellular cytoplasmic tail (ranging via 20 to 60 aa) that interacts with various proteins essential for regulations connected with integrin affinity as well as cytoskel et al integrity (Aplin *et al*., 1998; Hynes, 1999). They were initially described as cell matrix adhesion molecules (Tamkun *et al.,* 1986), but later it was found they are additionally crucial signal transducers (Clarkand Brugge, 1995; Giancotti and Ruoslahti, 1999). Characteristically, while specific ligands like ECM components bind to a particular integrin to form a cluster, the integrin induces a cascade of intracellular events termed outside in signalling. Reciprocally, signals from inside of the cell modulate the integrin-binding affinity to ligands, which convert the integrin from a low-affinity or resting state

to a high-affinity or primed (also known as activated) state (inside-out-signalling; Giancotti and Ruoslahti, 1999). Additionally, divalent cations such as Ca^{2+}, $Mg^{2+}$and Mn^{2+} modulate integrin-ligand binding; Ca^{2+} inhibits these interactions and stabilises a low-affinity conformation, while Mn^{2+} stabilises a high-affinity conformation (Mould *et al.,* 1995). An additional essential feature can be that a single integrin binds to one or various ligands and that a single ligand interacts with various heterodimers of integrin subunits, one example is, that many of integrin could recognise a specific ECM component in the competing or cooperative manner alone and at the same time; one particular integrin can recognize various ECM components (Stupack and Cheresh, 2002). Various integrins strongly bind the tripeptide Arg-Gly-Asp (RGD) inside particular ECM proteins like vitronectin, fibronectin and other proteins. Moreover, RGD presenting receptor subfamilies, Laminin receptors, collagen receptors and a set of leukocyte particular integrins may also be present in vertebrates (Hynes, 2002) (Figure 1.14).

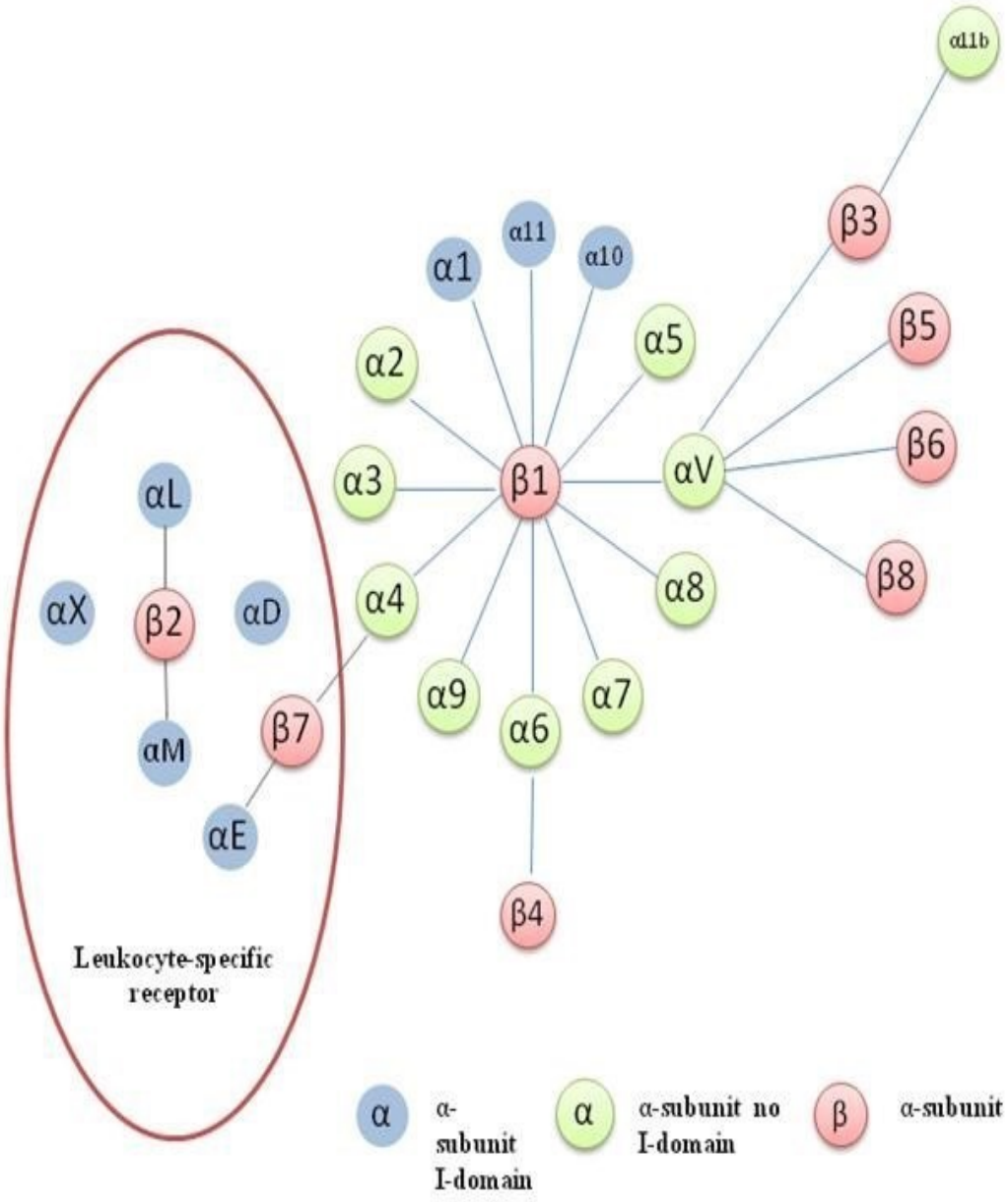


Figure 1 .14 The integrin receptor family. Integrins are made of α and β subunits. 18 α subunits and 8 β subunits associate together in different combinations resulting in at least 24 integrins. These can be subdivided into subfamilies based on evolutionary relationship, ligand specificity and restricted expression pattern such as β2 and β7, which are specifically expressed in white blood cells. Vertebrates have a set of collagen receptors with I/A domains (α1, α2, α10 and α11) and a pair of related integrins (α4β1, α9β1), which are restricted to chordates. Laminin receptors (purple) and RGD binding receptors (blue) are present throughout the metazoa. Asterisks (Adapted from Hynes, 2002) denoted splice variants in cytoplasmic domains.

1.17 Cytoskeletal proteins

The internal framework of a cell composed of a network of protein filaments and extending throughout the cytosol. The cytoskeleton contains mainly of actin filaments and microtubules and plays vital role in cell movement, shape, growth, division, and differentiation, as well as in the movement of organelles within the cell. All eukaryotic cells have a cytoskeleton.

1.17.1 Vinculin

Vinculin is a 1066 amino acids cytoskeletal protein that has a molecular weight of 117-kDa, that is localized on the cytoplasmic face of integrin-mediated cell–extracellular matrix junctions (focal adhesions) and cadherin-mediated cell–cell junctions; all the evidence suggested that vinculin is required to maintain the integrity of adherens junctions. The interaction of vinculin with the LD1 and LD2 motifs of paxillin regulate cell survival (Turner *et al*., 1999, Humphries *et al*., 2007). It has been proposed that the tail domain of vinculin competes with FAK for paxillin binding and thereby promotes ERK signalling through FAK or paxillin to inhibit apoptosis (Subauste *et al*., 2004).

1.17.2 Paxillin

Paxillin a multi-domain 68-kD protein (Glenney and Zokas, 1989) is a cytoskeletal component that localizes to the focal adhesions at the ends of actin stress fibres in chicken embryo fibroblasts. Paxillin is phosphotyrosine-containing protein that may play a role in several signalling pathways (Schaller, 2001). Paxillin binds to many proteins that are involved in changes the organization of the actin cytoskeleton, which are necessary for cell motility associated with embryonic development, wound repair and tumor metastasis (Legate *et al*., 2009).

1.17.3 Talin

Talin, a 270-kDa cytoskel et al protein is thought to be a key player in the formation of focal adhesions, implicated in integrin-mediated cellular interactions with the extracellular matrix.Talin is a ubiquitous cytosolic protein that is found in high concentrations in focal adhesions. It is capable of linking integrins to the actin cytoskeleton either directly or indirectly by interacting with vinculin and alpha-actin (Alan, 2006).

1.18 Collagen receptor integrins

The collagen receptor integrins initiate several processes within normal and pathological states. Collagen receptor integrins are usually structurally different in comparison to the other ECM binding integrins. They have a particular 'inserted' site as well as and I domain inside the β-propeller domain on the N-terminus as part of α subunit which usually mediates ligand binding. Belonging to this group is α1β1, α2β1, α10β1 and α11β1 (Briesewitz *et al*., 1993; Camper *et al.,* 1998; Velling *et al*., 1996). On top of the αI domain is a cation-binding site (MIDAS) that is formed by three loops of the domain coordination a metal ion. MIDAS is the major ligand-binding site in integrins possessing a αI domain (Michishita *et al*., 1993). Also specific to collagen binding integrin αI domains is the existence of an additional α helix, the αC-helix located on the top face of the domain. It is responsible for developing a groove on the leading area of the domain name that increases communications with collagen (Emsley *et al.*, 2000; Emsley *et al*., 2004). Collagen-integrin interactions include essential physiological functions such as, proliferation, migration, differentiation, ECM assembly, angiogenesis, and pathological ailments such as thrombosis and tumour metastasis (Hynes, 1992; Senger *et al*., 1997). Integrins α1β1, α2β1 show the broadest tissue expression during embryonic development (Gardner *et al*., 1996).

The α2β1 integrin is also expressed on endothelial cells and fibroblasts as well as epithelial cells (Camper *et al.,* 2001).

1.18.1 Modulation of integrin mission by conformational modification

There is a necessity for integrin activation to occur under the best orders for physical response as well as a system in position for keeping them less active when they are not required. This sort of regulation is accomplished by conformational adjustments that circulate with the domain from cytoplasmic components to extracellular ones (inside-out) or ligand binding can induce changes to the αI domain that cause the separation of transmembrane and cytoplasmic parts and initiate a signalling cascade inside the cells (outside-in).

Two conformations of αI domain are now understood the shut or open kind that varies by the existence of the metalion (Lee *et al*., 1995).

1.18.2 Crosstalk in between integrin and growth factor receptors

Integrins co-operate with several growth factor receptors, which include EGFR, PDGFR, and fibroblast increase component receptor (FGFR) and induce different cell signalling activities (Giancotti and Ruoslahti, 1999; Huynh-Do *et al*., 1999).

For example, αvβ3 integrin clusters with PDGF receptor or insulin receptor and form complexes (Bartfeld *et al.,* 1993; Schneller *et al*., 1997).

These complexes, established upon the integrin clustering, lead to crosstalk between two different receptors (Miyamoto *et al.,* 1996; Plopper *et al*., 1995). Due to the fact, several mechanisms between integrin and growth factor stimulation are identical; there is an opportunity for cross talk between these two pathways. One of the main converging levels with both of these pathways is usually focal adhesion kinase. While FAK phosphorylation stimulated by growth factors contributes to an increase in cell migration. FAK knockout cells do not migrate towards chemotactic response against PDGF that indicates FAK function is required and essential for PDGF stimulated migration (Sieg *et al*., 2000). This kind of impaired motility could be rescued through re-expressing FAK cells. Integrins promote signalling pathways such as MAP kinase pathway even in the absence of growth factor, the integrin binding to ECM protein trigger the MAP kinase pathway (Moro *et al*., 1998; Schlaepfer *et al*., 1994). Interestingly, growth factors do not stimulate those pathways in suspension cells underlying the importance of cell adherence to ECM proteins via integrins (Aplin and Juliano, 1999; Aplin *et al*., 2001; Assoian and Schwartz, 2001; Howe *et al*., 2002; Schwartz and Assoian, 2001).

It is an established fact that growth factors such as PDGF modulate the expression of a variety of proteinases including MMPs. Interruption of these kinds of signalling pathways by anti-PDGF prevent VSMC migration by controlling MMPs. These kinds of observations proposed that integrin initiated FAK signalling and growth factor cooperative signalling may have an important influence on cell migration (Howe *et al*., 2002).

1.19 Growth Factors

1.19.1 Vascular endothelial growth factor

VEGF-A is an essential regulator of physical angiogenesis and belongs to a gene family that includes placental growth factor (PIGF), VEGF-B, VEGF-C, VEGF-D and VEGF-E. At least six different VEGF-A isoforms have been identified through alternate splicing of exons including VEGF121, VEGF145, VEGF165, VEGF183, VEGF189 and VEGF206. VEGF121, VEGF165 and VEGF189 are the major isoforms detected in most cells whereas VEGF145 and VEGF206 are rare. VEGF expression is up regulated by hypoxia (Minchenko *et al.,* 1994, and Ferrara N., *et al*., 2003), which increases hypoxia inducible factor-1 (HIF-1) (Jiang *et al.,* 1996, and Brahimi-Horn *et al*., 2005) and induces the binding of HIF-1 to the hypoxia response element in the VEGF gene promoter region (Liu *et al*., 1995). Numerous growth factors and cytokines such as EGF, FGF-2, PDGF, TGF-α, TGF-β, FGF-4, keratinocyte growth factor (KGF/FGF-7), insulin-like growth factor 1(IGF-1), IL-1α, IL-1β, IL-6 also increase expression of VEGF mRNA or induce VEGF release (Brogi *et al*., 1994,and Nina*, et al.,* 2009).

VEGF121, VEGF165 and VEGF189 are widely expressed, whereas VEGF145 and VEGF206 are uncommon. VEGF binds to two receptor tyrosine kinase VEGFR-1 (Flt-1) and VEGFR-2 (Flk1/KDR). Both receptors have seven extracellular immunoglobulin (Ig)-like domains followed by a transmembrane region and a cytoplasmic tyrosine kinase domain (Ferrara, 2001; Petrova *et al.,* 1999). Growth factors including VEGF and FGF-2 increase VEGF receptor expression (Wilting *et al*., 1996; Shen *et al*., 1998, and Narita *et al*., 2009). As for other receptor tyrosine kinases (RTKs), ligand binding induces homodimerisation of VEGF receptors and phosphorylation of their intracellular domain. VEGFR-1 has a higher affinity for VEGF than VEGFR- 2. However, no apparent proliferation, migration or cytoskeletal effects mediated by VEGFR-1 were observed in ECs. It has been suggested that VEGFR-1 may not function as a signalling receptor, but rather as a decoy receptor to negatively regulate VEGF activity on the endothelium by sequestering the ligand (Seetharam *et al*., 1995, and Presta, *et al*., 2005). In ECs, VEGFR-1 is involved in up-regulated

expression of tissue factor, urokinase-type plasminogen activator and plasminogen activator inhibitor 1 (Clauss *et al*., 1996).

VEGFR-2 is phosphorylated more efficiently upon ligand binding and is thought to be the key receptor to transmit VEGF signalling. VEGF-dependent association and/or activation of a number of signalling proteins have been reported, including Grb2, Nck, PLCγ, Ras GAP, PI3 kinase, Src family tyrosine kinases, PKB/Akt, FAK, SHP1 and SHP2 (Matsumoto and Claosson-Welsh, 2001). Activation of different signalling pathways such as PI3 kinase/Akt, p38 mitogen-activated protein kinase or ERK, transmits functions of VEGF in endothelial survival, proliferation, migration, vascular permeability and gene expression. Heparan sulphate proteoglycans (HSPG) may act as co-receptors for VEGF to facilitate its receptor binding and bioactivity (Cohen *et al*., 1995; Gengrinovitch *et al*., 1999).

1.19.2 Fibroblast growth factor

In the early 1970s, Gospodarowicz purified a protein from bovine pituitary gland, which was able of inducing proliferation and phenotypic transformation of BALB/c 3T3 fibroblast (Gospodarowicz, 1975). This protein was named "basic fibroblast growth factor" (bFGF). Human FGFs are 18-25 KDa polypeptides and have a 120 amino acid residue core, is a strong promoter of fibroblast proliferation (Pey *et al*., 2007). This has been isolated from various neural tissues including the pituitary, adrenal cortex, corpus luteum and placenta (Omar *et al.*, 2004). FGF-2, a ubiquitous angiogenic and heparin-binding growth factor (HBGF), plays important roles in angiogenesis and tumour growth, (Beenken and Mohammadi, 2009) FGFs play major roles in normal development; wound healing, tumour development and progression. The 23 members of the FGFs family have been identified and characterised (Itoh, and Ornitz, 2004). They all have high affinity for heparin, heparan sulfate proteoglycans (HSPG) which are thought to facilitate dimerisation, and activation of FGFRs. FGF-2 has four isoforms derived from alternative translation initiation (Florkiewicz *et al*., 1989) and does not contain a signal sequence for secretion. While the 18-kDa form of FGF-2 is secreted by an unknown mechanism, the higher molecular weight forms are predominately localised to the nucleus. FGF-2 exerts its biological functions by binding to and activating the transmembrane receptor tyrosine kinases. FGF-

2 is found in storage form in ECM. Heparin sulphate proteoglycans present in the ECM protect the FGF-2 from activation within the matrix. Therefore, if ever there is an ECM breakdown FGF-2 can be released by soluble heparin. The FGF-2 receptors are integral membrane proteins with a single trans-membrane helix. The FGFR intracellular domain is largely composed of a tyrosine kinase domain, and is classified as a classical tyrosine kinase receptor (Harmer *et al.*, 2004). Four FGF receptors nominated as FGFR-1 to FGFR-4, which share 55% to 72% homology (Johnson *et al*., 1993). FGF binding leads to receptor dimerisation and tyrosine phosphorylation. The FGFR-1 has been studied in detail and several potential tyrosine phosphorylation sites in the cytoplasmic part have been described (Klint and Claesson-Welsh, 1999). FGF-2 induces proliferation, migration, proteinases production and expression of specific integrins in cultured endothelial cells (Klein *et al*., 1996). FGF-2 plays key roles in development, remodelling and disease states in almost every organ system (Bikfalvi *et al*., 1997). The various effects displayed by FGF-2 indicate the importance of the growth factor in regulation of developmental and physiological activities. Unexpectedly, the bFGF knockout mice lacking all isoforms are viable, fertile and are phenotypically similar to the wild type mice. However, the knockout animals have some mild defects including delayed wound healing, decreased neuronal density in the motor cortex and reduced blood pressure (Ortega *et al*., 1998; Miller *et al.,* 2000).

1.19.3 Other growth factors

Several other factors take part in vascular development and growth. Plateletderived growth factor (PDGF) plays a role in embryonic development, cell proliferation, cell migration, and angiogenesis. It had been initially identified in platlets but has since been found in many other cell types, including fibroblasts, keratinocytes, myoblasts, epithelial cells, macrophages and endothelial cells (Heldin and Westermark, 1999; Hoffmann *et al*., 2005).

Transforming growth factor-β (TGF-β) as well as its receptor is also expressed in endothelial cells as well as pericytes (Massague, 1990). TGF-β is chemotactic for many cells and establishes structural integrity of newly formed vessels during angiogenesis through synthesis of ECM components and proteases (Wahl *et al*., 1987). Angiopoietins, a family member of 4 secreted proteins and their

Tiereceptors, play a crucial role in angiogenesis. Angiopoietin-1 (Ang-1) binding to receptor Tie2 on endothelial cells is an anti-permiability elements as well as functions as a chemoattractant. Ang1 stimulates Tie2 receptor autophosphorylation, while Ang2 has been reported that Ang2 may function as an endogenous antagonist of Ang1. However, it is both Ang1 and Ang2 potentiated VEGF-induced angiogenesis *in vivo* (Maisonpierre *et al*., 1997; Koblizek *et al*., 1998; Wakui *et al.*, 2006).

Ephrin ligands are divided into an A and a B class according to their sequence, and they stimulate Eph RTKs. Many ephrins and Eph receptors are upregulated in tumours, especially in the more aggressive stages of tumour progression. Ephrins and the Eph-receptors are understood to play roles in neural and vascular development; ephrin-A1 was revealed to be upregulated throughout *in vitro* capillary tube development and induce angiogenesis (Holzman *et al*., 1990; Pandey *et al*., 1995). Ephrin-B2 marks arterial endothelial cells and Eph-B4, the receptor, marks veins (Wang *et al.,* 1998; Iso *et al*., 2006)..

1.20 Endothelial cell migration

During the first steps of angiogenesis, ECs need to reduce inter-EC contacts and detach peri-EC support, to destabilise mature vessels. Vascular permeability increases and the blood clotting protein fibrinogen leaks out of the vascular bed and forms a fibrin gel. For invasion into the underlying interstitium, ECs need to degrade and invade through insoluble fibrin, the underlying basement membrane as well as the interstitial matrix.

The basement membrane degradation results in the release of cytokines and growth factors from ECs and other cell types (Plank *et al*, 2003). As the gap between ECs increases, they form finger-like projections known as EC cords, which initially travel as a solid cord followed by lumen formation (Folkman and Haudenschild, 1980). The EC sprout elongates and fusion of microvilli at the end of the sprout forms a vascular network. Studies have shown that adhesion molecules of the select in, integrin, and cadherin families play an important role in capillary morphogenesis (Brooks *et al*., 1994; Stromblad and Cheresh, 1996). Despite the ability of the endothelium alone to form capillary networks *in vitro*, capillary morphogenesis is a complex process of interaction between EC

response and the paracrine effects of blood constituents, stromal cells and PCs (Gerhardt and Betsholtz, 2003; Bergers and Song, 2005). The following step is the migration and proliferation of the now *in situ* ECs. As the ECs leave the original place they become activated ECs and exhibit numerous changes. The ECs experience alterations in the expression of cell-cell and cell-matrix adhesion molecules, reorganisation of cytoskeletal elements, and express cell surface adhesion molecules such as integrins, members of the Ig supergene family, selectins, and components of the ECM (Polverini, 1995; Polverini, 1997). Integrins are expressed by ECs, which in turn bind to ECM proteins. One integrin of particular importance in angiogenesis is integrin αv-β3, which binds to MMP-2. Integrin αv-β3 causes MMP-2 matrix degradation of the EC surface (Brooks *et al*., 1994). Generation of proteolytic enzymes occurs due to the activation of the ECs, which in turn degrade the ECM and migrate away from the original vessel. Because of this in angiogenesis, capillary buds are produced. In addition, growth factors sequestered in the basal lamina and surrounding ECM are released to stimulate further angiogenesis steps. Furthermore, there is also induction of EC-derived cytokines and growth factors that play an important role in the later stages of the angiogenic response (Polverini, 1995; Polverini, 1997).

1.21 Endothelial cell filopodia

Endothelial cell motility is a critical aspect of angiogenesis, but only a limited number of molecules have been identified as specific regulators of endothelial cell. The regulated polymerization of actin filaments result in two morphologically distinct protrusive structures, lamellipodia and filopodia, at the leading edge of motile cells. (Nambiar *et al.,* 2010). The lamellipodia are thin (thickness 0.1–0.2 μm) fan-like projections composed of a branched network of actin. (Pollard and Borisy 2003). In contrast, filopodia are slender, finger-like extensions (diameter 0.1–0.3 μm), often emanating from lamellipodia, made up of tight bundles of actin filaments. (Faix and Rottner 2006; Mattila and Lappalainen 2008). With respect to directed cell migration, filopodia are particularly important, probing and sensing the pericellular environment for chemotactic and other molecular cues in the ECM that guide the direction and movement of the cell. They also act as points of attachment to the ECM for the cell, generating tension that may act to pull the cell forward and/or stabilize the cell as it moves. Given these roles, it is not surprising that

filopodia contain diverse receptors for ECM proteins and an array of signaling molecules.

1.22 Endothelial cell proliferation

When there is a 'disturbance' between the various cell types previously mentioned in section 1.17, ECs degrade and travel through their basement membrane and ECM. The release of growth factors (which include angiogenic growth factors) causes the activation of further 'proliferating' ECs to migrate behind the degraded ECs (Polverini, 1995; Polverini and Nickoloff, 1995). Angiogenic factors first target post-capillary venules and small terminal venules. It is contain ECs that are flattened and rest upon a basal lamina. The flattened ECs are bounded by a sporadic layer of PCs and SMCs and are invested into the matrix (Polverini, 1995; Polverini and Nickoloff, 1995). There are a number of angiogenic growth factors that act specifically on ECs. These include VEGF and angiopoietins. The production of cytokines and growth factors has been suggested for efficient functioning of ECs, such as control of EC growth, elongation, orientation and mutation of ECs as they organise into functioning vessels (Polverini, 1995).

1.23 Protein phosphorylation in cell signalling

Intracellular signalling controls cell survival, proliferation, differentiation and apoptosis. The importance of protein phosphorylation for cell signalling started to emerge with the findings that reversible phosphorylation can alter the activity of glycogen phosphorylase (Hunter *et al*., 1995; Hunter *et al*., 2000). It has been approximated that human genome consists of at least 518 protein kinases genes, representing about 1.7% of all human genes (Manning *et al*., 2002). In a typical vertebrate cell, around 90% of the proteins are serine phosphorylated compared to tyrosine phosphorylation of only approximately 0.05% of the proteins. Consequently, tyrosine phosphorylation enables a high degree of specificity in cell signalling through regulation of the activity of tyrosine kinases, and the counteracting tyrosine phosphatases, which remove phosphate groups from target tyrosine residues (Fischer, 1999).

1.23.1 Phosphorylation by tyrosine kinases

A tyrosine kinase is an enzyme that can transfer a phosphate group from ATP to a protein in a cell. It functions as an "on" or "off" switch in many cellular functions. Tyrosine kinases are a subclass of kinase. The phosphate group is attached to the amino acid tyrosine on the protein. Tyrosine kinases are a subgroup of the larger class of protein kinases, which attach phosphate groups to other amino acids (serine and threonine). Phosphorylation of proteins by kinases is an important mechanism in communicating signals within a cell (signal transduction) and regulating cellular activity, such as cell division. Approximately 2000 kinases are known, and more than 90 Protein Tyrosine Kinases (PTKs) genes have been found in the human genome. They are divided into two classes, receptor and nonreceptor PTKs. At present, 58 receptor tyrosine kinases (RTKs) are known, grouped into 20 subfamilies, controlling developing cell growth and differentiation, as well as remodelling and regeneration of adult tissues, are moderated by polypeptide growth factors and their transmembrane receptors, numerous of which are RTKs (Mustonen and Alitalo, 1995; van der Geer *et al*., 1994). Several families of receptor tyrosine kinases are characterised and some of them are strictly endothelial cell-specific. Growth factor receptors with protein tyrosine kinase activity have a similar molecular topology. They consist of a large glycosylated extracellular domain defining the receptor binding characteristics, with a hydrophobic transmembrane region anchoring the receptor in the plane of the plasma membrane.

The kinase domain of subclass III RTKs is divided into two halves by insertions of hydrophilic amino acid residues. The role of this kinase insert region is to modulate receptor interactions with certain cellular substrates. Lastly, they contain the carboxy-terminal tail, which interacts with the substrate binding sites of the protein tyrosine kinase region, modulating the capacity of the tyrosine kinase (TK) region to interact with exogenous substrates (Ullrich and Schlessinger, 1990). RTKs are activated by polypeptide ligands commonly known as growth factors or cytokines. Signalling involves ligand binding, which induces a conformational change in the external domain of the receptor resulting in its dimerisation (Ullrich and Schlessinger, 1990). This event results in receptor transphosphorylation at specific tyrosine residues and activation of the catalytic

domains for the phosphorylation of cytoplasmic substrates. These phosphorylated tyrosine residues may serve to control the kinase activity of the receptor, and to create docking sites for the cytoplasmic signalling molecules, which are often substrates for the kinase. These molecules are adapters or enzymes themselves, linking RTKs to different signalling pathways. The interaction of these proteins with the activated RTKs can initiate signalling pathways leading to the nucleus or other cellular targets (Heldin, 1995). Following ligand binding and dimerisation, receptors are internalised for degradation or recycling in order to attenuate signalling (Cadena and Gill, 1992).

1.23.2 FGF-2 signalling pathways

After binding to its receptor, FGF-2 activates a number of intracellular pathways (Nugent and Iozzo, 2000). The proteins FGF receptor substrate 2 (FRS2) and SHC bind to the phosphotyrosine deposit of the bFGF receptor and feature as docking protein for the GRB2-SOS complex, which can activate RAS and subsequently initiate the Raf-MEK-MAPK cascade (Pintucci *et al*., 2002). The MAPK translocates to the nucleus, where it activates transcription factors by phosphorylation. Moreover, PLCγ binds also to the phosphotyrosine residue and activated during FGF-2 receptor binding. By activation it initiates a signalling cascade via hydrolysis of phosphatidylinositol to inositol-3-phosphate and diacylglycerol (DAG) leading to calcium release and activation of protein kinase C (PKC), which is implicated in a variety of cell functions. Moreover, FGF-2 has also been shown to be internalised via the cell surface receptors and subsequently translocated and accumulated in the nucleolus, where it is, among others, thought to stimulate the transcription of ribosomal genes during G0→G1 transition in the cell cycle (Chen *et al*., 2004). The binding of FGF-2 to its receptor as well as internalisation of the growth factors is significantly reliant after binding to HSPG.

1.24 The optical system

1.24.1 The eye

The eye is composed of three different layers: exterior layer (cornea and sclera), intermediate layer (iris-ciliary physical body and choroid), and interior layer (retina) (Duvvuri *et al*., 2003) and is filled of two sorts of fluids: liquid humor between the cornea and the iris and vitreous humor between the lens and retina (Figure 1.15).

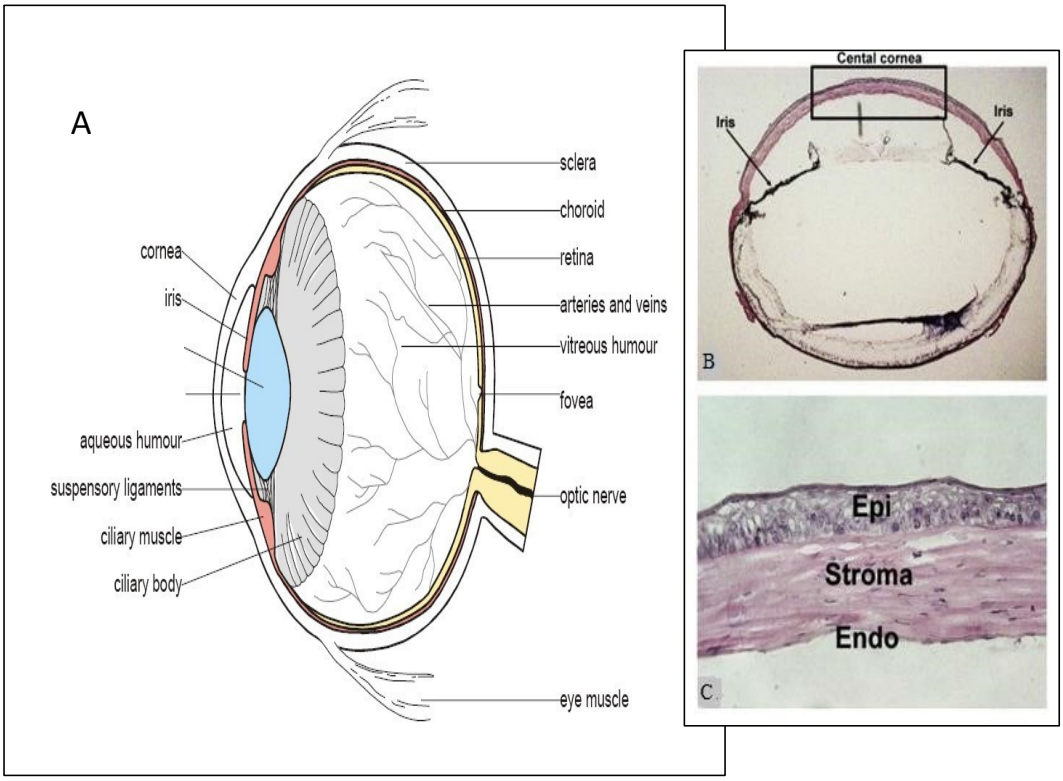


Figure 1 .15 The human eye.(A) Shows the anatomical structure of Cross section of eye, (B) The black rectangle indicates the central cornea where all analyses were carried out, (C) The corneal stroma from the central cornea (Shoujun *et al*., 2014).

This cornea is an optically transparent tissue that enables light to reach the retina. This tissue is non-vascular, and nutrients and oxygen are supplied by diffusion from lachrimal fluid and aqueous humour as well as from blood vessels located at the junction between the cornea and the sclera. The cornea is composed of five layers: epithelium, Bowman's membrane, stroma, Decement's membrane, and endothelium (Duvvuri *et al*., 2003). The epithelium has tight junctions and is a

hydrophobic layer that makes it a crucial barrier to medicine distribution. The Bowman's membrane separates the epithelium and the stroma. The stroma comprises 90% of the corneal thickness. This region is hydrophilic because 85% of the stroma is water. The Decement's membrane lies between the stroma and the endothelium. The endothelium contacts the aqueous humour and is responsible for controlling corneal hydration. The sclera forms about five-sixth area of the outer envelope of the eye (Boubriak *et al.,* 2000). The sclera is a fibrous tissue extending from the cornea to the optic nerve. It is a protective layer usually referred to as the "white of the eye". Nearly 70 % of the sclera is water, and the remaining components are mostly fibrillar collagen (predominately collagen typeI) and proteoglycans containing glycosamino glycan side chain (Boubriak *et al*., 2000).

The coloured part of the eye is the Iris and controls light entering eye, and divides the anterior humour chamber from the posterior humour chamber. The ciliary body produces the aqueous humour at rate of 2.2×10^{-3} ml/min (Friedrich *et al*., 1997). 1-2% of aqueous humour production flows in the posterior direction into the vitreous (Maurice *et al.,* 1987) and the remaining flows in the anterior direction and drains through the Schlemm's canal. The choroid is vascular and pigmented tissue between the retina and the sclera.

The retinal pigment epithelium (RPE) is located between the choroid and the retina and the superchoroidal space is between the choroid and the sclera. The choroid is composed of layers of blood vessels that nourish the sub retinal region. The retina is a multi-layered membrane occupying the internal space of the posterior portion of the eye wall. One side of the retina contains the rod, cones, and is adjacent to the retinal pigment epithelium and the choroid; the other side of the retina faces the vitreous. Photoreceptor cells are located in the sub retinal region near to the choroid. The macula is the central of retina that serves fine central vision and colour perception. The fovea is a depression at the centre of macula. The vitreous is a transparent substance that fills the centre of the eye between the lens and the retina. It is composed of hyaluronic acid and collagen (Huges *et al*., 1972) in 98% water. Aqueous humour is the fluid that fills the space between the cornea and the iris and nourishes the cornea and the lens.

1.24.2 Vitreous balance

That vitreous is now considered an important ocular structure with respect to both normal physiology (Foulds *et al*., 1987) and several important pathologic conditions of the posterior segment (Sebag *et al*., 1989). Vitreous hormone balance has actually been thoroughly evaluated by (Sebag, 1998; Bishop, 2000) (Figure.1.16). **A.Hyaluronan**: is a main macromolecule of vitreous. It is found throughout the body and was initially separated from bovine vitreous in 1934 by Meyer and Palmer. Hyaluronan is a long, unbranched polymer of repeating disaccharide (glucuronic acid β (1, 3)-N-acetylglucosamine) moieties linked by β 1– 4 bonds (Balaz, 1984). The sodium salt of hyaluronan has a molecular weight of 3 to 4.5 × 10^6 in normal human vitreous. Hyaluronan is not normally a free polymer *in vivo*, but it is covalently linked to a protein core, called a proteoglycan.

B.Collagen: the vitreous contains collagen type-II, is a hybrid of types V/XI, and type-IX collagen in a molar ratio of 75:10:15, respectively (Bishop, 2000). In the whole body, only cartilage material has as higher a percentage of type-II collagen as vitreous, explaining why certain inherited mutations of type-II collagen metabolism affect vitreous as well as joints. Vitreous collagens are organised into fibrils with type V/XI residing in the core, type-II collagen surrounding the core, and type-IX collagen on the surface of the fibril. The fibrils are 7 to 28 nm in diameter (Seery *et al.*, 1991).

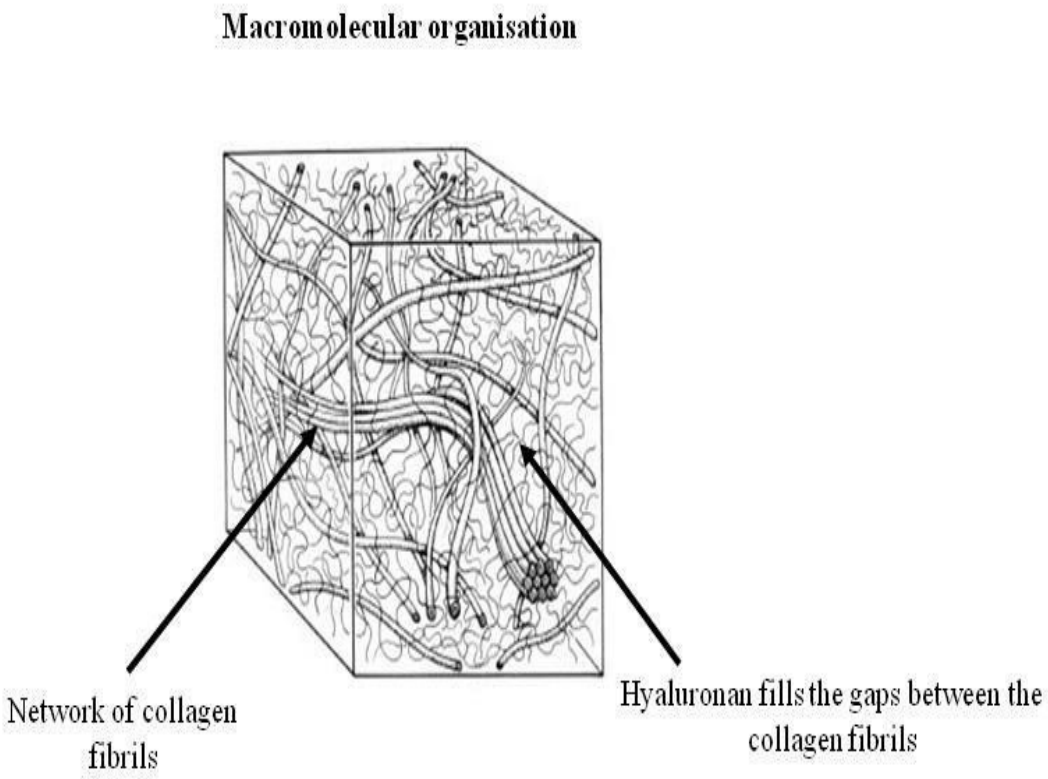


Figure 1 .16 The structure of the vitreous, maintained by a dilute dispersion of unbranched collagen fibrils with hyaluronan filling the space in between.

1.24.3 Vitreous supramolecular organisation

The vitreous has been described as a dilute meshwork of collagen fibrils interspersed with extensive arrays of hyaluronan molecules (Mayne, 2002). If collagen is removed, the remaining hyaluronan forms a viscous solution; if hyaluronan is removed, the gel shrinks but is not destroyed. Comper and Laurent proposed that electrostatic binding occurs between the negatively charged hyaluronan and the positively charged collagen in vitreous (Comper *et al.,* 1996). Bishop has proposed that to appreciate how vitreous is organised and stabilised requires an understanding of what prevents collagen fibrils from aggregating and by what means the collagen fibrils are connected to maintain a stable gel structure (Bishop, 2000). Some studies indicated the chondroitin sulfate chains of type-IX collagen bridge located between adjacent collagen fibrils in a ladder-like

configuration spacing them apart (Scott *et al*., 1992), this type of spacing essential for vitreous transparency, so can keeping vitreous collagen fibrils separated by as a minimum one wavelength of incident light minimises light scattering, allowing the unhindered transmission of light to the retina for photoreception. Bishop proposed that the leucine-rich repeat protein opticin is the predominant structural protein responsible for short-range spacing of collagen fibrils (Bishop, 2000). Concerning long-range spacing, it has been suggested that hyaluronan plays a pivotal role in stabilising the vitreous (Scott *et al.,* 1992; Mayne *et al*., 1997).

1.24.4 Inhibition of general cell development by the vitreous humor

The vitreous body is resistant to endothelial cell invasion and the disturbing consequences of neovascularisation that may occur under pathological conditions (Neely and Gardner 1998; Campocchiaro, 2000). The regression and prevention of vessel invasion may partly be due to the influence of angiogenic inhibitors (Lutty *et al*. 1985), as several studies have assigned anti-angiogenic properties to the vitreous humour. Tumour vascularisation and development within the vitreous physical body is prevented (Brem *et al*., 1976), draws out from it hinder endothelial cell expansion and stability *in vitro* (Lutty *et al*., 1985; Zhu *et al*., 1997) and suppress tumour-induced neovascularisation (Felton *et al*. 1979) and angiogenesis in the chick CAM assay *in vivo* (Lutty *et al*., 1985). Putative antiangiogenic molecules present in the vitreous humour have been described in some studies and these inhibitory factors are thought to derive from hyalocytes (Raymond and Jacobson 1982; Zhu *et al.,* 1997), such as chondromodulin-1 (Funaki *et al*., 2001), thrombospondin-1 (Sheibani *et al*., 2000), a pigment epithelial-derived element (Dawson *et al*., 1999), and changing growth factors (Eisenstein and Grant-Bertacchini, 1991).

1.25 Ocular conditions

Table 1.3 shows data concerning the patients suffering from eye disease (Clark *et al*., 2003). Among ocular diseases, the posterior segment ocular diseases, agerelated macular degeneration, diabetic retinopathy, and glaucoma, are the main causes of irreversible blindness in developed countries.

Table 1 .3 Leading causes of visual impairment and ocular discomfort (Clark *et al*., 2003)

Disease	Number of patients
Cataract	6-19 % of patients over 43 years of age
Age-related macular degeneration	11-28 % of patients over 65 years of age
Glaucoma	1-4 % of patients over 65 years of age
Diabetic retinopathy	71-90 % of patients over 10 years of age
Dry eye (US)	50-60 million (~10-15% of US population)
Ocular allergy	~25 % of US population
Retinitis pigmentosa	1 in 3000-5000 people

1.26 Glycation General Introduction

Glycation is a non-enzymatic reaction that adds carbohydrates, especially glucose, to proteins, lipids and nucleic acids througha series of parallel and sequential reactions (often termed the Maillard reaction). Louis Maillard (1912) (Chichester, 1986), who observed browning of amino acids when they were heated with sugars. The 'Maillard reaction' was not shown to be important outside the food industry for many decades. The first protein demonstrated to be glycated

in vivo was haemoglobin Bookchin and Gallop, (1968) showed that there was a hexose molecule linked to the protein N-terminal. Their further rearrangement, gives rise to a stable Amadori product. These products degrade into a variety of compounds that are much more reactive than the sugars from which they were derived (Wautier and Schmidt, 2004). These propagators again form yellow-brown, often fluorescent, irreversible compounds, usually called Advanced Glycation EndProducts (AGEs).

1.27 Diabetes mellitus

Diabetes mellitus (DM) is a complex and heterogeneous disease characterized by chronic hyperglycaemia with instabilities in carbohydrate, fat and protein metabolism due to a deficiency of insulin secretion and/ or reduction in the biological effectiveness of insulin (Michael *et al*., 2004). The most common symptoms of hyperglycaemia are polydipsia, polyuria, weight loss, blurred vision and polyphagia (Sharma and McGowan, 2000). Diabetic patients suffer from many complications leading to increased morbidity and mortality (Ahmad and Ahmed, 2006), including micro and macro vascular complications which involve renal failure, nerve damage, blindness, increased atherosclerosis, embryopathy and many others (Ahmed and Furth, 1990). The majority of diabetic complications are due to chronic hyperglycaemia (Browniee, 2001). It affects not only quality of life, but also poses a great burden upon healthcare authorities in both developed and developing nations (Goh and Coopr, 2008). Although the etiology of this disease is not clear, autoimmunity, viral infection, environmental and genetic factors all appears to play a crucial role in the pathogenesis of diabetes mellitus (Notkins and Lernmark, 2001). The current classification tries to include both etiology and clinical stages of the disease and is useful clinically. It includes four main kinds or classes. The two main types are, type 1, which accounts for 5–10% of all cases, and type 2, consider; 90-95% of all individuals with diabetes. The third group contains of other less common types of diabetes that are caused or linked with assured specific conditions and/or syndromes. The last group comprises diabetes diagnosed during pregnancy, called gestational diabetes mellitus (GDM). Besides; there is an intermediate group of individuals whose glucose level, although not meeting standards for

diabetes are still too high to be considered normal. This group comprises of individuals that have impaired fasting glucose (IFG) and/or impaired glucose tolerance (IGT) and is stated to as “pre-diabetes” as progression to overt diabetes is common, particularly when therapeutic interventions such as lifestyle changes or medications are not provided (Gavin *et al*., 2003).

1.28 Diabetes mellitus types:

1.28.1 Type 1 diabetes mellitus:

Type1, IDDM or Juvenile diabetes this type of diabetes usually develops in children or young people, but may occur in any age. Type1 diabetes usually develops when the immune system destroys the insulin-producing cells (beta cells in the pancreas). This is called an autoimmune response. Type-I diabetes can develop in people with a family history of type1 diabetes, but it also develops in people with no family history of diabetes. This process occurs over many months or years, and there may be no initial symptoms of diabetes.

1.28.2 Type 2 diabetes mellitus:

Formerly called non insulin dependent diabetes mellitus (NIDDM, type-II or adult-onset); is characterized by insulin resistance in peripheral tissue and an insulin secretory defect of the beta cell. Characterised by insulin resistance, and considered most common type, also known as non-insulin dependent in which the cells fail to use the insulin properly. This type quite common in adult over 40 years of age (Roglic, 2005).

1.29 Diabetic complications

All forms of diabetes are characterized by chronic hyperglycaemia and the development of diabetes-specific microvascular pathology in the retina, skin, renal glomerulus and peripheral nerves. The global prevalence of diabetes is rapidly rising at an alarming rate (Rolo and Palmira, 2006; Anitha *et al.*, 2008). Patients with diabetes mellitus are prone to developing diabetic complications, which ultimately represent a major cause of morbidity and mortality.The biochemical pathways linking chronic hyperglycaemia and tissue damage are not completely understood.

Virtually every major organ system in the body is damaged by diabetes such as:

1.29.1 Short term complications

Hyperglycemia, Diabetic ketoacidosis (DKA), Hyperosmolar Non-ketotic (HONK) state, Lactic Acidosis, Infections etc.

1.29.2 Long Term Complications

Eye disorders, Diabetic retinopathy, Cataracts, Diabetic neuropathy, Macro vascular Disease, Heart disease, High blood pressure, Stroke, Kidney Disease (End stage renal disease), Feet problem and Amputation of extremities, Early loss of teeth, Impotence, Pregnancy complication, Congenital malformations in babies, Death of the newborn etc.

1.29.3 Diabetic cataract

Cataracts are the main cause of visual destruction and blindness worldwide, and are characterised by pacification of the lens. Chronic hyperglycaemia represents a risk factor for cataract genesis (Crabbe, 1998). However, the main mechanism responsible for diabetic cataract formation is unknown. Currently, the glycation theory is widely accepted as playing a key role in cataract development in the lens, and numerous studies report that the accumulation of AGEs on lens crystalline may lead to protein cross-linking, oxidation and formation of high molecular weight aggregates responsible for cataract formation (Truscott, 2005;Yamagishi *et al.,* 2007).

1.29.4 Diabetic nephropathy

Diabetic nephropathy is the most common cause of end-stage renal disease in the world (Yamagishi *et al.,* 2007). It develops in 40–50% of all diabetic patients (Berger *et al.,* 2003; Wolf, 2004), and is characterised by glomerular and tubular basement membrane thickening and mesangial expansion, which eventually results in vascular occlusion, microvascular damage, and fibrotic changes, thereby leading to glomerulosclerosis. Significantly, the accumulation of AGEs in patients with nephropathy may be due to decreased clearance in the kidney, rather than increased production by glycation. More evidence suggests that the interaction of AGEs with RAGE is involved in nerve dysfunction, which may

play an inflammatory role in peripheral nerve damage, although their precise role remains unknown (Huijberts *et al.,* 2008; Rong, 2005).

1.30 Advanced glycation endproducts and macrovascular complications

The term ‘‘macrovascular complications’’ in diabetes involves a broad spectrum of pathological changes affecting major blood vessels and leading to structural and functional abnormalities. Structural changes result mainly from glycation of wall components and functional changes which originate in endothelial dysfunction, and which increase stiffness of the arterial wall or reduce vascular compliance. Macrovascular disease in diabetes comprises an accelerated form of atherosclerosis, which is believed to be fundamentally responsible for several vascular diseases, namely stroke, myocardial infarction and peripheral vascular diseases (Laakso, 1999; Rahman, 2007).

1.31 Diabetes and atherosclerosis

Atherosclerosis is a complex multifactorial disease caused by excessive accumulation of lipids in the vascular wall, and eventually limits blood flow (Mallika *et al.,* 2007). Atherosclerotic arterial disease may be clinically manifested as cardiovascular disease (CVD), where more risk in diabetic people. Several studies report that AGEs may play a role in the development of atherosclerosis in diabetic patients (Bierhaus *et al.,* 1998; Jandeleit-Dahm, 2008). Advanced glycation endproducts may accelerate atherosclerosis through several mechanisms, including the cross-linking of proteins, the modification of matrix components and abnormal lipoprotein metabolism (Goh and Cooper, 2008).

1.32 Obesity metabolic syndrome and insulin resistance.

One of the obesity-associated health risks is development of metabolic syndrome. According to the WHO, metabolic syndrome is characterized by the presence of central obesity, dyslipidemia (elevated triglycerides and decreased HDL cholesterol), impaired glucose tolerance or insulin resistance, type 2 diabetes and high blood pressure. Randle proposed that fatty acid (FA) compete with glucose as anenergy substrate in rat heart and diaphragm muscle, and speculated that increased fatty acidoxidation was responsible for the obesity-

induced insulin resistance (Randl *et al* 1965). It has been shown that an increase in the intracellular concentration of FA metabolites (diacylglycerol, fatty acyl CoA) leads to activation of PKC, c-Jun N-terminal kinase (JNK) and inhibitory kappaB kinase (IKK), which ultimately block the insulin signaling pathway through functional inhibition of insulin receptor substrate-1 (IRS-1), (Blaschke F et al 2005). Therefore a person with severe untreated diabetes mellitus suffers rapid weight loss and asthenia (lack of energy) despite eating large amounts of food (polyphagia).It accounts for 80–85 per cent of the overall risk of developing Type2 diabetes and underlies the current global spread of the condition (Hanuer H, 2010). Almost two in every three people in the UK are overweight or obese (62 per cent of women and 66 percent of men).

1.33 Introduction of Diabetic retinopathy

Diabetic retinopathy occurs when diabetic complications lead to destruction of the very small blood vessels in the retina. In earlist stage of diabetic retinopathy, called non-proliferative retinopathy, where blood vessels become leaky and weak. This is followed by proliferative retinopathy, where fragile new blood vessels increase around the retina and in the vitreous humor. Retinal oedema, haemorrhage, anemia, microaneurysms, and neovascularization are characteristics of diabetic retinopathy. The Diabetes Control and Complications Trial (DCCT) determined hyperglycaemia as a significant threat for the progression and development of retinopathy; however, the pathogenic mechanisms of DR are not fully understood. Basement membrane thickening and pericyte loss have been recognised as the histological hallmarks of early DR (Kohner, 1989). Mainly, DR consider as disease of the retinal capillary endothelial cells (ECs) (Kohner, 1993; Archer, 1999). Since the retina is one of the few tissues that do not require insulin to transport glucose into the cell, hyperglycaemia leads to high intracellular glucose levels. In hyperglycaemia, ECs respond to abnormally high blood glucose values, and with rapid normalisation of blood glucose, ECs undergo apoptosis (Li *et al*., 1996; Mizuntani *et al*., 1996; Joussen *et al*., 2001) leading to eventual blood-retinal barrier breakdown (Poulaki *et al*., 2002). The consequences of retinal microvascular cell apoptosis can account for various features of DR (Lorenzi and Gerhardinger 2001). It has been shown that prolonged exposure to

hyperglycaemia leads to progressive dysfunction of the endothelium through a number of potential pathways (Barnett, 1993; Funatsu and Yamashita, 2002). These biochemical mechanisms include sorbitol (polyol) pathway, nonenzymatic protein glycation (advanced glycation end products, AGEs), oxidative stress (generation of reactive oxygen species, free radicals, and impaired antioxidant mechanisms), protein kinase C beta (PKC-β) and the rennin-angiotensin system (RAS) (see figure 1.17) these abnormal pathways work in a network may influence various vasoactive factors and cytokines to mediate functional and structural changes of DR (Candido and Allen, 2002). For instance, AGEs cause increased oxidative stress and leading to cell death. Moreover, retinal ECs are much more disposed to oxidative stress and increased vascular permeability when compared by brain-derived ECs (Grammas and Rideen, 2003). It has being found not only the pathogenesis of DR does not only affect retinal vessels. In addition, other cell types in the retina are affected early by diabetes (Lorenzi and Gerhardinger, 2001).

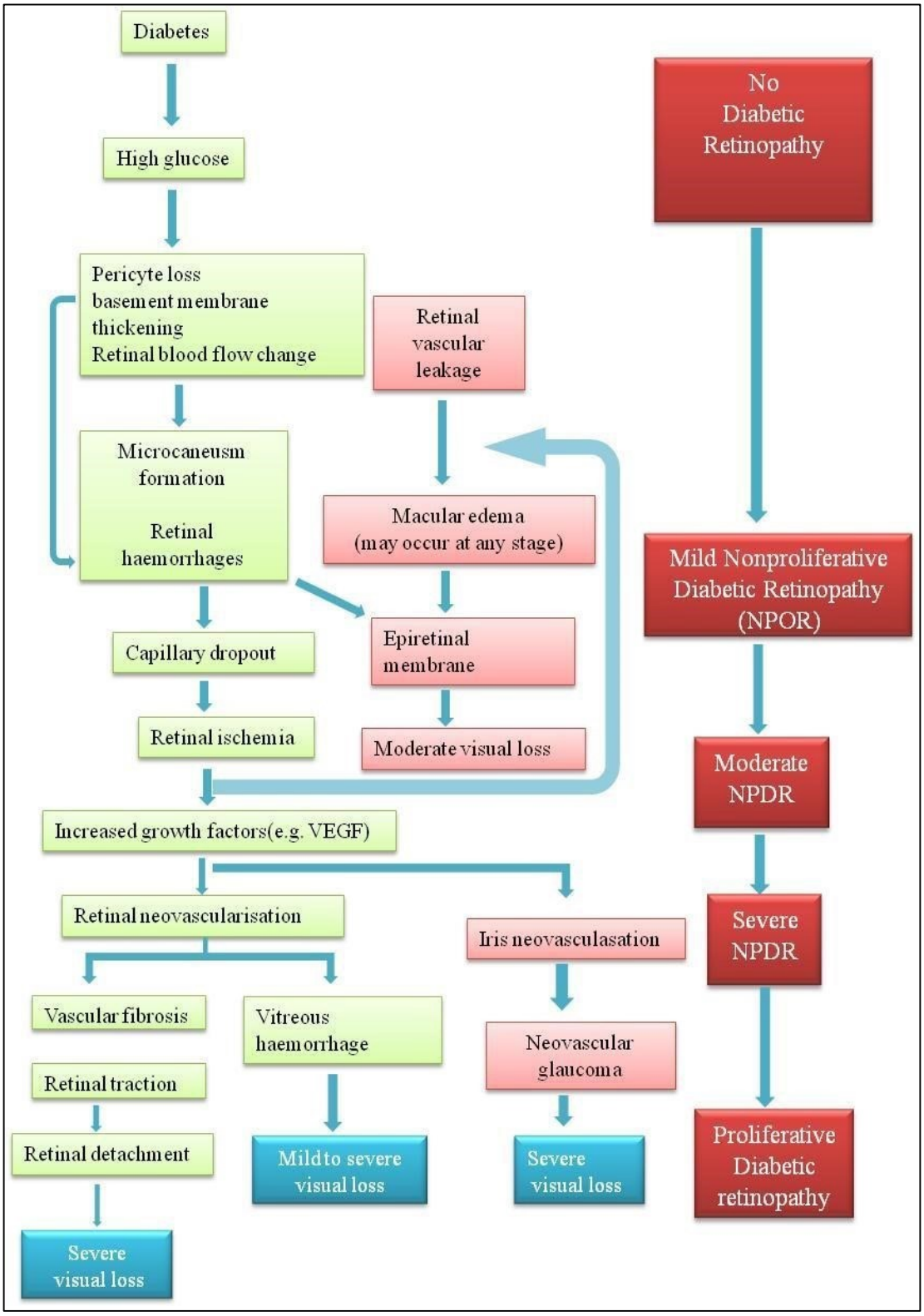


Figure 1 .17 Schematic diagram of the pathogenesis of diabetic retinopathy. NPOR start with microcaneusm formation to retinal ischemia. PDR; sever visual loss due to retinal detachment, and neovascular glaucoma, mild to sever visual loss due to vitreous haemorrhage.

1.34 Epidemiology

International epidemiological studies indicate that over the last 50 years there has been progressive raise in diabetes incidence. Diabetes mellitus has becoming increasingly widespread in recent years. While Diabetes consider as common chronic disease, which consider to be the fifth leading cause of death in 2000. Additionally, it became the 3rd most common killer of mankind, after cancer, and cardiovascular and cerebrovascular diseases (Li, 2004). World Diabetes Day 2013 marks the release of the International Diabetes Federation's 6th edition of the Diabetes Atlas.According to the new studies, the number of people living with diabetes is expected to increase from 382 million in 2013 to 592 million by 2035.The highest number of people with diabetes is from 40 to 59 years of age, in main time 175 million people with diabetes are undiagnosed due to many reason. 80% of people with diabetes belong to low and middle-income countries North America have highest prevalence of diabetes is in where it reached 10.2% of adult population while in Europe the prevalence is 6.9% of population aged 20 to 79 years. Mainly worrying is continuous increase in diabetes incidence of both type 1 as well as type 2 in developmental age population. (Jarosz-Chobot *et al*., 2011; D'Adamo and Caprio, 2011). Since of increase in diabetes, prevalence there is higher number of patients will have microangiopathic complications including DRin children and youth and in adults (Cho *et al*., 2011; Rosenson *et al*., 2011). Chronic complications decrease the quality of life and are a main cause of disability. DR has become a most important reason for blindness and visual impairment in developed countries and is continually increasing (Fong *et al*., 2004). Near all patients with type 1 diabetes will develop some appearance of DR, whereas in type 2 diabetic patients 80% of insulin- dependent patients and 50% of patients not requiring insulin therapy will have DR within 20 to 25 years following disease onset (Lamoureux and Wong, 2011). The prevalence of any retinopathy was 8% at 3 years, 25% at 5 years, 60% at 10 years, and 80% at 15 years. The prevalence of PDR was 0% at 3 years and increased to 25% at 15 years. The prevalence of proliferative diabetic retinopathy (PDR) was 0% at 3 years and increased to 25% at 15 years (National Health and Nutrition Examination Survey, 2006). It is predictably estimated that 20% of all diabetes cases are undiagnosed, so the actual prevalence is likely significantly higher (Philip Hooper *et al*., 2012).

1.35 Eipdemiology of Diabetes in UK.

2.6 million People have been diagnosed with diabetes in the UK (2009). By 2025, there will be more than four million people with diabetes in the UK. In 2008, 145,000 people were diagnosed with diabetes in the UK. Diabetes is the leading cause of blindness in people of working age in the UK. It is estimated that there are 4,200 people in England who are blind due to diabetic retinopathy.This increases by 1,280 each year. (Scanlon, 2008), within 20 years of diagnosis nearly all people with Type 1 and almost two thirds of people with Type 2 diabetes (60 percent) have some degree of retinopathy. (Scanlon, 2008). People with diabetes are twice as likely to suffer from cataracts or glaucoma as the general population. (Petit and Adamec 2002). More than one in ten (11.6 per cent) deaths among 20 to 79-year-olds in England can be attributed to diabetes. If current trends continue, one in eight (12.2 per cent) deaths among 20 to 79-yearolds will be attributable to the condition by 2010. (Yorkshire and Humber Public Health Authority 2008). Life expectancy is reduced, on average, by more than 20 years in people with Type 1 diabetes up to 10 years in people with Type 2 diabetes.

1.35.1 Background Diabetic Retinopathy (BDR)

This condition appears in people who have had diabetes for a long time, two types of diabetic retinopathy, which can damage the sight. Both involve the fine network of blood vessels in the retina.

1.35.2 Proliferative diabetic retinopathy (PDR)

This condition is growth of new vessels from the retina or optic nerve,mainly occurs when many of the blood vessels in the retina become obstructed, stopping blood flow, to repair the damage to the area where the original vessels closed, and the retina responds by growing new blood vessels. This is called neovascularisation. However, these new blood vessels are often very weak and do not supply the retina with sufficient blood flow, growing in the wrong place on the surface of the retina and inside the the vitreous. The new vessels are also often accompanied by scar tissue that could cause the retina to wrinkle or detach. PDR is a major cause of blindness, and its earliest signs include damage to the blood vessels and the formation of lesions in the retina. Automated detection and

grading of hard exudates from the fundus imaging is a critical step in the automated screening system for diabetic retinopathy (Bala and Vijayachitra, 2012). Small bulges, known as microaneurysms, leave behind metabolic waste products known as exudateslead capillaries to leak. The fluid causes the retina to swell (edema) and on top ofthat leave behind metabolic waste products known as exudates. Additionally, if the small retinal vessels block off (capillary closure or capillary drop out) the retina will become oxygen starved (ischemic). When this happens, white patches of oxygen-starved retina (cotton wool spots) appear. In addition, vessels can form scar tissue, which the vitreous can pull. This can cause bleeding into the vitreous and/or cause retinal detachment.

1.36 Differences between the PDR and BDR

Diabetes has an effect on circulatory method in the retina.The intial step of the disease is recognized as backdrop diabetic retinopathy (BDR). In this step, only the arteries inside the retina become weakned and leak, forming small, dot-like haemorrhages, which will lead to retinal detachment and blindness. These kinds of leaky often produce swelling as well as oedema in the retina and decreased vision. The following stage is recognized as proliferative diabetic retinopathy (PDR). In this stage, circulation problems lead to areas of the retina to become oxygen-deprived or ischemic. New, fragile, vessels develop as in (Figure 1.18), formed (proliferative retinopathy). Haemorrhage from these vessels could cause acute loss of sight, which may obvious.

Organisation from the haemorrhage leads to fibrosis within the retina, (http://www.stlukeseye.com/Conditions/DiabeticRetinopathy.html).

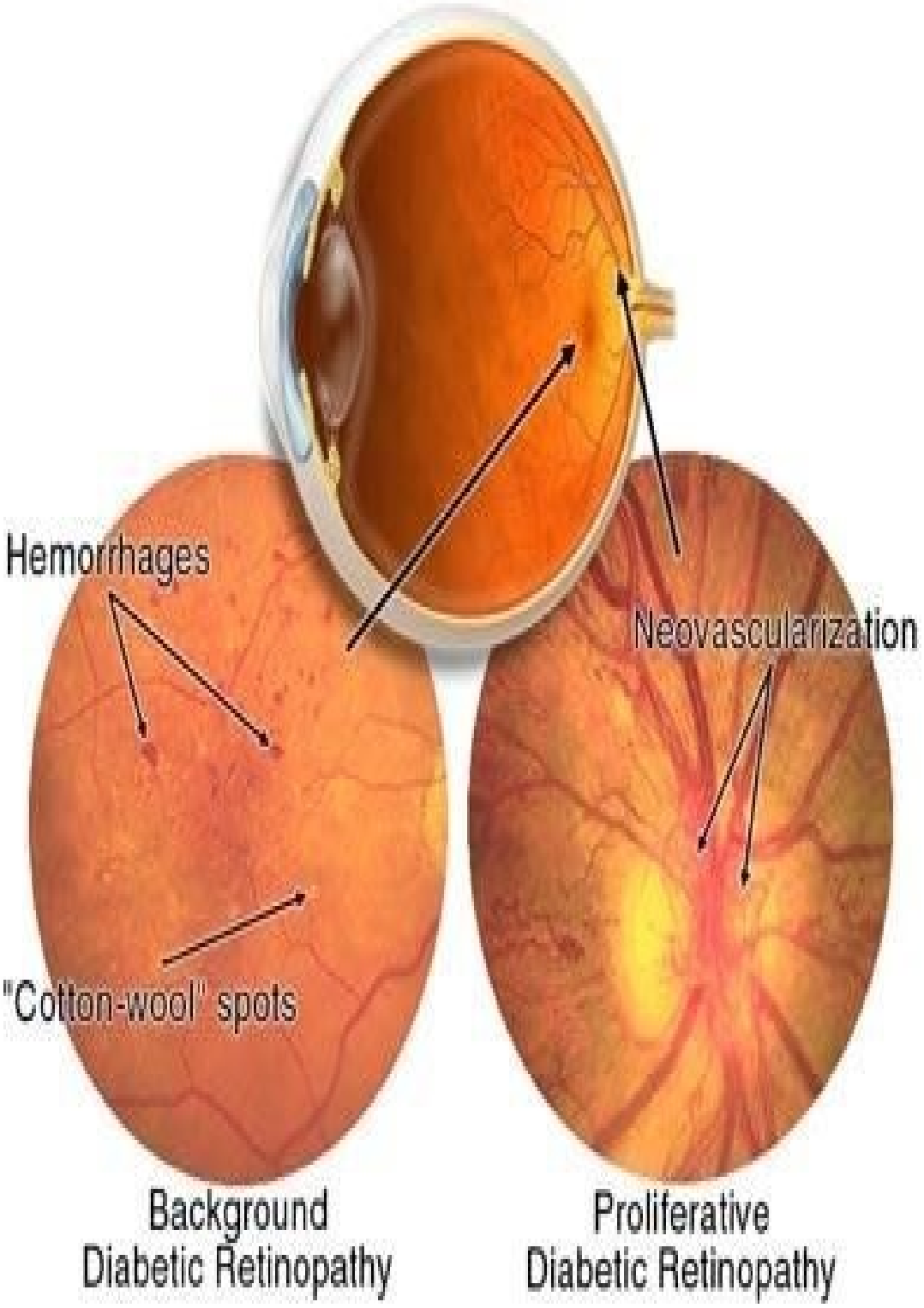


Figure 1 -18 Diabetic retinopathy: New thin walled and fragile blood vessels are formed .Organisation of the haemorrhage causes fibrosis within the retina. (http://www.stlukeseye.com/Conditions/DiabeticRetinopathy.html).

Circulatory system attempts to maintain appropriate oxygen amounts nside the retina. This is called neovascularization. Unluckily, these types of new vessels are delicate, and haemorrhage easly. Blood may possibly leak in to the the retina and vitreous, producing spots or floaters, along with decreased visionIn the advanced phases of the disease, continued abnormal vessel growth and scar tissue formation may cause severe problems include ingretinal detachment and glaucoma.

1.37 Pathophysiology of Diabetic Retinopathy

The mechanism of DR is still unclear, but some studies suggest that chronic hyperglycemia is a major cause of damage to multiple organs. Complications of chronic hyperglycemia will lead to inadequate perfusion due to vascular organ tissue damage, including damage to the retina itself. There are four biochemical processes that occur in chronic hyperglycemia and are related to the onset of diabetic retinopathy, among others.

1.37.1 Accumulation of Sorbitol

Excessive production and accumulation of sorbitol because of the activation of the polyol pathway occurs due to increased activity of the enzyme aldose reductase that is present in nerve tissue, retina, lens, glomerulus, and the walls of blood vessels. Sorbitol is a sugar compound and alcohol, which cannot pass through the basal membrane and will accumulate in large amounts in the cell. Cell damage caused by the accumulation of sorbitol, which is hydrophilic, occurs when the cells become swollen due to osmotic process. In addition, sorbitol also increases the ratio of NADH / NAD+ thereby reducing the uptake mioinositol. Mioinositol serves as a precursor for the synthesis of phosphatidylinositol modulation of the enzyme Na-K-ATPase that regulates nerve conduction. In short, the accumulation of sorbitol can cause disruption of nerve conduction. Experiments on animals have shown the enzyme aldose reductase inhibitor (sorbinil) that inhibits the formation of sorbitol, can reduce or slow the onset of diabetic retinopatic. However, clinical trials in humans have not shown a slow down of progressive retinopathy (Susan, 1993; Nagoya, 1997).

1.37.2 Formation of protein kinase C (PKC)

There are currently at least 13 known isoenzymes of the PKC family are expressed preferentially in different tissues of the body. The vascular, retinal and renal tissues express PKC- I and PKC- II more so than other isoenzymes (Nagpala *et al*., 1996). Under conditions connected with hyperglycaemia, PKC activity in the retina and the vascular endothelial cells is elevated because of increased *de novo* synthesis of diacylglycerol that is a regulator of glucose cyclic citrullinated peptide (CCP). PKC could have an influence on platelet aggregation, vascular permeability, synthesis of growth factors and vasoconstriction.Increase in CCP improves diabetic complications, simply by interfering with vascular permeability and retinal the circulation of blood flow (Helen and Christopher 2007). Increased vascular permeability will cause plasma extravasation, therefore increasing intravascular blood viscosity, and is accompanied with an increase in platelet aggregation causing thrombosis. Moreover, the growth factors formation will produce an increase in vascular smooth muscle cell proliferation and extracellular matrix including fibrous tissue; subsequently, there will be thickening of the vascular wall combined with the activation of endothelin-1, which is a vasoconstrictor that narrows vascular lumen. These effects combined will eventually cause retinal vascular occlusion (Lee *et al*., 1989; Daria *et al.,* 2012).

1.37.3 Formation of Advanced Glycation End Products (AGE)

Glucose binds to the amino group in a non-enzymatic manner to form a covalent bond. The process will ultimately result in AGE compounds. The effects of AGE are mutually synergistic with the effects of PKC, causing increased vascular permeability, synthesis of growth factors and endothelin activation whilst, inhibiting the activation of nitric oxide by endothelial cells. The process will increase the risk of retinal vascular occlusion (Sho-ichi and Takanori, 2009). AGE contained inside and outside cells, correlated with glucose levels. AGE accumulation that precedes cell damage. The level of AGEs is 10-45X higher in DM than non-DM patients in 5-20 weeks. In patients with diabetes, the glucose rise induced AGE accumulation is substantial, and this accumulation is faster in the intracellular than extracellular compartment. (Sho-ichi and Takanori, 2009)

1.37.4 Formation of Reactive Oxygen Species

Reactive Oxygen Species (ROS) is a phrase used to express a number of reactive molecules and free radicals derived from molecular oxygen. ROS formed from oxygen to the m et al ion catalyst or enzyme that produces hydrogen peroxide (H2O2), superoxide (O2-). Recent evidence has shown that ROS play a key role as a messenger in normal cell signal transduction and cell cycling. In case of Autooksidasi increased ROS generation through glucose on polyol pathway and degradation of AGE. ROS accumulation in tissues would lead to oxidative stress, which adds to cell damage (Hancock *et al*., 2001)

1.38 AGE-driven angiogenesis *in vitro*

Yamagishi *et al*. (1997) studied AGE-driven angiogenesis in vitro, and tested BSA- AGE on the growth and tube-formation of human microvascular endothelial cells. Moreover, Kim *et al*. (2008) suggested that AGE-induced proliferation of cells via binding of RAGE-AGE occurs through signalling pathways. During treatment of cells with AGE, cell proliferation was significantly increased compared to control cells *in vitro*, as shown in figure 1.19 (Yamagishi *et al*., 1997; Kim *et al*., 2008).

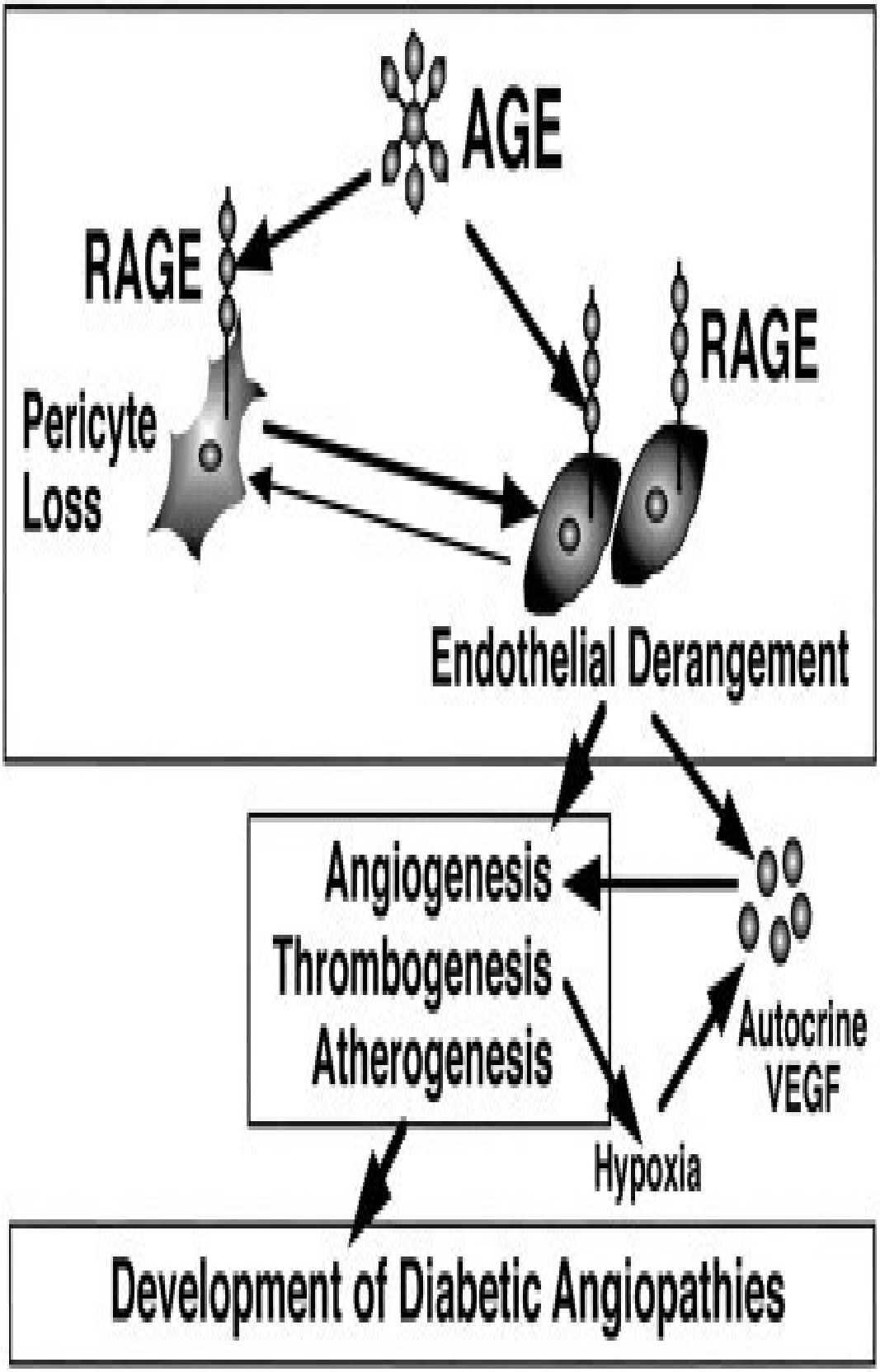


Figure 1 .19 shows the probable mechanism of the development of diabetic microangiopathy. (Yamagishi *et al*., 1997)

1.39Pharmacologic Management of DR

Although effective in stopping the progression of DR, a procedure for retinal photocoagulation surgery does not destroy tissue and improve vision.

A. Corticosteroids: These agents possess anti-inflammatory properties and the ability to down regulate VEGF, thereby reducing leakage from damaged retinal capillaries. Safety studies are ongoing for this class of drugs.

B. VEGF Inhibitors: VEGF is a suitable therapeutic target because it is upregulated in DR and contributes to neovascularization and capillary leakage in severe DR. Because VEGF inhibitors are administered intravitreally, adverse systemic effects are minimized (Simó and Hernández, 2009). **C. Renin-Angiotensin System (RAS)**:

Inhibitors**:** Angiotensin-converting enzyme (ACE) inhibitors and angiotensin II receptor blockers (ARBs) reduce DR and VEGF levels in PDR (Leal *et al*., 2005).

D. Antiplatelet Drugs:

Platelet aggregation contributes to capillary occlusion and microaneurysm formation in DR. The antiplatelet drug ticlopidine has an off-label indication to delay the rate of microaneurysm formation in NPDR (Knight, 2003). **E. Protein Kinase C (PKC) Inhibitors:**

The beta isoform of PKC modulates and up regulates the expression of VEGF, which, in turn, contributes to microvascular DR complications (Shen, 2003).

F. Advance Glycation End-Product (AGE) Inhibitors:

Accumulation of AGEs in the diabetic retina appears to contribute to the endorgan damage detected in DR. Pimagedine, also known asamino guanidine, is an oral AGE inhibitor under investigation for its ability to prevent end-organ complications. Adverse reactions and oral dosing have not been fully elucidated (Brownlee *et al*., 1986; Vasan *et al*., 2003).

1.40 Diabetic retinopathy treatment

The best treatment for diabetic retinopathy is to prevent it, during the first three stages of diabetic retinopathy, no treatment is needed and before macular oedema; people with diabetes should control their levels of blood sugar, blood pressure, and blood cholesterol. Treatment usually will not cure diabetic retinopathy nor does it usually bring back normal vision, but it could slow the progression of vision loss. Without treatment, diabetic retinopathy progresses steadily from minimal to severe stages.

1.40.1 Laser surgery

The laser is traveling throughout the clear cornea, lens and vitreous without affecting them at all. Laser surgery minimizes abnormal new vessels and decreases macular swelling. Treatment is usually recommended for those who have macular edema, proliferative diabetic retinopathy (PDR) and neovascular glaucoma. However, it is nessary to understand the Laser surgery does not cure diabetic retinopathy and does not always prevent further loss of vision.

1.40.2 Medication injection

In many circumstances, medication can often help care for diabetic retinopathy.Occasionally a steroid medication can be used. In other cases antiVEGF medication used. This substance contributes to abnormal blood vessel growth in the eye. An anti-VEGF drug can help decrease the growth of these abnormal blood vessels. The medication reduces the swelling, leakage, and growth of unwanted blood vessel growth in the retina, and may improve the eyesight. Medication treatments may be given once or as a series of injections at regular intervals, usually around every four to six weeks.

1.40.3 Vitrectomy surgery

Vitrectomy is a surgery where in operating microscopes and smaller precise equipment areused to remove blood, and scar tissue that accompany abnormal vessels in the eye. By removing, the vitreous haemorrhage the obstacle will eliminate and allows light rays to focus on the retina again.Vitrectomy usually prevents further vitreous haemorrhage by removing the abnormal vessels that

caused the bleeding. Elimination of the scar tissue allows the retina go back to its normal location.

1.41Small leucine-rich proteoglycans (SLRP)

SLRPs are comprised of a small leucine-rich protein core approximately 40 KDa, consist of 17 members of secreted proteins divided in five classes based on their structural composition and chromosomal location (Figure 1.20).(Iozzo, 1999; Henry *et al.,* 2001; Dellett *et al*., 2012). The core proteins contain a number of leucine-rich repeats (LRRS), which consist of the consensus sequence LXXLXLXXNXL, where X is any amino acid, L is leucine or valine, and N is asparagines, cysteine or threonine. The N-terminal domain contains the negatively charged and covalently attached glycosaminoglycan (GAG) chains, and the central domain of the protein comprises approximately 80 % of the molecule, composed of tandem repeats of leucine-rich regions. The first class includes decorin (Krusius and Ruoslahti, 1986). The second class includes lumican (Blochberger *et al*., 1992), fibromodulin (Oldberg *et al*., 1989), keratocan (Corpuz *et al*., 1996), praline, arginine-rich end leucine-rich repeat protein (PRELP) (Bengtsson *et al*., 1995), and osteoadherin/osteomodulin (Sommarin *et al.,* 1998). The third class within the SLRP family comprises epiphycan/proteoglycan-Lb (shinomura and Kimata, 1992), mimican/osteoglycin (Madisen *et al*., 1990) and opticin/oculoglycan (Reardon *et al*., 2000; Friedman *et al.* 2000).

The fourth class includes Chondroadherin, Nyctalopin and Tsukuhi (Ohta *et al*., 2004), and fifith class includes Podocan (Ross *et al*., 2003), and Podocan-like 1 Protein (Mochida *et al*., 2011). More recently, as a function of their various proteins cores and glycosaminoglycans side chains, SLRPs have ability to bind to various cell surface receptors, growth factors, cytokines and other ECM components resulting in the ability to influence various cellular function (Dellett *et al.,* 2012).

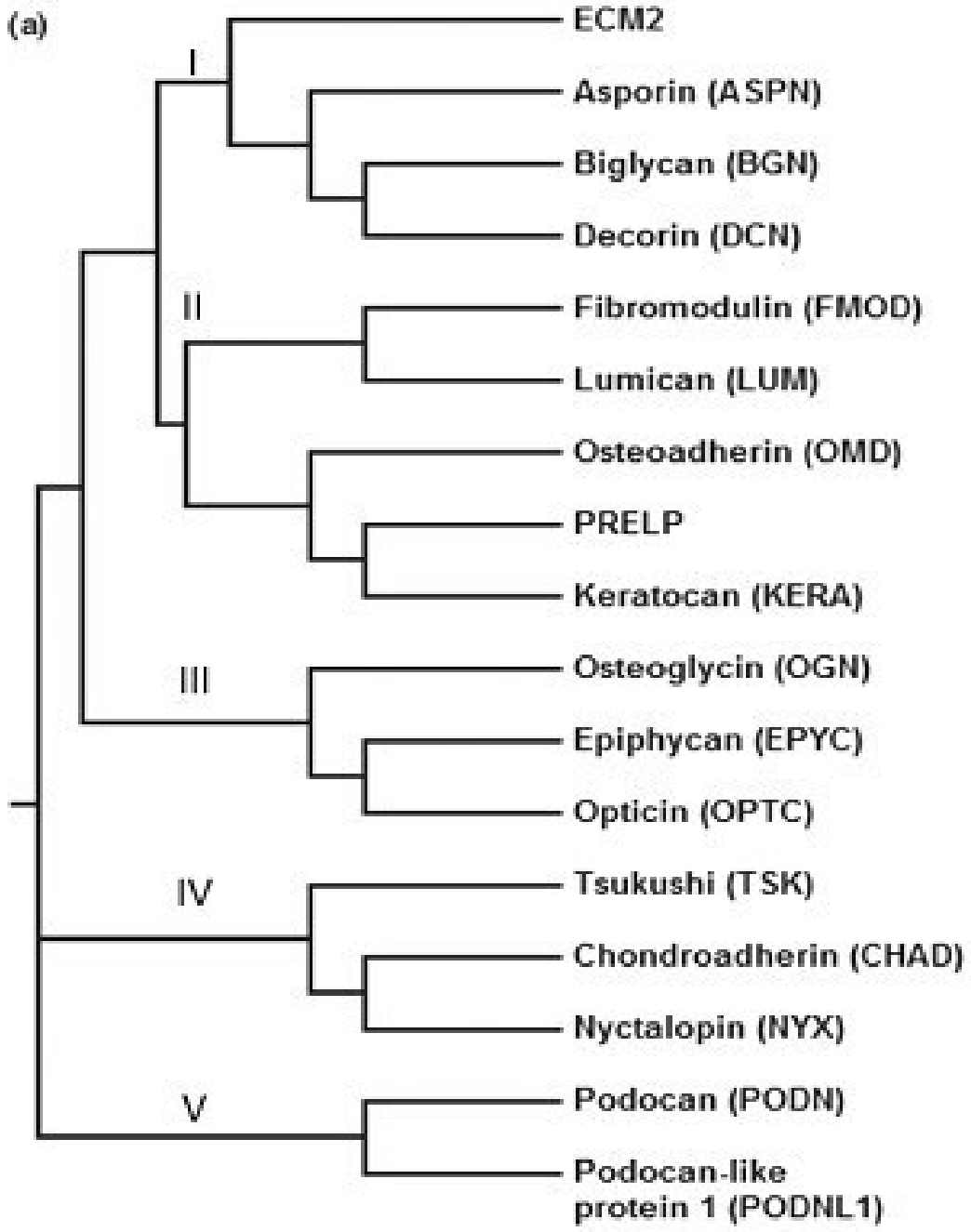


Figure 1 .20 Dendrogram representing the evolutionary relationship between the members of the SLRP family (Dellett *et al.*, 2012)

Horizontal distances are proportional to the evolutionary distance and based on human protein sequences. This family is mainly subdivided into three main classes (Classes I, II, III, IV and V) based upon the genomic organisation, the number of LRRs and the spacing of the four cysteine cluster.

1.42 Opticin

Reardon and colleagues discovered opticin in an eye-specific molecule (Reardon *et al.*, 2000, Pattwella *et al*., 2010) by using a 4 M guanidine hydrochloride extract from bovine vitreous collagen fibrils to prepare peptides as a starting point for molecular cloning. Another group of researchers discovered an iris specific molecule, which they termed oculoglycan (Friedman *et al*., 2002), and was later on found to be identical with opticin. Opticin expression is believed to be mostly specific to the eye since human opticin sequence can be found in only eye specific EST libraries (Hobby *et al*., 2000; Wistow *et al*., 2002). Using *in-situ* hybridisation and immunohistochemical studies they showed opticin was especially immunolocalised to the vitreous humor where labelling was most intense in the basal and cortical vitreous gel and less intense in the central vitreous. The findings suggested that opticin is secreted by the non-pigmented ciliary epithelium into the vitreous cavity where it associates with vitreous collagen and adjacent basement membranes (Ramesh *et al*., 2004).Furthermore, opticin was found in various ocular tissues, mainly the ciliary body, the iris, and the anterior vitreous close to the pars plana.

Fovola *et al*., isolated a chicken homologue of opticin (cOptc), using in situ hybridisation, cOptc was found to be expressed in the brain and neural tube from developmental stage 9 onwards. At later stages, cOptc expression was found strongly in the non-pigmented ciliary epithelium. Expression of cOptc was analysed from stages 4–26 with opticin mRNA been detected in not only the ciliary epithelium but optic stalk, neural tube, brain including Rathke's pouch, pharyngeal pouches, nasal pit and optic vesicle (Fovola *et al*., 2004).

Opticin is a proteoglycan of the small leucine rich repeat (LRR) family located in the ECM. The sequence of opticin and glycation target amino acids is shown in Figure1.21.

Figure 1.21 The amino acid sequence of opticin chain,taken from the Q9UBM4 entry in Swiss-Prot/TrEMBL protein database. It consists of 332 amino acid residues.48 leucine amino acids (L), and 13 valine amino acids (V).Glycated target amino acids are26 arginine amino acids (R), 8 lysine amino acids (K), and 6 cysteine(C),(Adapted from;Swiss-Prot/TrEMBL protein database).

Opticin has been shown bind to glycosaminoglycans (GAGs) including heparin and chondroitin sulphates (Hindson *et al.,* 2005), suggesting a role in stabilising vitreous structure and maintaining vitreoretinal adhesion. Bishop first suggested a function in maintaining gel stability and structure (Bishop, 2000). The vitreous is composed mainly of a type-II collagen scaffold full with a matrix of hyaluronan. Other non-collagenous proteoglycans and proteins for instance versican and fibulin could be involved in stabilisation of this delicate structural network (Bishop, 2000). It is suggested that opticin is bound to the surface of chondroitin sulphate proteoglycan type-IX collagen and is covalently linked to their surface. Chondroitin sulphate chains of the type-IX collagen on one fibril could link to opticin on the surface of adjacent fibrils explaining the ladder like conformations of chondroitin sulphate GAG chains seen bridging adjacent fibrils observed in ultra-structural studies of the vitreous using cationic dyes like Cupromeronic Blue (Le Goff and Bishop, 2006).

Opticin is the only member of the LRR family of proteins present in the vitreous binds to heterotypic vitreous collagen fibrils. One of the proposed functions of

this family of proteins is the prevention of lateral association, or aggregation, of collagen fibrils, and its abundance in the vitreous may be important to the determination of appropriate short-range spacing of the thin collagen fibrils of the vitreous essential to permit light transmission. In this sense then, these proteins are regulators of supramolecular organisation of tissues and include other proteins such as decorin and lumican. These have importance to spacing of other critical collagenous matrices for example the corneal stroma (lumican knockout mice have opaque corneas) and skin matrix.

Coating of vitreous collagen fibrils with molecules for instance opticin and typeIX collagen through its chondroitin sulphate chains could thus have a dual purpose: first allow structural integrity of the vitreous give link to form a contiguous collagen network; second, these molecules also might avoid aggregation of the vitreous fibrils, which would destabilise the gel. During ageing or disease, mainly after cellular infiltration of the gel, these molecules are likely to be damaged or degraded and thus lead to collagen fibril aggregation and gel condensation, clinically known as vitreous syneresis. The vitreous is normally anti-angiogenic, but becomes pro-angiogenic in many diseases for instance proliferative diabetic retinopathy, however the vitreous maybe has multiple mechanisms for avoiding angiogenesis because they are essential for vision and hence survival. Opticin continues to secrete into the vitreous cavity throughout life, therefore unlikely to be purely structural. Another role for opticin has been suggested, namely, as a repository for growth factors (Sanders *et al*., 2003). Binding of growth factors by matrix molecules is well recognised. For example, vitreous type-II collagen binds TGF-β and BMP-2 (Zhu *et al*., 1999; Fukui *et al*., 2003). Fibroblast growth factor among many other factors is stored extracellularly in basement membranes bounce to a heparan sulphate proteoglycan (syndecan) (Li *et al.,* 2003). Opticin also appears to bind growth hormone (Sanders *et al.,* 2003). Growth hormone has been implicated in new vessel growth either directly or through its mediator insulin-like growth factor 1 (IGF-1), mainly during development, and matrix molecules for instance opticin may have a role in ensuring an enough supply of growth hormone for ocular vasculogenesis. This could beapplying to the retina and other ocular tissues since opticin appears to be widely distributed in the eye. Thus, it may have more

functions besides promoting the development of the hyaloid vascular system during embryogenesis.

1.43 Methylglyoxal

1.43.1 Functionality involving methyalglyoxal

Methylglyoxal (MG) research started late in the 19th century with the research focus being synthesis, and understanding the reactions of MG (Kalapos, 1999). MG, often known as pyruvaldehyde, is remarkably reactive aldehyde compound with carbonyl groups and occurs as a yellowish fluid with a strong odour.

Methylglyoxal production has been discovered in all mammalian cells and it is essentially generate from glucose metabolism through the glycolytic pathway (Phillips and Thornalley, 1993). In this pathway, the usual of MG is thought to be formed by the non-enzymatic and / or enzymatic elimination of phosphate from dihydroxyacetone phosphate (DHAP) and glyceraldehyde 3-phosphate (G-3-P)

1.43.2 MG and AGEs

AGEs, a group of brown colored compounds with intra- or inter-molecular crosslinking, are formed by the Maillard reaction of aldehydes and ketones with amino groups of proteins (Singh *et al*., 2001). The formation of AGEs is age-correlated (Li *et al.,* 1996) and they are found to accumulate slowly in vascular and renal tissues. However, the accumulation of AGEs is accelerated in conditions such as complications of diabetes (Brownlee *et al*., 1988). As the most active AGE precursor, MG reacts with residues of different proteins including arginine, lysine and cysteine and therefore forms different AGEs, such as argpyrimidine, (Dhar *et al*., 2008). Many studies, both *in vivo* and *in vitro* have proved that increased MG leads to an increase in AGEs formation. In cultured VSMCs, CEL and CML production detected using immunohistochemistry increased significantly after treatment with MG for 3 hours (Dhar *et al*., 2008). In the spontaneously hypertensive rat (SHR) kidney, immunoreactivity to CEL and CML increased at 8, 13 and 20 weeks groups compared to age-matched Wistar-Kyoto (WKY) rats, but not in the 5 week group, following the same pattern as MG levels in the plasma (Wang *et al*., 2004; Wang *et al.,* 2005).

Medical information indicates that plasma MG concentrations are 5-6 folds higher in patients with Type 1 diabetes mellitus and 2-3 fold in those with type 2 diabetes mellitus (McLellan *et al*., 1994).

1.44 Protein glycation

1.44.1 Chemistry of protein glycation

Nonenzymatic glycation, in which reducing sugars are covalently attached to free amino groups and ultimately form AGEs, has been found to occur during the normal aging and at accelerated rate in diabetes mellitus. They preferentially occur on lysine and arginine amino acids. Oxidation, accompanying glycation *in vivo*, further supports chemical modifications. AGE formation and protein crosslinking are irremediable processes that modify the structural and functional properties of proteins, lipid components and nucleic acids. Moreover: A general pattern of the chemistry involved in the glycation reaction is outlined in Figure 1.22. Protein glycation divided into three different steps:

1. Early stage, Protein glycation: This ends in Amadori products.

2. Intermediate stage, after the occurrence of Amadori products. Over a period of time, this rearrangement results in more reactive products, such as dicarbonyl compounds including glyoxal, MOG and deoxyglucosones.

3. An advanced stage, which ends with the formation of heterogeneous compounds known advanced glycation end products or (AGEs).

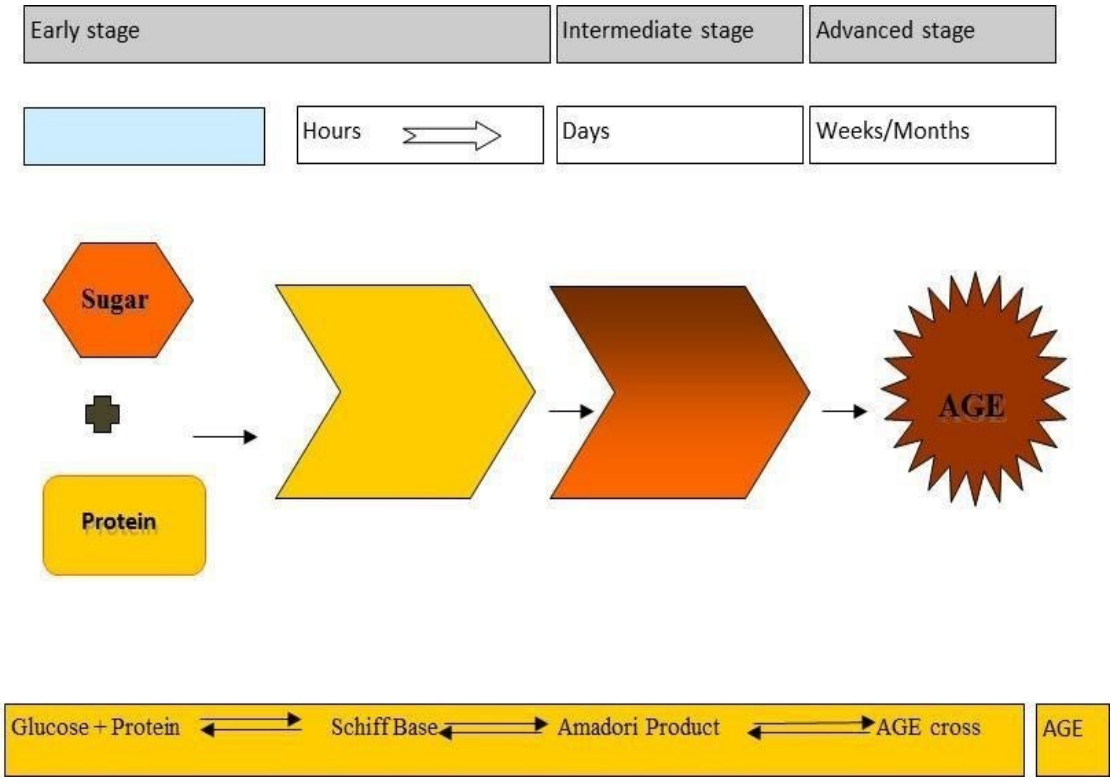


Figure 1.22 Formation of advanced glycation products
(Harding and Ganea, 2006)

1.44.2 Advanced Glycation end products

They interact with nearby proteins to produce pathological cross-linkages that harden the tissue. According to the glycation hypothesis, accumulation of AGEs alters the structural properties of tissue protein and reduces their receptiveness to metabolism (Peppa and Vlassara 2005).

1.44.3 AGEs groups:

a. Fluorescent cross-linkingAGEs.

b. Non-Fluorescent cross-linking AGEs.

c. Non-Cross-linking AGEs.

Metabolic intermediates and intracellular reducing sugars other than glucose are largely responsible for the formation of AGEs. One of the most reactive metabolic intermediates is MOG. Alternatively, AGEs can enter the body

exogenously through diet and smoking. One of most common AGEs found in tissues is Necarboxymethyllysine (CML). Another pathway of protein modification by glucose involves the auto oxidation of glucose or the Schiff base to form low weight carbonyl compounds such as glyoxal (GO), MOG, and glycolaldehyde (GLA), which can modify lysine as well as arginine residues.

1.44.4 RAGE (receptor for advanced glycation end products)

RAGE was first identified as a cell surface receptor for the products of nonenzymatic glycation and oxidation of proteins, the advanced glycation products (AGEs). Several AGE receptors have been characterized on the surface membranes of monocytes, macrophage, endothelial, neuronal, mesangial and hepatic cells including macrophage scavenger receptor type-I and type-II, oligosaccharyl transferase-48 (AGE-R1), 80 K-H phosphoprotein (AGE-R2) and Galectin-3 (AGE-R3) and RAGE, a member of the immunoglobulin superfamily which has been found to have a wide distribution in tissues(Li *et al.,* 1996). The RAGE gene was first cloned and characterized from mouse lung in 1992.The principal means through which AGEs influence cellular actions is by binding to specific receptors. (Christiane *et al.,* 2014).

Moreover, RAGE is a transmembrane receptor comprising 394 amino acids with a single hydrophobic transmembrane domain of 19 amino acids, and a short cytoplasmic tail of 43 amino acids, which is essential for intracellular signalling as figure1.23.

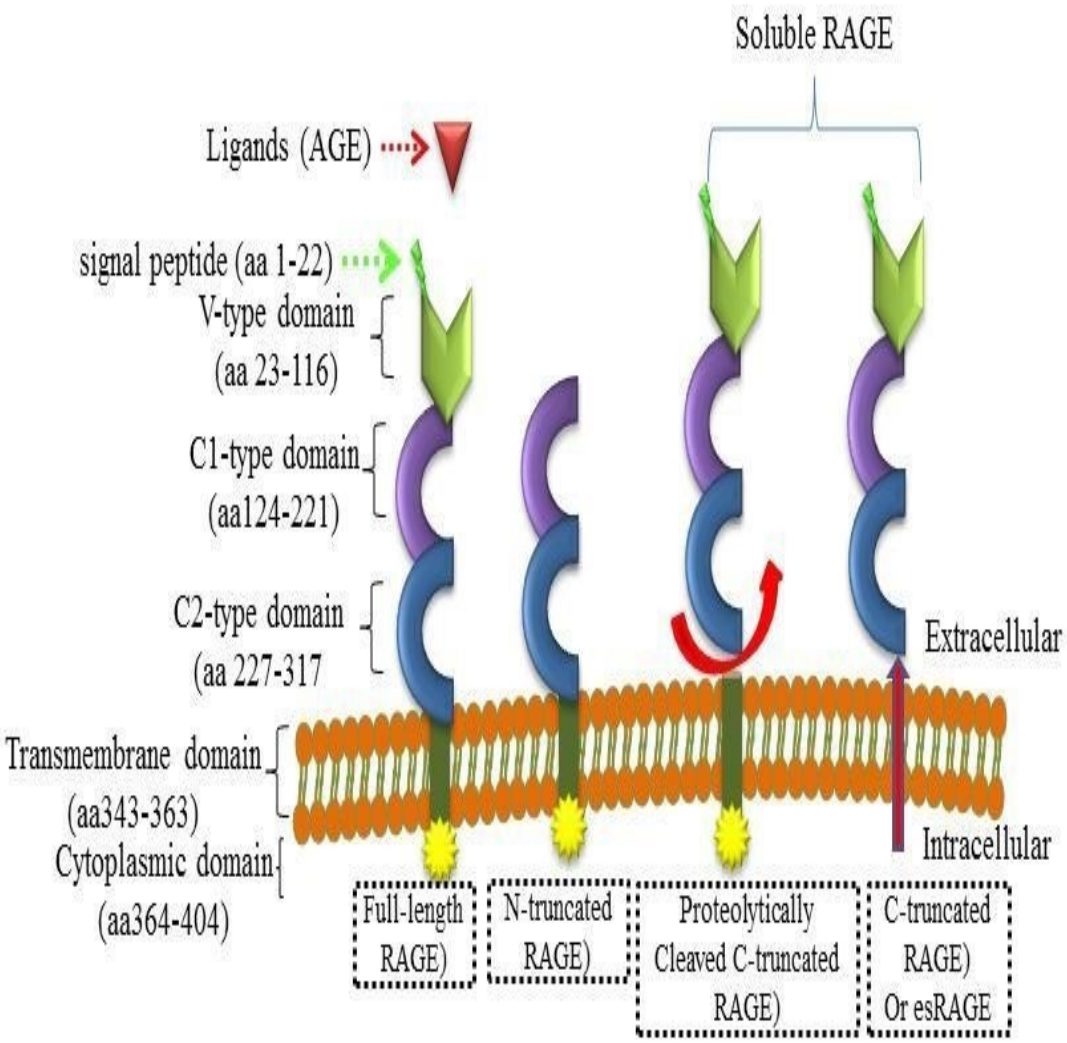


Figure 1 .23 Schematic representation of RAGE.

Schematic representation of RAGE and the generation of some of its isoforms. RAGE is composed of an intracellular tail, a transmembrane domain, and an extracellular domain consisting of three immunoglobulin-like domains, one Vtype followed by two C-type (C1 and C2) domains. The V-type domain is essential for ligand binding, and deletion of this domain results in an N-truncated form. The C-truncated, circulating soluble RAGE corresponds to the extracellular domain of RAGE lacking the intracellular tail and transmembrane domains. It may derive via proteolytic cleavage of full-length RAGE from the cell surface (cRAGE) or via alternative splicing of RAGE mRNA (esRAGE). C: constant; V: variable. (Yaw Kuang *et al*., 2013).

1.45 Sources of AGEs

1.45.1 Exogenous sources of AGEs

The formation of AGEs is usually endogenous, but can be derivative from exogenous sources (Figure 1.24), such as tobacco smoke and food (Nicoll ID, 1998). Numerous studies propose that introducing exogenous AGEs into the biological systems may have significant impacts in terms of the progression of disease processes. Although it is well recognised that cigarettes can lead to vascular disease, Tobecco derived AGE have been observed in the lens crystallin and conary artery walls (Nicoll, 1998), what role exogenous AGE have in contributing to this process, especially in diabetes, is not known. Heat treatment of food results in non-enzymatic browning; it has been proposed that the accumulation of food derived AGE in cause glomerus damage. (Ross, 1993). Evidence has accumulated that dietary AGEs are partially absorbed (Forster *et al.,* 2005) and either retained in the body or excreted in the urine (Erbersdobler and Faist, 2001). These dietary AGEs represent an important source for circulating AGEs under *in vivo* conditions (Vlassara, 2002; Uribarri *et al*., 2003).

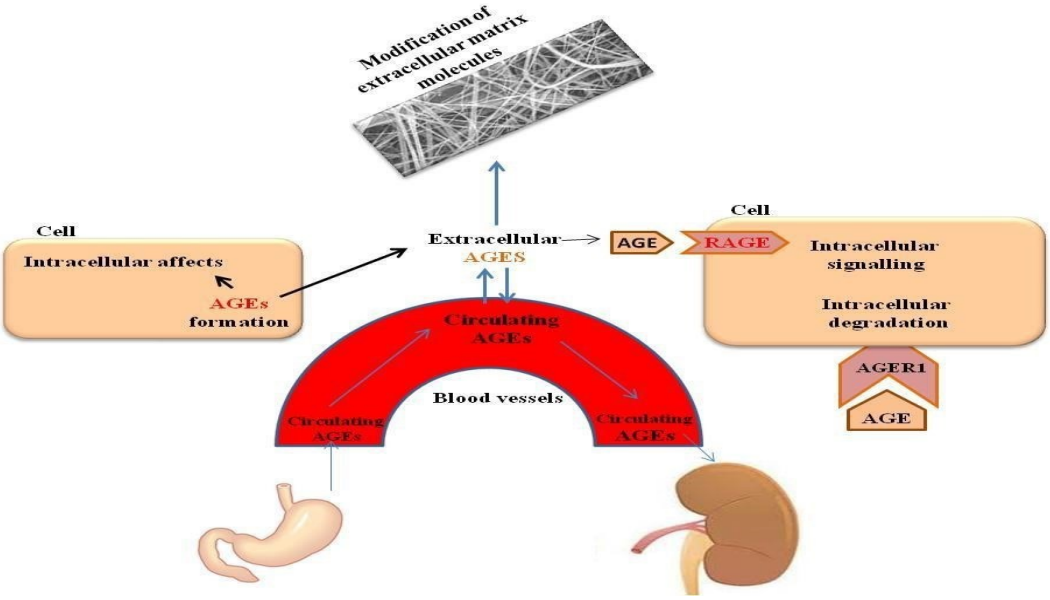


Figure 1 .24 The cycle of endogenous and exogenous AGEs. Adapted from (Ali *et al.*, 2014).

1.45.2 AGEs inhibitors

Attempts have been made to pharmacologically manipulate the process of nonenzymatic glycation in order to prevent or slow down the formation of glycation products. AGE inhibitors can be classified into six categories or types. Pyridoxamine (PM) is an in vitro inhibitor of the formation of AGEs by blocking the reaction at the Amadori intermediate. The exact mechanism of how PM functions as a post-Amadori inhibitor is unknown. However, it has been determined that PM scavenges reactive carbonyl products of glucose degradation along with interfering with the catalytic role of redox metal ions in glycoxidative reactions. Guanidine compounds have the capacity to trap α, β-dicarbonyl compounds, which can subsequently stop reactions from occurring with protein amino groups. Noteworthy, a guanidine compound was presented as being a nucleophilic hydrazine compound concerned with entrapping those carbonyls created during the process of the glycation reaction particularly Amadori products; thereby hindering the formation of AGEs.

1.45.3 RAGE blockers

Moreover, the inhibition of glycation reactions, another approach to reduce AGEmediated damage may be adopted by preventing cellular actions of AGEs. These compounds exert their actions via several different mechanisms, such as blocking the interaction between AGEs and RAGE, or inactivating either AGEs or RAGE and thereby preventing their interaction, or by the inhibition of the intracellular signalling transduction mediated by the AGEs-RAGE interaction. The prototype agent for the trapping of AGE ligands is soluble RAGE (sRAGE), which is the extracellular ligand-binding domain of RAGE. The use of sRAGE to interfere with the binding of RAGE in diabetic animals has been reported to prevent several effects of hyperglycaemia, such as diabetic nephropathy and macrovascular injury. It is also known to restore effective wound-healing and decreased levels of cytokines, such as TNF-α, IL-6 and of metalloproteinases. Recently, a novel splice variant coding for a soluble RAGE protein, called endogenous secretory RAGE (esRAGE) (Shigeru *et al.,* 2003), has been identified in human vascular cells. It neutralises AGEs and exerts protection against AGEinduced endothelial cell injury.

1.45.4 Measurement of glycation reaction products

Determination of protein modification has always been a challenge for biochemists. Due to the AGEs are found as a mixture of different structures, their measurement has always been difficult to achieve, the absence of internal standards leaves assays open to error, which requires a high degree of accuracy and reproducibility of each sample. So far, a measure of AGEs measurement is limited to laboratory investigation, and there is no commonly accepted method for estimating AGE, or any commercially available kit.

Several tests have been employed both qualitatively and quantitatively to determine the level of active AGE. Predictably, AGEs have specific fluorescence characteristics and the detection of AGEs by fluorescence spectroscopy one of the main methods of measuring *in vitro* and *in vivo* are used.

Assay methods of separation such as SDS-PAGE, were also used during the search of glycation. In addition, immunochemical methods, using mainly ELISA monoclonal and polyclonal antibodies specific for defined age structures, enable highly sensitive and quantitative measures of AGE. Recently, mass spectrometric techniques have been widely introduced in the search for glycation. It was reported that the use of matrix-assisted laser desorption ionization-time mapping of flight mass spectrometry of peptides (MALDI-TOF-MS) is a valuable tool for the determination of specific protein glycation products, the both *in vitro* and *in vivo.*

Moreover, the measurement of the reaction products of glycation can be divided in to three different samples and experiment all largely biological, and includes the measurement of early glycation products, the measurement of intermediate glycation products, and measurement advanced glycation products.

1.45.4.1 Measurement of early glycation products

The measurement of early-stage glycation products (Amadori products) is generally used to evaluate metabolic controls in diabetic patients. The two parameters regularly used are glycated haemoglobin (HbA1c) and glycated serum proteins.

1.45.4.2 Measurement of intermediate glycation products

Advanced glycation endproduct accumulation is enhanced in diabetes, with concomitant increases in the concentration of dicarbonyl compounds, such as glyoxal, MOG and 3-deoxyglucosone. Estimation of dicarbonyl compound levels is one of interest in terms of evaluating oxidation processes occurring in glycated proteins. Such compounds may be quantitatively determined using mass spectrometric techniques of diabetic patients.

1.45.4.3 Measurement of advanced glycation products

Advanced glycation endproducts were initially measured by spectroscopic and fluorimetric methods. However, these methods are not very specific, and can only provide mere indications on the general trend of the glycation process. More recently, however, various investigators have developed ELISA methods using poly-and monoclonal antibodies. The limitation of this current methodology is, however, that there is no absolute AGE standard for quantitative analysis. Advanced glycation endproducts are currently being identified as biomarkers for specific disease processes. Amongst those found *in vivo*, are CML and pentosidine.

1.45.5 Methylglyoxal reacts with proteins to form AGEs

MOG is an α-dicarbonyl compound that reacts with lysine, arginine and histidine residues in proteins to form AGEs, such as hydroimidazolone, argpyrimidine and MOG lysine dimer (MOLD). MOG is derived mostly from triose phosphate intermediates of glycolysis by non-enzymatic mechanisms *in vivo*. AGE formation from MOG occurs predominantly in arginine residues of proteins. Examples of arginine-derived AGEs caused by MOG glycation are argpyrimidine and hydroimidazolone.

1.45.6 Reactive carbonyl species

Carbonyl species are compounds containing the (C=O) carbonyl functional group. As the oxygen atom in the carbonyl group is more electronegative than the carbon atom, it pulls the electrons from the carbon atom and thereby increases the polarity of the carbonyl group. The carbonyl carbon is electrophilic in nature and readily reacts with nucleophiles. The carbonyls methylglyoxal (MG),

glyoxal, acrolein, hydroxyhexenal, hydroxynonenal (HNE) are highly reactive compared to other carbonyls produced in the biological system (Ellis, 2007). These carbonyls are collectively called reactive carbonyl species (RCS). RCS are more stable than the free radicals, and can diffuse over long distances to reach its target molecules (Lesgards *et al*., 2011).

1.46 Carbonyls in pathological conditions

Persistent hyperglycaemia in diabetic patients causes metabolic alterations, leading to over production of a variety of intermediary metabolites. One of the metabolites is the reactive αβ-dicarbonyl, MG, which are both an aldehyde and a ketone. MG has been attributed to be responsible for the development and progression of diabetes and diabetes associated vascular complications (Bourajjaj *et al.*, 2003) including atherosclerosis, ischaemia, hypertension, retinopathy and nephropathy (Brownlee, 2001). All of the diabetes associated vascular complications are characterized by a variety of changes in the micro and macro vasculatures. Most of these changes are due the impairment of the appropriate response of the vasculatures to vascoactive substances, hormones, neurotransmitters, cytokines 2 etc. The changes in the vasculature can be one of the following or a consortium of many abnormal functions of the vasculature and high potential for thrombosis can lead to atherosclerosis. Inflammation, presence of high levels of oxidants and stiffening of the vasculature and improper expression of surface markers are also markers for vascular complications (Creager *et al*., 2003). In Alzheimer's disease an increase in the levels of acrolein, HNE and acrolein, modified proteins have been identified (Lovell *et al.,* 2001). Increased levels of HNE and HNE modified proteins have also been identified in hypertension (Asselin *et al.,* 2006) and HNE have also been identified in atherosclerotic plaques (Leonarduzzi *et al.,* 2005). MG is also thought to be present in the amyloid plaques (Kuhla *et al.,* 2005).

Aims

Protein glycation is a feature of chronic diabetes and can result in modified cellular function. It was before shown that opticin is an important constituent of the eye and may be liked to modulation of vascular growth and subsequent PDR in diabetic patient. Therefor in this project aim to:

1- Identify the characteristics and glycation pattern of opticin.

2- Measure the effect of opticin glycation on:

A-Modulation angiogenesis *in vitro*.

B-Signal transduction capacity.

3- Demonstrat its potential important in determining vascularization associated with PDR.

Chapter 2:
Material and Methods

2 Material and Methods

All the reagents and chemical used in this thesis were of cell culture grade as appropriate.

2.1 Material

- 2-Mercaptoethanol (Sigma, UK)
- ABTS peroxidase substrate: 2, 2-Azino-bis (3-ethylbenz-thiazoline-6sulphonic acid) (Vector, UK).
- Acrodisc 32mm syringe filter with 0.2 µm membrane (Pall Corporation).
- Acrylamide/Bis-acrylamide solution 40% (Bio-Rad Laboratories, Germany).
- Alexa fluor green fluorescence dye (Sigma.
- Ammonium persulphate (Sigma, UK).
- Anti- fibroblast growth factor antibody (Santa Cruz Biotechnology) Antiactin (α-actin), rabbit polyclonal antibody (Sigma) Anti-AGE (Abcam).
- Antibiotics: L-glutamine, pencilline, and streptomycin solution in 0.9%NaCl (Sigma).
- Anti-human VLA-2integrin (Millipore).
- Anti-mouse polyclonal IgGperoxidase conjugate (Sigma).
- Anti-Opticin (Abcam).
- Anti-Paxillin (Millipore).
- Anti-Talin (Millipore).
- Anti-Vinculin (Millipore).
- Basic fibroblast growth factor; bovine (R & D systems).
- Blotting papers (Whatman).
- Bovine aortic endothelial cells, secondary cell line.
- Bovine serum albumin (Sigma).
- Bromophenol blue (Serva, Germany).
- Coomassie brilliant blue (Sigma, UK).
- ECL and ECL^{+} kits (Amersham Biosciences, Buckinghamshire, UK).
- Flk-1 (C-20): VEGFR-2 antibody (Santa Cruz Biotechnology, Inc).
- Fo et al bovine serum (Cambrex Bioscience).
- Gelatine, 0.1% w/v (Sigma).
- Glacial acetic acid (Fisher Scientific International, UK).

- Glycerol (BDH, UK).
- Glycine (BDH, UK).
- Horse-radish peroxidase conjugated goat anti-rabbit IgG, whole molecule (Sigma).
- Hydrochloric acid (HCl) (BDH, UK).
- Isopropanol (Sigma, UK).
- Lysozyme (Sigma, UK).
- Matrigel TM basement membrane reduced (Becton Dickinson).
- Methanol (Fisher Scientific International, UK).
- MOGMG (Sigma, UK).
- N, N, N′, N′-tetramethylethylenediamine (TEMED) (Sigma, UK).
- Nitrocellulose membrane, protan nitrocellulose (Schleicher & Schuell).
- Opticin (Manchester University).
- Para formaldehyde (Sigma).
- Peroxidase conjugated rabbit anti-mouse immunoglobulin (DAKO).
- Protein molecular weight marker for SDS-PAGE (Sigma, UK).
- Recombinant human FGF-2 (R & D System).
- Silver stain (sigma, UK).
- Six-well, 24-well, 48-well and 96-well plates, 96-well EIA/RIA plates (Nunc corporation).
- Sodium azide (Sigma, UK).
- Sodium bicarbonate ($NaHCO_3$) (Sigma, UK).
- Sodium dodecyl sulphate (SDS) (BDH, UK).
- Sodium hydroxide (NaOH) (BDH, UK).
- Staining solutions: methylene blue, giemsa (Sigma).
- Sterile needle (Terumo).
- Thermanox plastic cover slips, 13mm diameter (Nunc Corporation).
- Thymidine (Sigma).
- Tissue culture flasks; T-25, and T-75 (Nunc).
- Tris (hydroxymethyl) methylamine (BDH, UK).
- Trypan blue (Sigma).
- Trypsin-10X (Sigma), trypsin-EDTA solution, trypsin blocking solution, buffered saline rinsing solution (TCS Cell Works).

- Tween 20, glycine, sodium dodecylsulphate, NaCl (Sigma).

2.2 Solutions

- Acrylamide/bisacrylamide solution (Bio-Rad Laboratories).
- Antibody dilution buffer: primary and secondary antibody diluted 1XX bloking solution.
- APS (10%): 100mg was dissolved in 1mL dH2O.
- Bicarbonate/carbonate coating buffer (100mM): 3.03g Na2CO3, 6.0g. NaHCO3 and 1 L dH2O PH 9.6.
- Blocking solution: 1%BSA in PBS.
- Bovine serum albumin (1%): 1g BSA to 100 mL TBS-tween. The pH adjusted to 7.4. The buffer was stored at 4°C for one week.
- Destianing solution: Methanol (250 ml) was mixed with 70 ml of acetic acid and the volume was made up to 1 L with dH_2O.
- Electrophoresis buffer: 12.02g Tris-base, 4g SDS and 57.68g glycine were dissolved in 2 L dH2O. The buffer was kept at RT.
- Milk (5%): 5g milk to 100ml TBS-tween. The pH was adjusted to 7.4 and the buffer stored at 4°C for one week.
- Phosphate buffer solution (0.1 M) (pH 7.4): the acid component of the buffer (sodium dihydrogen phosphate) and the basic component (sodium were weighed out in a certain ratio according to the Henderson).
- Hasselbalch equation. The desired volume was then accordingly adjusted with distilled water and the solution was mixed. The pH of sodium phosphate buffer was adjusted to 7.4. Sodium azide was added to the mixture to prevent any bacterial growth. Then stored at 4 °C.
- Phosphate buffered saline (PBS): one tablet of PBS was dissolved in 100 ml H2O and autoclaved at 121°C for 15 min.
- Radioimmunoprecipitation assay (RIPA) buffer: contain 50mM Tris- HCL, 0.25% SDS, 1% Triton X-100, 0.15M NaCl, 1mM EDTA, 1% sodium Deoxycholat 1 mM sodium orthovanadate, 1g/mL pepstatin and 0.5 mM PMSF, pH 7.2.
- Running buffer: Tris-base (1.5 g), glycine (7.2 g) and (0.5 g) of SDS were dissolved in 500 ml distilled water.

☞ Sample treatment buffer: Sodium dodecyl sulphate (0.1 g) and 0.1 ml of 2 mercaptoethanol were added to 1 ml of solution C (what is solution C) and 2ml of glycerine and the volume was made up to 10 ml with distilled water.

☞ Separation gel: In a universal tube; 2.5 ml of separating solution was added to 6.1 ml of distilled water and mixed with 1.45 ml of 40% cold acrylamide solution, 100 µl of APS was added to the mixture. Gel polymerization was initiated by adding 10 µl of room temperature TEMED and allowed to polymerize for 10-15 min.

☞ Stacking gel: 2.5 ml of separating solution in 4.2 ml of distilled water was mixed with 3.3 ml of 40% cold acrylamide solution. 100 µl of APS was added to the mixture. Polymerization was initiated by adding 10 µl of TEMED. The gel was allowed to polymerize for 7-10 min, the solution was stored at 4 °C.

☞ Towbin buffer: 1.51g Tris-base, 7.2g glycine and 0.167g SDS were dissolved in 400 mL distilled water and 75mL methanol. The pH adjusted to 8.3 and adjusted to 500 ml with distilled water. Moreover, the buffer kept at room temperature.

☞ Wash solution: PBS containing 0.05% Tween 20 (TBST).

2.3 Equipments

❒ Blotter (Semi-Phor, Hoefer Scientific Instruments)

❒ Centrifuges (Eppendorf and Laborzentrifugen 3K10, Sigma)

❒ CO_2 incubator (Lab Impex Research)

❒ Cross power 500 for electrophoresis (Scientific laboratory Supplies Ltd, England).

❒ Cryo vials (Nunc)

❒ De-ionizer (Millipore)

❒ Digital multi channel pipits (Eppendorf)

❒ Dual gel casting electrophoresis chambers (Scientific Laboratory Supplies Ltd, England)

❒ Fluorescence microscope

❒ Freezer (-80°C) (Juan Quality Systems)

❒ Ice maker (Borolab Ltd)

❒ Immunofluorescence microscope (Zeiss Axo imager Z1)

❒ Inverted phase contrast microscope

❒ Laminar flow hood or safety cabinet: tissue culture grade (Walker Safety Cabinet Ltd)

❒ Luminescence spectrometer model LS 30 (Perkin Elmer).

❒ Magnetic stirrer hotplate (Stuart Scientific Co, England).

❒ Media card reader (MISCO, UK)

❒ Microplate reader, 96-well (Spectramax)

❒ Orbital shaker (Denley)

❒ Single threshold coulter counter (Beckman Coulter)

❒ Sonicator

❒ Universal model 200 laboratory, pH meter (Medical scientific instruments, England).

❒ Vortex (Chiltern)

2.4 Methods

2.4.1 Dialysis procedure

To remove unbound MG before protein assay, MG-treated opticin were subjected to extensive dialysis against distilled water by using dialysis cassette. Cassette was placed in warm water for 5 minutes. The Protein samples were delivered into dialysis cassette after the air was removed by pressing on the membrane. Dialysis cassette was placed into 2L beaker containing distilled water. A few drops of chloroform were added to prevent any contamination. Dialysis was carried out by stirring the samples at 4 ºC for 4 – 5 changes over 1 to 2 days. At the end of dialysis, the dialysed samples were transferred into clean tube and stored in freezer until used. (Figure 2.1).

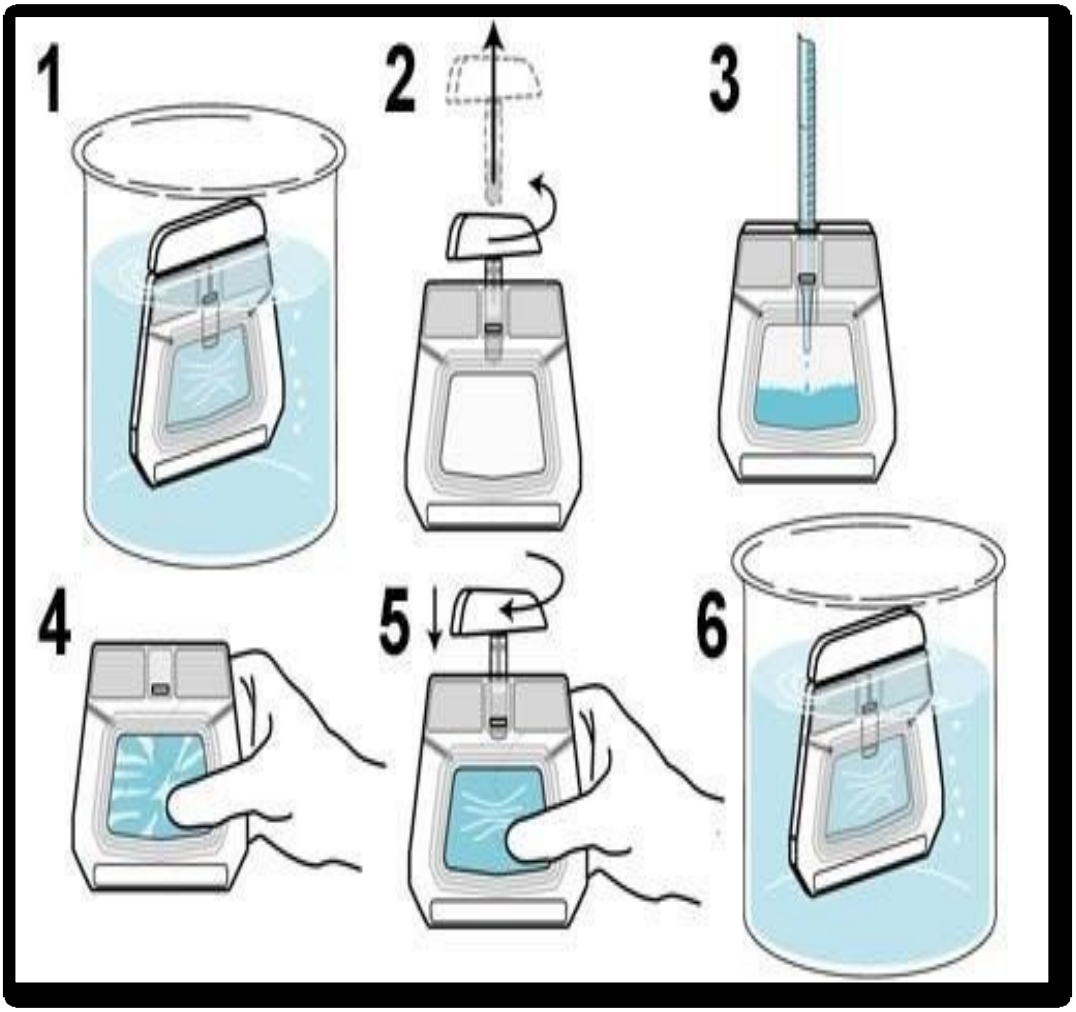


Figure 2.1The dialysis process by using the dialysis cassette

2.4.1.1 Coomassie brilliant blue

The stain dissolved in 500 ml of methanol and 100 ml of acetic acid. The volume was made up to 1 L with distilled water and then filtered. The staining solution was stored at room temperature. The colloidal Coomassie brilliant blue staining of polyacrylamide gels was performed with the Roti-Blue kit (Carl Roth GmbH). After SDS-PAGE, the gels were incubated with Roti-Blue staining solution for 215 h with constant agitation. The gels were then incubated in destining solution until the background of the gels appeared nearly transparent. Then the gels were washed with dH2O several times.

2.4.2 MALDI TOF Mass Spectrometry

This work has done by Kratos Analytical Limited Company, Manchester.UK. All samples were supplied in PBS buffer at 2.5mg/ml concentration but PBS buffer should be removed from the samples because of the damaging effect of this buffer on the MALDI ionisation process. Therefore, there are two methods to clean up the samples including drop-dialysis using 0.025 μm VSWP membrane and C18 ZipTip (both are from Millipore corp. USA). However, in this test just one of the clean up methods was successfully utilised, which was the ZipTip method. The next stage, 5uL of each of the samples was dialyzed against millipore H_2O using drop dialysis disks (millipore) for 60 minutes. The samples (1ul) were then spotted directly on to the MALDI plate with Sinapinic acid matrix (1uL) and left to dry. Each sample was analyzed in linear mode using an Axima Confidence MALDI-TOF instrument (Shimadzu/Kratos) with a standard raster containing 121 points.

2.4.3 Sliver staining of polyacrylamide gels

Silver staining is a highly sensitive method for detecting proteins and nucleic acids in polyacrylamide slab gels. The Bio-Rad Silver Stain, derived from the method of Merril, 1 is 10-50 fold more sensitive than Coomassie brilliant blue, and we used Modified Silver Stain Protocol as in (Bio-Rad Laboratories protocols). This modified protocol, optimized for mini gels (~7 cm x 8 cm x 0.75 mm), gives clear backgrounds and consistent results in less time than the standard protocol.

2.4.4 **Silver Stain Protocol**

Fixative: 40% methanoland 10% acetic acid (v/v).

Oxidizer: 10 ml of oxidizer and 90-deionizer water.

Silver Reagent: 10 ml of reagent and 90 deionised water.

Developer: 32 grams of developer per litre of deionised water.

Stop: 1% acetic acid (v/v):200 ml of acetic acid and 800ml deionised water.

The protocol for mini-gels (up to 1.0 mm thick). When gel removed from glass and place in several gel volumes of milli-Q water shake it for 30 minutes. Then, discard water and add Fixing Solution (approx. 100 mls for a small gel). Shake for 10minutes. After replace fixing solution with Rinse Solution. Shake for 5minutes.Replace rinse solution with Sensitizer. Shake for 2 minutes, and discard sensitizing solution and replace with dH2O. Shake for 2minutes, and wash with dH2O three times for 5 minutes. After second wash, add developers shake for 1minute and repeat it until the bands visualized. Last step discard developer and add Stop Solution. Shake for at least 5 minutes. Wash gel with dH2O.

2.4.5 Image analysis of SDS-PAGE gels

The gels were analyzed using either laser densitometer 2202 Ultroscan or photographed using Gene snap programme from G Box Chem HR16. Bands were compared within the same gel.

2.5 Preparations of samples

2.5.1 Preparation of MG-treated opticin

Opticin (1mg/ml) was incubated with MG (0.1M) in 0.1M sodium phosphate buffer containing 3mM sodium azide, pH 7.4 at 37 °C for 0, ½ hour and, 1, 3, 6, days at 37 ºC. After incubation, un-reacted MG was removed by dialysis against distilled water for 2 days at 4 ºC and samples were stored at -20 °C. Control opticin was incubated under the same conditions without the addition of MG.

2.5.2 Detection of AGE using MALDI/TOF.

Bruker Autoflex MALDI-TOF-MS (Bruker Daltonik, Germany). All samples were supplied in PBS buffer at 2.5mg/ml concentration but PBS buffer should be removed from the samples because of the damaging effect of this buffer on the MALDI ionisation process. Therefore, there are two methods to clean up the samples including drop-dialysis using 0.025 μm VSWP membrane and C18 Zip Tip that both are from (Millipore corp. USA). However, in this test just one of the clean-up methods was successfully utilised, which was the ZipTip method.

For MALDI analysis, 1 uL of sample was loaded onto the sample plate with 0.5uL of sinapinic acid matrix (saturated sinapinic acid in 50% Acetonitrile/50% 0.1% TFA) and allowed to co-crystallize on the sample target. The mass spectrometer is equipped with a nitrogen laser (337 nm, 3ns pulse width). All measurements were performed in positive ion linear mode using delayed ion extraction. The delay time was adjusted according to the mass of the protein. The ion acceleration voltage was set to 20 kV. In all MS/MS experiments, helium was used as the collision gas.

2.5.3 Cell culture

All tissue culture experiments and procedures were carried out in a sterile environment in a class II biological safety cabinet. Ethanol [70% volume/ volume (v/v)] was used for cleaning all surfaces before commencing work. Only sterile plastics were used and all media were pre-warmed by placing in a water bath at 37ºC for 30 minutes before use.

2.5.3.1 Routine culture of bovine aortic endothelial cells

Bovine Aortic Endothelial Cells (BAEC) characterized by the presence of von Willebrand factor and the uptake of Di-labeled acetylated low density lipoprotein were previously isolated at Manchester Metropolitan University by Dr Slevin. They were routinely cultured in Dulbecco's modified Eagle's medium containing 15 % foetal Bovine serum (FBS) (DMEM/FBS), 1.5 mM glutamine, 100 IU/ml penicillin, and 50 ng/ml streptomycin (complete medium). Culture flasks were maintained at 37 °C in a humidified incubator (5 % CO2 and 95 % air). Cells were passaged once per week at a ratio of 1:3 and used within ten passages in all experiments. Cells at concentration of 1.5 x 10^5 and 5 x 10^5 in 10 to 15 ml of

complete medium were seeded into T-25 and T-75 flasks, pre-coated with 0.1% gelatine (Sigma, UK) and cultured in DMEM (Lonza, UK) supplemented with 15% fo et al bovine serum (FBS, Cambrex, UK), 2 mM glutamine and 1% antibiotics (100 µg/ml streptomycin, 100 U/ml penicillin). Cells were put in saturated air humidity 5% CO2-incubator at 37°C. Growth medium was changed every 2-3 days, when the cells were confluence; they were sub-cultured by enzymatic digestion with 0.05% trypsin / 0.02% EDTA and split at a ratio of 1:3.

2.5.3.2 Sub-culturing of endothelial cells

Medium was discarded from the flasks, and the cells washed twice with 10-15 ml sterile PBS at 37 ºC and incubated with 5-10 ml of 1 % (v/v). Trypsin / Ethylene Diamine Tetra Acetic acid (Trypsin/EDTA; 4 ml) solution for 2 to 5 min. Detachment of cells was checked by phase contrast inverted microscope. Upon cell detachment, 5-10 ml of complete medium was added immediately to the flask to neutralise the trypsin. The cell suspension was transferred to a 25 ml centrifuged tube, and centrifuged at 1500 rpm for 5 min. The supernatant was discarded and the pellet was resuspended in 1 ml fresh medium at 37 ºC. For a T75 culture flask, it was pre-coated with 2 ml of 0.1% gelatin.

2.5.3.3 Trypsinization

Medium from the flask was discarded and washed twice with 10 ml sterile PBS and incubated with 5 to 10 ml of 1 × trypsin solution for 2 to 5 minutes. 8-10 ml of complete media was added immediately to the flask to neutralize the trypsin. The resulting cell centrifuged at 1500 rpm for 5 minutes. The supernatant was discarded and the pellet was suspended in 1 ml fresh medium, and the cell number was determined.

2.6 Storage of cells in liquid nitrogen

Cell pellets were resuspended in freezing medium composed of 10 % DMSO in FBS. Approximately 3.5×10^5 cells in cold freezing medium were transferred to labeled cryotubes on ice and placed at -20 for 1 h, then transfer into the upper

level of the gaseous phase of liquid nitrogen for another 1 h and 30 min after that transferred to the liquid nitrogen for long term storage.

2.7 Thawing Frozen Cells

The vial was removed from liquid nitrogen, thawed immediately in a water bath at 37°C, and transferred to an empty sterile 15mL Falcon tube. DMEM media containing 15% was added drop wise to a volume of 10mL. The tube was centrifuged at 1500 rpm for 5 minutes at room temperature. The Pellets were then resuspended in fresh complete medium and seeded in T75 cm^2 culture flasks.

2.8 Cell counting

Under experimental conditions medium was aspirated and the cells were rinsed in PBS and detached using a 1 % trypsin (v/v) solution. 0.1 ml of cell suspension was added to 20 ml Isoton solution and transferred to a counting chamber; and was mixed and counted at least three times using a Beckman Coulter counter. The following equation was used when 0.5 ml of trypsinzed cell suspension was added to 20 ml of isotonic solution and cells were counted as cells/ml:

X (cell number) × (20/0.5) = X × 40 (dilution factor)

2.9 Preparations of medium

2.9.1 Preparation of complete medium (15% FBS);

Pre-warmed sterile FBS was denatured at 65-70 ºC for approximately 30min and other reagents were mixed as described below (Table 2.1)

Table 2.1 Preparation of 15% FBS culture medium

Reagents	Volume of Medium (100ml)	Volume of Medium (500ml)
DMEM	84 ml	420 ml
FBS	15 ml	80 ml

2.9.2 Preparation of 2.5 % FBS and 0.1% FBS

Serum-poor medium containing 2.5% or 0.1% FBS was prepared by diluting 15% FBS with DMEM as indicated in (Table 2.2)

Table 2 .2 Preparation of 2.5% and 0.1% FBS culture medium

Reagents	0.1% FBS (120 ml)	2.5% FBS (120 ml)
DMEM	118.2 ml	99 ml
15%FBS	0.8 ml	20 ml
PSG	1.0 ml	1.0 ml

2.10 Proliferation assay

BAEC cells (3×10^4 cells/ml) in 0.5 ml of culture medium (15% FBS) were seeded in 24-well plates and allowed to adhere for approximately 4 hours. The complete medium was discarded and the cells were washed gently with PBS twice. Fresh media supplemented with 2.5% FBS was added to each well. Compounds at various concentrations, were incubated with and without growth factors, FGF-2 (25 ng /ml), opticin (25µg/ml) and FGF-2 + opticin (native or glycated), or opticin (native or glycated) controls wells were prepared appropriately. The contents of the plate were gently mixed to avoid possible dislodging of cells and incubated for a further 72 hours. Cells were washed twice with PBS and 250µl of trypsin was added to each well. The number of cells was counted using a Coulter counter. Experiments were carried out in triplicate wells, and repeated three times independently. The statistical analysis was carried out. All results are represented by means ± S.D. analysed t test between two groups, (*), ($p<0.05$) statistically significant.

$$\% \text{ change} = 100 - \left(\frac{\text{Number of cells/ml (Test)}}{\text{Number of cells/ml (Control)}} \text{ X } 100 \right)$$

Equation for determination of % change; Percentage change in the presence of growth factors was calculated using the above equation.

2.11 Wound healing assay

Thermanox plastic coverslips were placed in a 24-well plate. BAECs at a concentration of 2.4×10^5/ml were added to each well in complete medium and incubated for 24-48 hours. When confluent, the medium was replaced with (0.1% FBS, DMEM) and cells were incubated for a further 24 hours. Cells were washed with PBS (3-4 times) and wounded with a sterile razor blade producing uniform and straight edged cuts. The wounded cell monolayer was washed with PBS to remove cellular debris or dislodged cells and placed in a fresh 24-well plate containing serum poor medium (SPM) (0.1% FBS). Factors pre-incubated with FGF-2 (25ng/ml), opticin (native or glycated) were added to the test wells. For each experimental condition, cells were treated in triplicate. Plates were incubated in 5% CO_2 incubator at 37 °C for 24 hours. Pilot studies demonstrated that BAEC wounded under these conditions underwent negligible proliferation up to 24 hours; however, cell movement resulting in wound closure was statistically significant in cells treated with growth factor after 24 hours. The coverslips were rinsed 3 times in PBS, fixed in 100% ethanol for 5 minutes, and allowed to air dry. Cells were stained with methylene blue for 30 seconds and excess stain was removed with distilled water. The plates were left to air dry. Pictures were taken using phase contrast microscopy (40×) for 10 random areas of each cover slip. Movement of cells into the denuded area was quantified by counting numbers of migrated cells in each field of view. For each coverslip, 10 fields of view (each ~2 mm by 1.45 mm) were examined at random. To evaluate cell migration, two parameters were taken into account: the number of migrated cells counted under a light microscope (×40) into five random fields of denuded area and the distance of migration from the wound edge obtained by Image J, image analysis software (http:rsb.info.nih.gov//ij/). The number of migrated cells to the denuded area was determined by counting the number of cells in the injured area. Statistical significance was determined by one-way analysis of variance. Cell migration was examined from the “wound” edge by the aid of a microscope and photographed using a digital camera. Each photomicrograph was analysed with Image analysis software. A representative example is shown (Figure2.2).

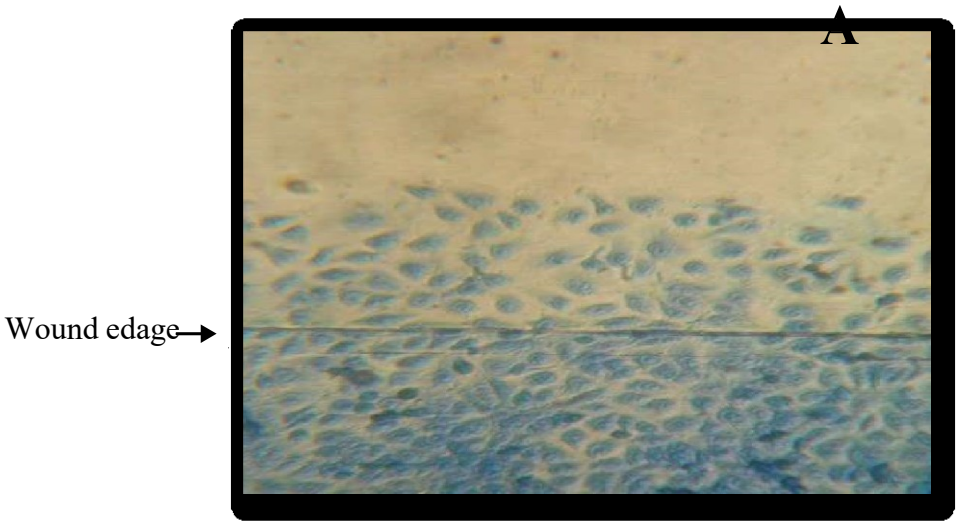


Figure 2 .2 BAEC migration after 24 hours incubation.

2.12 Boyden chamber

BAEC migration was examined *in vitro* using TranswellCostar® porous membranes (Milian Laboratory Products, Gahanna, OH, USA; 8-mm pore filter) in a 24-well plate. Inserts were coated overnight with 100µl sterile 0.1% gelatine each insert and air-dried. BAEC were seeded at a concentration of (7.3×10^4 cells/ ml) in 100 mL of serum-poor medium, on the upper part of the filter and factors at different concentrations with and without chemo attractants; FGF-2 (25ng/ml), opticin (25ug/ml: native or glycated) and their combination were added. Basal medium was supplemented with 0.1% FBS. For each experimental condition, cells were treated in triplicate. Cells were incubated in 5% CO_2 incubator at 37ºC for 24 hours. After incubation, the cells that did not migrate on the upper surface of the membrane were removed with a cotton swab soaked with PBS then wiped with a dried cotton swab. The cells that had migrated were fixed with 4% Paraformaldehyde, left to air-dry and stained with Giemsa stain and five microscopic fields from each membrane counted with an optical microscope. Cells migrations in triplicate wells were determined by counting the number of cells in the lower side of the filter using optical microscopy (10X objectives)

2.12. A. Expression and purification of recombinant bovine Opticin

Opticin was provided by Professor Paul Bishop (Welcome trust centre for cellmatrix research, Manchester University, UK). Briefly, full-length recombinant bovine Opticin was expressed in 293-EBNA cells (human embryonic kidney cells, Invitrogen) using pCEP-pu expression vector. This expression vector is a modified version of the pCEP4 expression vector from Invitrogen. The expression and the purification of the recombinant protein were performed as previously described (Le Goff *et al*., 2003) using a combination of anion-exchange (DEAE) chromatography and Jacalin lectin affinity chromatography.

2.13 Formation of tube-like structures in Matrigel™

EC/matrigel suspensions 1:1 (v/v) containing 1.5×10^6 cells/ml (BAEC and HUVEC) and 1.0×10^6 cells/ml (HREC) were prepared in triplicate and supplemented with (1) PBS, (2) Opticin (25 µg/ml), (3) FGF-2 (25 ng/ml), (4) FGF-2 (25 ng/ml) and Opticin (25 µg/ml), and spotted into 48-well plates and incubated for 1 hour at 37 ºC to allow the gel to polymerise. For BAEC and HREC, 0.5 ml of DMEM supplemented with 2 % (v/v) FCS and the appropriate treatments (1-4) listed above, and for HUVEC, 0.5 ml of large vessel basal growth medium (with growth supplements) supplemented with 2 % (v/v) FCS and appropriate treatments (1-5) were added to the wells. The gels were incubated for 24 h at 37 ºC to allow capillary morphogenesis. The Matrigel gels were then fixed with 4 % (v/v) PFA in PBS for 10 minutes at RT. The tube-like structures were examined and photographed using a phase contrast microscope (Nikon TMS and Nikon coolpix 4500 Digital camera). The total length of tube-like structures was measured in 5 fields of view for each experimental condition. Statistical significance was determined by student's t-test.

2.14 Formation of tube-like structures in type-I Collagen

Prior to starting the experiment ECs were cultured in SPM containing 0.1 % FCS (v/v) (BAEC and HREC) and 2 % FCS (v/v) for 48 h. Cells suspensions at a concentration of 4.5×10^5 (HREC) and 7.5×10^5 (BAEC) cells/min in medium containing 0.1 % and 2 % FCS l were mixed with collagen at a concentration of

300 µg/ml and supplemented with (1) PBS (2) Opticin (25 µg/ml) (3) FGF-2 (25 ng/ml), (4) FGF-2 (25 ng/ml) and opticin (25 µg/ml), and spotted in triplicate into 48-well plates and allow to polymerise for 1 hour at 37 ºC. After polymerisation, 0.5 ml of medium containing 0.1 % or 2 % FCS (v/v) supplemented with treatments 1-5 was added to the corresponding wells in the 48-well plate and the gels incubated for 72 hours at 37 ºC to allow capillary morphogenesis. The gels were fixed with 4 % (v/v) PFA in PBS for 10 minutes at RT and dehydrated with 70 % ethanol for 30 minutes followed by 100 % ethanol for 5 minutes at -20°C. The collagen gels were allowed to dry overnight and stained with Giemsa. The total length of tube-like structures was examined using a phase contrast microscope (Nikon TMS) and photographed using a digital camera (Nikon coolpix 4500). The total lengths of the tube-like structures were measured in 5 fields of view for each experimental condition. Statistical significance was determined.

2.15 SDS-PAGE

Semi-confluent BAEC were cultured in 6 well plates in 2 % (v/v) FCS for 48 h then pre- before the addition of FGF-2 (25 ng/ml) ± Opticin (25µg/ml), and incubated at 37 ºC for 10 min and 24 h. Total cell lysates were collected in RIPA buffer, centrifuged at 10,000g for 15 min at 4ºC to remove insoluble debris and stored at –70 ºC until use. Protein concentration of cell lysates was determined using a modification of the Bradford assay (Bio-Rad, California, USA). Equal quantities of protein (10 ug) were mixed with 2 x Laemmli sample buffer, vortex mixed and boiled in a water bath for 15 minutes. SDS-PAGE was carried out using 4-12 % Bis-Tris gradient gels (Invitrogen). Samples were separated along with pre-stained molecular weight markers (20-200 kDa) or LI-COR's pre-stained Western Blotting

Proteins were transferred onto nitrocellulose filters for 1 hour 30 min and blocked for 1 hour at room temperature in TBS-Tween (50 mM Tris-HCl, pH 8.0, 0.15 M NaCl containing 0.1 % (w/v) Tween-20/BSA). Filters were stained with the following primary antibodies diluted in blocking buffer overnight at 4ºC on a rotating shaker; rabbit monoclonal antibody to ERK-1/2 (1:1000), mouse monoclonal antibody to phospho-ERK-12, rabbit monoclonal antibody to p38 (1:500), mouse monoclonal antibody to phospho-p38, rabbit monoclonal

antibody to JNK (1:500), mouse monoclonal antibody to phospho-JNK, rabbit monoclonal antibody to MEK-1/2 (1:1000), goat monoclonal antibody to phospho-MEK-1/2, rabbit monoclonal antibody to AKT-1, goat monoclonal antibody to phospho-

AKT-1 and rabbit polyclonal α-Actin (1:1000). After washing 5 × 10 min in TBS-Tween at RT filters were stained with rabbit anti-goat, rabbit anti-rat, and goat anti-rabbit or rabbit anti-mouse horseradish peroxidase-conjugated to secondary antibodies diluted in TBS-Tween containing 5 % (w/v) de-fatted milk (1:2000) for 1 hour at RT with continuous shaking on an orbital mixer. After a further 5 washes in TBS-Tween, proteins were visualised using ECL chemiluminescent detection.

2.16 Protein extraction for total proteins

BAEC (1.5×10^5 cell/ml) were plated into a 6-well plate (Nunc), in 2 ml of complete culture medium (15% FBS) DEME. When they were confluent (8090%), medium was discarded, and cells were washed with PBS. Fresh SPM (2.5% FBS) was added to each well, according to the following experimental conditions:- Control, opticin(25ug/ml :native or glycated), FGF-2 (25ng/ml), and their combinations (FGF-2 + opticin [native or glycated]), and the cells were incubated for different periods of incubation time (10 minutes, 24, 48 and 72 hours), in a 5% CO_2 incubator at 37°C. After 72 hours of incubation, the medium was discarded, and each well was rinsed with 500 µl of cold PBS. Ice cold radioimmuno precipitation buffer (RIPA) 400µl was added to wells and kept on ice. Gently the plate was shaken on ice for a few minutes, cells were scraped using a cell scraper. The total cells lysates were transferred to cold eppendorfs tubes on ice and stored at -20°C until used.

2.17 Protein estimation for Western blotting

Protein estimation was undertaken Bio-Rad protein assay: 1) 0.1% of bovine serum albumin (BSA) (1 mg/ml in distilled water) was prepared. 2) The Bio-Rad dye was diluted to 1:5 in distilled water. 3) A standard curve was prepared by measuring the absorbance at 570 nm. For Western blotting; the protein content

of each sample was normalised to 30µg then diluted half volume with the sample buffer, for a loading amount of 15µg (Table 2.3). Samples and sample buffer were mixed and boiled for 15 minutes, standard curve was calculated

Table 2 .3 BSA dilution to establish the standard curve.

BSA Standard (µg)	0	5	10	20	40
BSA volume added (1µg/ µl)	0	5	10	20	40
Volume of dH2O(µl)	100	95	90	80	60
Volume of Bio-Rad (1:5)(ml)	2				

2.17.1 Western blotting

The acrylamide gel was prepared by mixing 3.3 ml of 40% bis-acrylamide with 4.2 ml distilled water and 2.5 ml separating buffer in a universal tube. 100 µl of 10% ammonium persulphate (APS) solution was added followed by 10µl TEMED solutions and the gel left to polymerise for 20 minutes. A few drops of isopropanol were added on top of the gel. Isopropanol was removed after 15 minutes and rinsed with plenty of distilled water, for one minute. As much as possible, distilled water was removed by filter paper and then the stacking solution was prepared in a second universal tube, by combining 1.45 ml of 40% bis-acrylamide with 6.1 ml distilled water, and 2.5 ml stacking buffer. As before, 100 µl APS was added followed by 10 µl TEMED solution and the gel left to polymerise for a minimum of 20 minutes.

Slowly the combs, clamps, and gaskets were removed taking care not to damage the wells and the gel plates inserted into the electrophoresis chamber. The chamber was subsequently filled with reservoir buffer (25 mM tris, 192 mM glycine, 0.1% SDS, pH 8.3). A total of 500 ml-electrode buffer was used to fill

the tank, in the space between the two sets of glass plates. Take samples, sample buffers and molecular weight marker out of freezer and warm in room temperature. The protein concentration of each sample was determined by BioRad protein assay to facilitate equal protein loading/well. Equal parts of protein sample and sample buffer were mixed in eppendorfs tube, the tubes were placed in boiling water for 15 minutes, molecular weight marker 10 µl aliquots were boiled for one minute, and then sample buffer 20 µl, molecular weight marker 10 µl and the protein sample from plaques 20 µl were gently loaded. Sample buffer was added to the first and last well and in the second well molecular weight marker was added. The syringe was washed after loading the marker (Table 2.4).

Samples containing 15 µg protein (up to 20 µl solution), along with pre-stained molecular weight markers, were separated by SDS-PAGE (10% w/v) for~45 minutes at 60V (when samples were in stacking gel) and switched to 200V for separation until the dye, bromophenol blue, reached the bottom of the separation gel.

Table 2 .4 Preparation of stacking and separating gels for Western blotting.

	dH2O	Separating/Stacking buffer	Acrylamide 40% (4ºC)	Ammonium persulphate	TEMED
Separating solution	4.2ml	2.5ml	3.3ml	100µl	10µl
Stacking solution	6.1ml	2.5ml	1.45ml	100µl	10µl

2.17.2 Blotting

Two nitrocellulose membranes (NCM) and 12 pieces of blotting paper were soaking for 2 minutes in tow bin's buffer. Stacking gels were removed from the separation gels and discarded. The gels were sandwiched separately. The sandwich was assembled in an electro blotter for each gel as follows: Three pieces of blotting paper /NCM/gel/3 pieces of blotting paper. Any bubbles within the sandwiches were removed by rolling a clean 5 ml tip over the sandwich. Proteins were transferred to the membrane at 40 mA/gel for one hour.

2.17.3 Blocking

Membranes were blocked with 1% BSA incubation for 1 hour at room temperature on rotating shaker. BSA was discarded and membranes were incubated separately in 10 ml of primary antibody solution (1:1000) dilution (rabbit polyclonal anti-ERK 1/2 and mouse monoclonal antibodies to pERK1/2 at 4°C. After overnight incubation on a rotating shaker, primary antibody solutions were discarded (we can used another time), membranes were washed five times in tris buffered saline, and tween-20 (TBS-tween) for 10 minutes each at room temperature on rotating shaker. Membranes were incubated for 1 hour at room temperature in 10 ml of mouse anti-rabbit or rabbit anti-mouse horseradish peroxidase-conjugated secondary antibody diluted in TBS-tween containing 5% (w/v) de-fatted milk (1:1000) for ERK 1/2 and pERK1/2 respectively. After incubation, secondary antibody solutions were discarded and membranes were washed five times in TBS-tween for 10 minutes each at room temperature on a rotating shaker.

2.17.4 Developing

In a dark room, membranes were immersed in enhanced chemiluminescence (ECL) solution. To prepare the ECL solution, 1ml of solution-A was added to 1ml of solution-B and kept in the dark room for five minutes. Once the ECL solution was prepared, it was then poured on to the membranes for one minute, left in the dark room excess reagent was drained off, and membranes were quickly wrapped in cellophane films and kept in a box. The box was taken to the G-Box and the sample was measured as a chemiluminescent sample the intensities of bands on the membranes were quantified by image analysis software. The results were semi-quantitative and compared to the loading control.

2.18 .Binding assay opticin (native and glycated) binding to collagen type-I

Binding assays were performed using ELISA-based techniques. Briefly, 96-well plates were coated with collagen type-I at 50 µg/ml in PBS or with Tris-buffered saline, pH 7.4 (TBS), containing 2 mM $Mn2^{+}$ (TBS/$Mn2^{+}$). Each experimental well was repeated in triplicate. After washing with TBS containing 0.1% Tween 20 (TBS-T) or TBS-T/$Mn2^{+}$, the wells were blocked with 5% BSA. Increasing

concentrations of recombinant opticin or biotinylated collagen type-I was prepared in blocking buffer. These ligands were added to wells for 2 hours. After washing, bound opticin was detected using an affinity-purified anti-rabbit opticin antibody followed by an HRP-conjugated anti-rabbit antibody. Bound biotinylated collagen type-I was detected using HRP-conjugated streptravidin. After extensive washing, TMB substrate was added to the wells, and the absorbance was measured at 620 nm using a Multiscan plate reader. Absorbance obtained from wells coated only with BSA wassubtracted from the experimental wells. All these experiments were performed in triplicate.

2.19 .Binding assay with opticin and FGF-2/FGFR complex

The 96 well plate coated with 100ul/well of capture antibody (goat anti-FGFR1) in coating buffer at a concentration of 2 µg/ml. the plate were sealed and incubated at 4°C overnight. Aspirate well and wash 3 times with at least 300ul/well wash buffer. Inverted the plate and bloted on absorbent paper to remove any residual buffer. Wells blocked with 200µl/well of 1% BSA in PBS. Incubate at room temperature for 2 hours. The well aspirated and washed 3 times with at least 300ul/well by wash buffer. Inverted the plate, blot on absorbent paper to remove any residual buffer, and stored at 4 ℃ until use.

Added FGFR 2µg/ml 100ul per well. Added 100 µl well of antigen (Opticin used as different concentration 0-5-10-25-50-100 µg/ml added to 2 µg /ml FGF-2 to the wells. Plate sealed and incubated at room temperature for 2 hours. Aspirated well and washed 3 times with at least 300ul/well by wash buffer. Inverted the plate and bloted on absorbent paper to remove any residual buffer. Added 100ul/well of anti-FGF2 antibody diluted in blocking buffer at a concentration of 2 µg/ml. The plate sealed and incubated at room temperature for 1 hour.
Aspirated well and washed 3 times with at least 300ul/well by wash buffer. Inverted the plate and bloted on absorbent paper to remove any residual buffer, than Add 100ul/well of secondary antibodies conjugated to HRP anti-goat ANTIFGFR1 diluted blocking buffer at 1/500 dilution and incubate at room temperature for 45 minutes. After that well aspirated and washed 3 times with at least 300ul/well wash buffer. Inverted the plate and blot on absorbent paper to

remove any residual buffer. In this wash step, soak wells in wash buffer for 1 to 2 minutes prior to aspiration. Repeated for a total of 7 washes. Meanwhile, DAB substrate prepare during plate washing. Add 8 drops of DAB stock 100ul/well of ABTS substrate solution to each well. Incubated plate at 37■C for 2 to 10 minutes. The color development can be stopped by adding 100ul of stop solution 2MH2SO4, or use ABC Preoxidease staining kit lastly read plate at 405-450 nm.

2.20 The frozen section

The key instrument for cryosection is the cryostat, which is essentially a microtome inside a freezer. The mouse eyes was placed on a m et al tissue disc, which is then secured in a chuck, and frozen rapidly to about –20 to –30°C.The eyes was embedded in a gel like medium consisting of poly ethylene glycol and polyvinyl alcohol; this compound is known by many names and when frozen has the same density as frozen tissue. The sections cut at 4μm, each three sections placed on one slide, the slides saved in freezer until use.

2.21 Immunocytochemistry

Cover slides (treated in NaOH 1M for 30 min; wash H2O: Ethanol 100%) dry them than coated with and 0.1% gelatin in 24 well plate, BAEC were seeded at 105cells/ml in DMEM on cover slip on each well, the conditions were added to each well, and left for1 to three hours in incubated at 37°C after that they were fixed with 4% Para formaldehyde for ten minutes. The cells were washed 3 times/5 min in PBS and treated with 0.5% hydrogen peroxide for 30 minutes. Cells permeabilized with 0.1% Triton X100 (v/v in PBS) for 4 minute. Cells were washed again with PBS for 3 times/5 minutes. Nonspecific sites were saturated with blocking serum 1% BSA for 20 minutes at room temperature. cover slips were incubated with primary Antibody 150 μl each well (anti- Paxillin, antiVinculin, and anti-VLA-2 (α2β1).for 2-3 hours at room temperature or ■ overnight at 4C. wash the cells twice with PBS, for 5 mintes. Incubate the in the dark, with a 1:100 diluted of goat anti-mouse IgG fluorescein conjugated secondary antibody in 1% BSA in PBS for 1 hour at room temperature. Wash the cells three times with PBS, for 15 minutes. Examine the cells under a fluoresce microscope.

2.22 Immunohistochemistry

Cut sections were selected carefully; drew around the section with hydrophobic pen and fellow these steps: **A.Fixation**: The slides were incubated in coplin jar containing 4% Paraformaldehyde in 1X PBS for 5minutes. The slides were Rinsed in 1× PBTS for 3× 3 minutes. **B. Secondary Antibody Block**: The sections were Incubated in diluted blocking serum (10 % BSA diluted with PBST) for 30 minutes (constantly). The slides were rinsed in 1× PBST for 1× 5mins**.C**. **Primary Antibody binding**: The sectios were incubated with appropriately primary antibody (Anti-opticin 1:100 and Anti-AGE1:150)**.** The slides were rinsed in 1× PBST for 3× 3mins. **D. Fluorescent Secondary Antibody Binding:** The sections were incubated with 1:100 diluted secondary antibodies in block buffer for 60 minutes. Rinse slides 1× PBS for 3× 3mins and dry off excessive liquid. **E. Mounting (with DAPI counter stain for nuclei):** Few drops were added of Vector Hard mount over sections**.** The slids were covered with cover slip, (taking care not to produce bubbles under the cover slip). The slides were kipet for 15minutes to dry than keepet them covered in fridge. The sectios were visualized with a Zeiss fluorescent microscope.

2.23 Statistical analysis

Statistical analysis was made through using Microsoft Excel 2007 program. Data were expressed at mean ± standard deviation (SD). Statistical significance was assessed by Student's t-test. P-values below 0.05 were considered as statistically significant (*P ≤ .05, **P ≤ .01, ***P ≤ .001). Each experiment was repeated at least three times (n=3), and a representative example is shown.

Chapter 3: Results

Results
Section 1: Opticin glycation

3 Results

3.1 Glycation

3.1.1 Quantification and localisation of opticin glycation

Under hyperglycaemic conditions in diabetes, the level of dicarbonyl compounds including MG is increased and lead to the formation of AGEs (Brownlee 2001; Vander-Jagt 2008). A 5-6 fold increase of MG levels in the blood samples of insulin dependent (type-1) diabetic patients and a 2-3 fold increase in blood samples of non-insulin dependent (type-2) diabetic patients has been reported (McLellan *et al*., 1994). Preretinal neovascularisation is responsible for the start of diabetic retinopathy and ends in blindness. Collagen fibrils of vitreous humour offer a fundamental substrate for this process. These fibres are coated with opticin, a glycoprotein previously shown to be a potent endogenous inhibitor of angiogenesis (Le Goff *et al*., 2012). Here we show that exposure of opticin to MG produces glycation.

3.1.2 Opticin detection by SDS-PAGE

The opticin was donated by Manchester University following extraction from human vitreous collagen fibrils at 6.13mg/ml, following this opticin was diluted to 1mg/ml. The migration of opticin was visualized at 45 kDa as determinedby SDSPAGE following silver staining. This experiment was repeated three times and a representative SDS-PAGE gel is shown in figure 3.1.

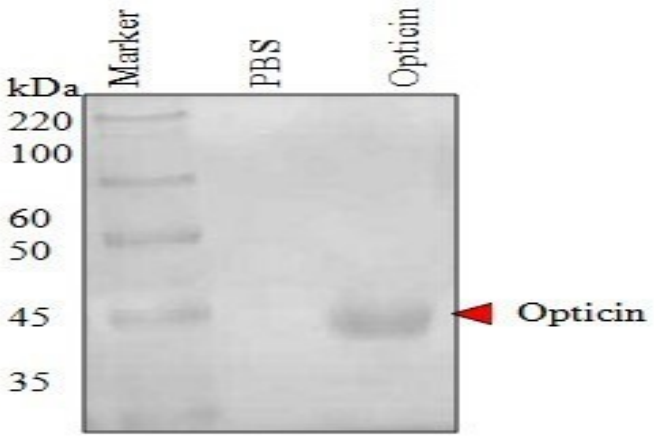


Figure 3 .1Opticin detection by SDS-PAGE.

The native opticin at (1mg/ml) was mixed with sample buffer, and PBS used as negative control, was electrophoresed on a 12% SDS-PAGE gel. The experiment was repeated three times and a representative SDS-PAGE gel is shown.

3.1.3 Elecreophoretic analysis of Glycated opticin

To study the (AGE-opticin) formation the different sugar types being used; MG, glucose, fructose, and ribose. SDS-PAGE is a method commonly used to separate unfragmented, glycated protein (Ahmed, *et al*., 1986). The glycation of opticin in presence of sugars as seen in diabetic patients with PDR is the first step to study the pro- and anti-angiogenic factors in the vicinity of preretinal neovascularization. MG (0.1M) selected for all our experiments while glucose, fructose, and ribose used only to confirm the glycation on SDS-PAGE.

3.1.3.1 Glycation of opticin *in vitro* using SDS-PAGE analysis

Opticin at (1mg/ml) incubated with MG (0.1M) for 24 hours, and opticin in ratio did show lighter cross-linked AGEs (Figure 3.2). The subunits of cross-linked AGEs caused the formation of dimers with a approximate molecular weight of 90 kDa, as determined using the molecular weight markers (lane M).The glycated opticin (Figure 3.2, lanes A) showed no glycation at time zero, in contrast, the opticin glycated when increase the time up to 24 hours (Figure 3.2, lanes; B, C, D). The native opticin where incubated alone used as control. The M_r of glycated opticin was approximately twice of the original. (Figure 3.2, lane M).

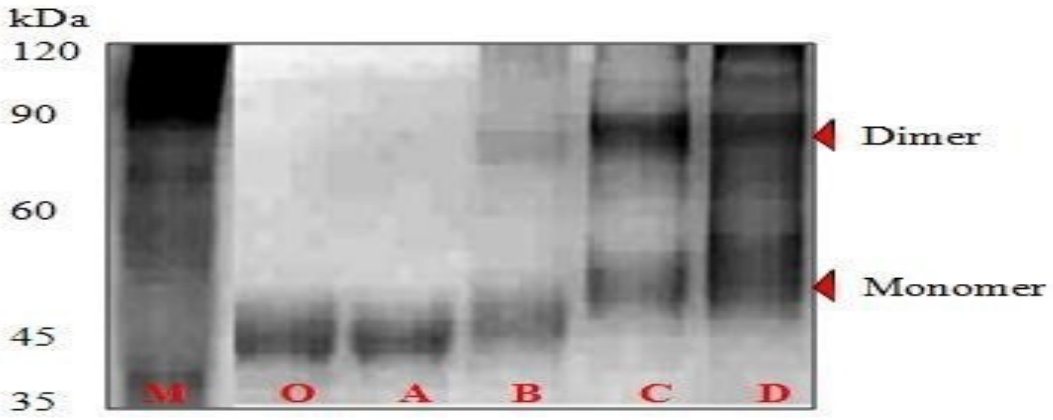


Figure 3.2The image is demonstrated glycation of opticin with MG (0.1M). Opticin (1 mg/ml) was incubated alone or in the presence of 0.1 M MG in 0.1 M sodium phosphate buffer, pH 7.4 at 37°C for different time courses. M: Marker; O: opticin alone; A: zero time; B: 1 hour; C: 6 hours; D: 24 hours. The cross linking was determined by silver stain. The experiment was repeated three times and a representative result is shown.

3.1.3.2 Glycation of opticin for up to 24 hours with MG

SDS-PAGE was used to study the effect of periods of incubation on AGE formation in vitro. Early glycation products were determined for varying time intervals as shown in figure 3.3, by using opticin at 1mg/ml and MG (0.1M) in 0.1 M sodium phosphate buffer (pH 7.4). Results showed a gradual increase in the molecular weight of dimers of opticin, which is a characteristic of AGE formation, reaching its highest level at 24 hours of incubation. Control sample (opticin alone), also at time zero showed no glycation compared to those incubated in the presence of MG (from 1, 6 and 24 hours). Formation of AGEs was linearly dependent on the reaction time.

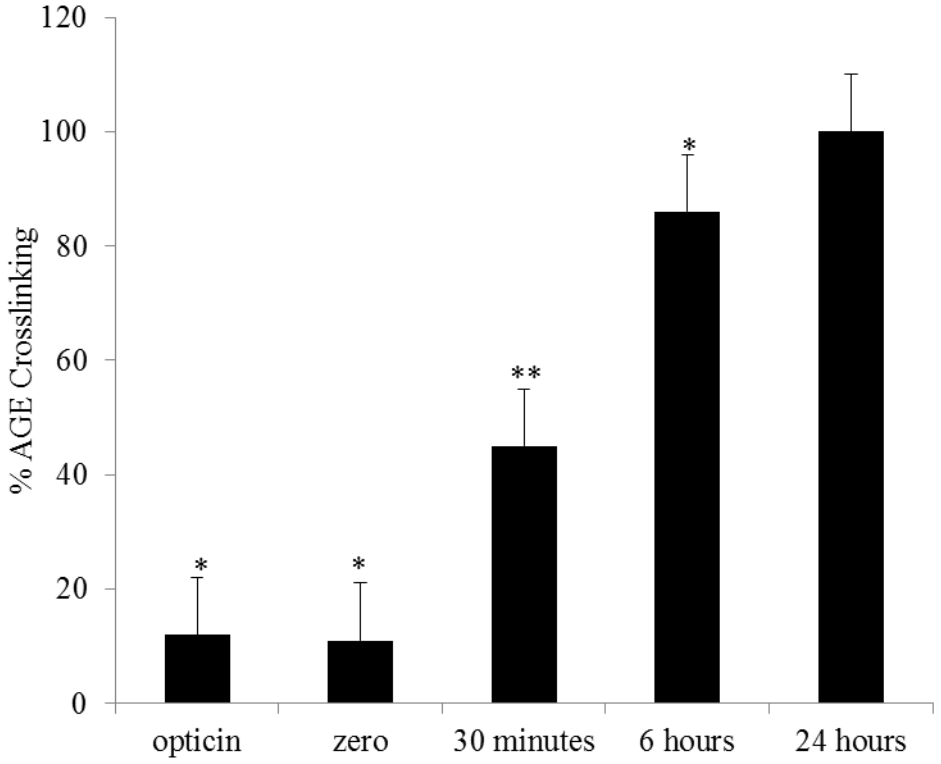


Figure 3.3 AGE formation in the opticin-MG system for 24 hours.
Opticin (1 mg/ml) was incubated with 0.1M MG in 0.1 M sodium phosphate buffer (pH 7.4) at 37 °C for 1, 6, and 24 hours. This experiment was repeated three times (n=3). The bar graph shows the mean ± S.D (*), and (**) signify a statistically significant difference ($p<0.05$ and $p<0,001$).

3.1.3.3 Glycated of opticin with MG in vitro using SDS-PAGE analysis The result shows the MG (0.1M) glycated opticin (1mg/ml) when incubated at different time courses, and opticin in ratio did show lighter cross-linked AGEs (Figure 3.4), the subunits of cross-linked AGEs cause the formation of dimers with a molecular weight of about 90 kDa. The glycated opticin (Figure 3.4), lanes A showed no glycation at time zero, in contrast the glycation increase when increasing the time up to 24 hours.

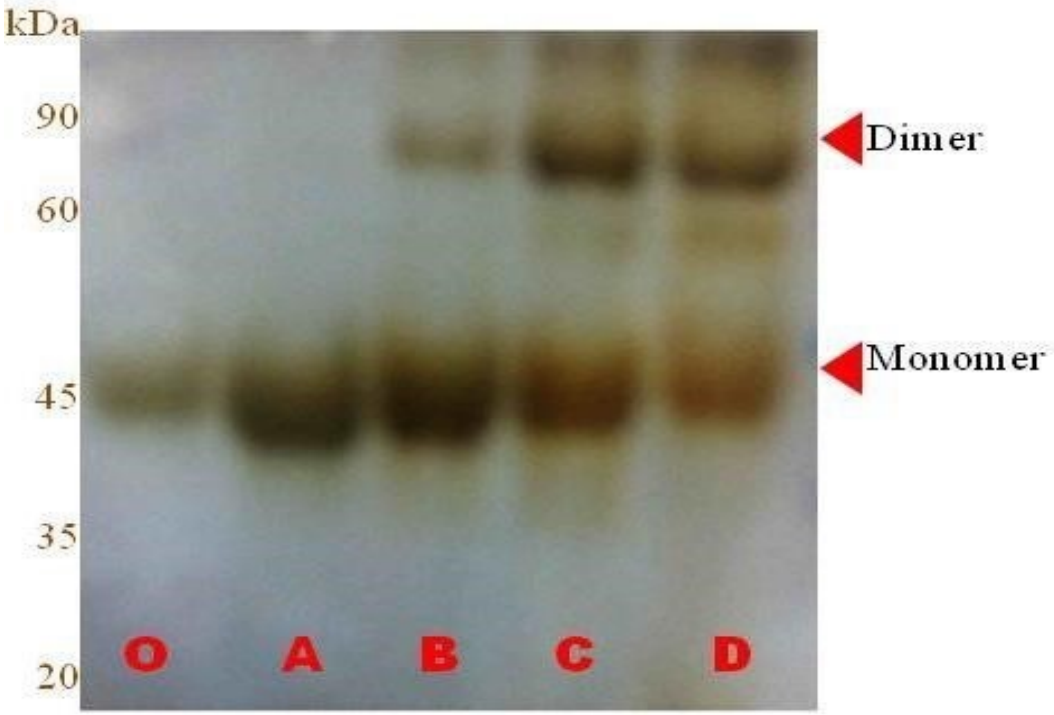


Figure 3 .4 Glycation of opticin (1 mg/ml) with MG (0.1M) up to 72 hours. The image is demonstrated glycation of opticin with 0.1M MG. Opticin (1 mg/ml) was incubated alone or in the presence of 0.1 M MG in 0.1 M sodium phosphate buffer, pH 7.4 at 37°C for different time.O: opticin alone; A: zero time; B: 30 minutes; C: 24 hours; D: 72 hours. The cross linking was determined by silver stain. This experiment was repeated three times and a representative SDS-PAGE gel is shown.

3.1.3.4 Glycation of opticin for up to 72 hours with MG

SDS-PAGE was used to study the effect of periods of incubation on AGE formation *in vitro*. Early glycation products were determined for varying time intervals as shown in figure 3.5, by using opticin at 1mg/ml and MG (0.1M) in 0.1 M sodium phosphate buffer (pH 7.4). Results showed a gradual increase in the molecular weight of dimers of glycated opticin, which is as a characteristic of AGE formation, reaching its highest at 72 hours incubation. Control sample without MG showed no glycation compared to those incubated in the presence of MG (0 time, 30 minutes, 24 hours and 72 hours.). Formation of AGEs was linearly dependent on the incubation time.

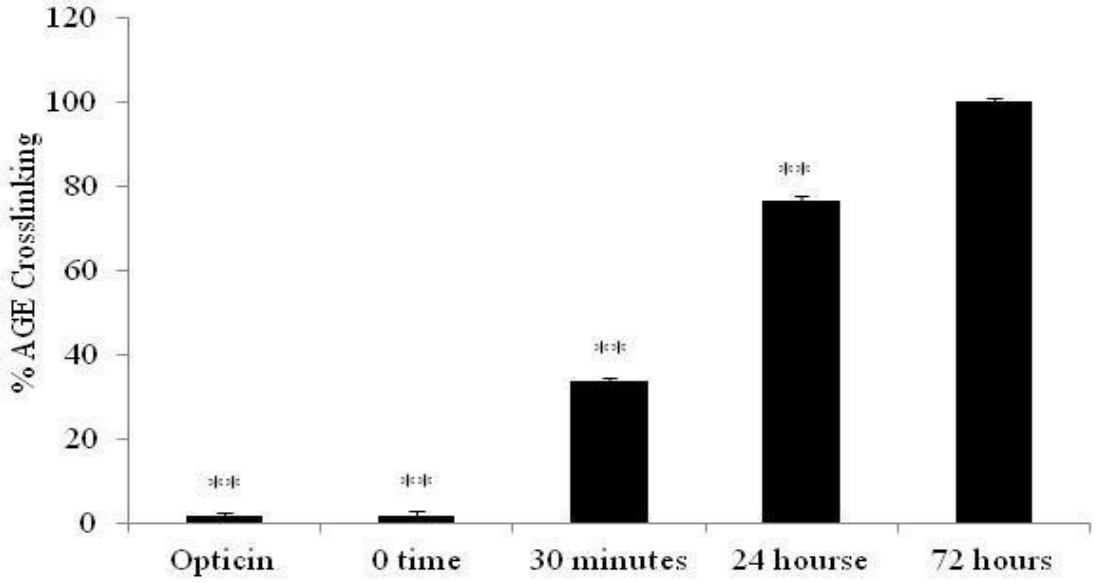


Figure 3 .5 Incubation of opticin for up to 72 hours with MG.
The effect of incubation for different time courses on AGE formation in the opticin-MG system. Opticin (1 mg/ml) was incubated with 0.1 MMG in 0.1 M sodium phosphate buffer of pH 7.4 at 37 °C for 30 minutes, 24, and 72 hours. This experiment was repeated three times (n=3). The bar graph shows the mean ± S.D (*) signify a statistically significant difference ($p<0.05$).

3.1.4 Glycation of opticin *in vitro* by glucose, fructose, and ribose

1mg/ml of opticin incubated with 0.5M glucose, 0.5M fructose, and 0.5M ribose, for zero time, 24 hours, and 72 hours. The subunits of cross-linked AGEs cause the formation of dimers with a molecular weight of 90 kDa respectively. As determined using the molecular weight markers (lane M). The glycated opticin (Figure 3.6, lanes O) showed no glycation at time zero (Figure 3.6, lanes;G1, F1, R1), in contrast, the opticin in the highest glycation when increase the time up to 72 hours with glucose, fructose, and ribose. Figure 3.6, lanes; G2, F2, R2. The native opticin was incubated alone and used as control. The M_r of glycated opticin was approximately twice of the original one (Figure 3.6, lane M).

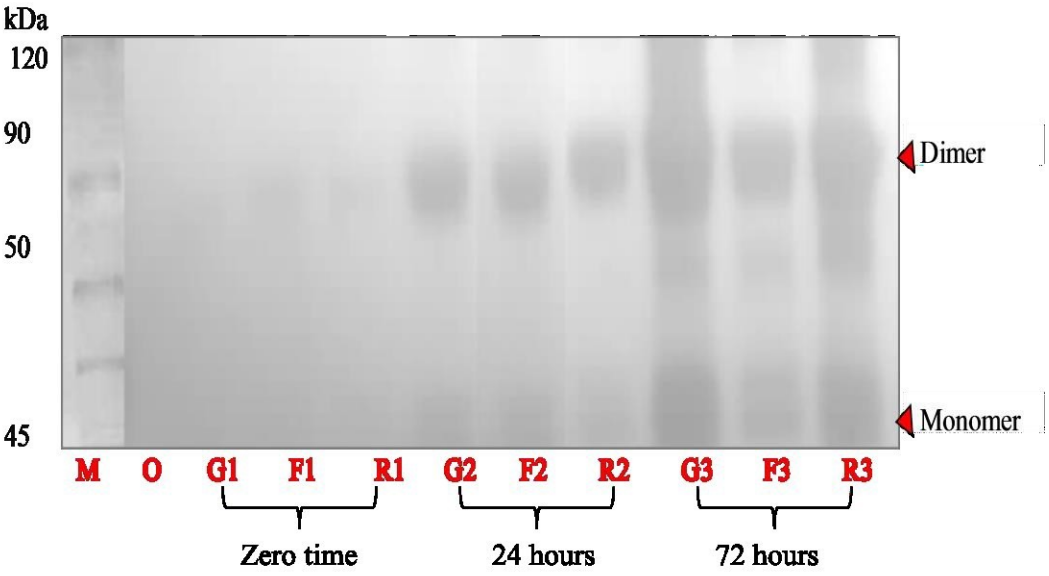


Figure 3.6 Glycation of opticin with glucose, fructose, and ribose for 72 hours.

The image demonstrates glycation of opticin by glucose, fructose, and ribose. Opticin (1 mg/ml) was incubated alone or in the presence of glucose, fructose, or ribose in 0.1 M sodium phosphate buffer, pH 7.4 at 37°C for different time courses. M: Marker; O: opticin alone; G1: glucose at zero time; F1: fructose at zero time; R1: ribose at zero time; G2: glucose at 24 hours; F2: fructose at 24 hours; R2: Ribose at 24 hours; G3: glucose at 72 hours; F3: fructose at 72 hours; R3: ribose at 72 hours. The cross linking was determined by silver stain. The experiment was repeated three times and a representative result is shown.

3.1.4.1 Glycation of opticin by glucose

SDS-PAGE was used to study the effect of periods of incubation on AGE formation *in vitro*. Early glycation products were determined for varying time intervals as shown in figure 3.7, by using opticin at 1mg/ml and 0.5M glucose in 0.1 M sodium phosphate buffer (pH 7.4). Results showed a gradual increase in the molecular weight of dimers of glycated opticin, which is as a characteristic of AGE formation, reaching its highest at 72 hours incubation. Control sample without glucose showed no glycation compared to those incubated in the presence of glucose (0 time, 24 and 72 hours.) Formation of AGEs was linearly dependent on the incubation time.

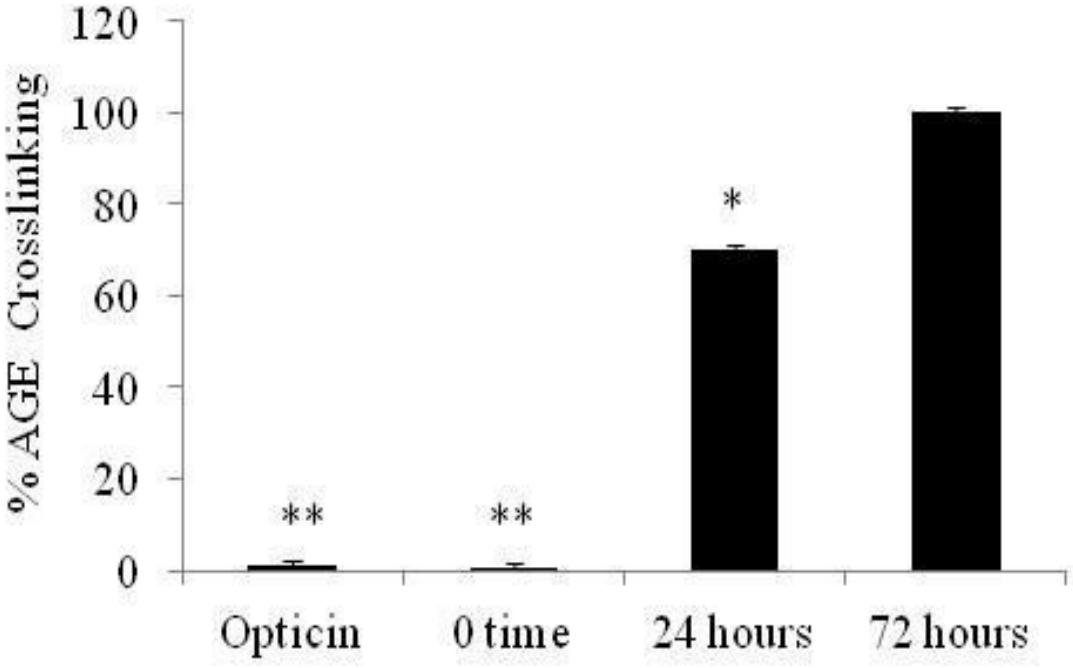


Figure 3.7 AGE formation in the opticin-glucose system.
The effect of incubation different time courses on AGE formation in the opticinMG system. Opticin (1 mg/ml) was incubated with 0.5 Mglucose in 0.1 M sodium phosphate buffer of pH 7.4 at 37 °C for 30 minutes, 24, and 72 hours. This experiment was repeated three times (n=3). The bar graph shows the mean ± S.D (*) and (**) signify a statistically significant difference ($p<0.05$, and $p<0.001$) compared with opticin.

3.1.4.2 Glycation of opticin by fructose

SDS-PAGE was used to study the effect of periods of incubation on AGE formation *in vitro*. Early glycation products were determined for varying time intervals as shown in figure 3.8, by using opticin at 1mg/ml and 0.5M fructose in 0.1 M sodium phosphate buffer (pH 7.4). Results showed a gradual increase in the molecular weight of dimers of glycated opticin, which is as a characteristic of AGE formation, reaching its highest at 72 hours incubation. Control sample without fructose showed no glycation compared to those incubated in the presence of fructose (0 time, 24 and 72 hours.). Formation of AGEs was linearly dependent on the incubation time.

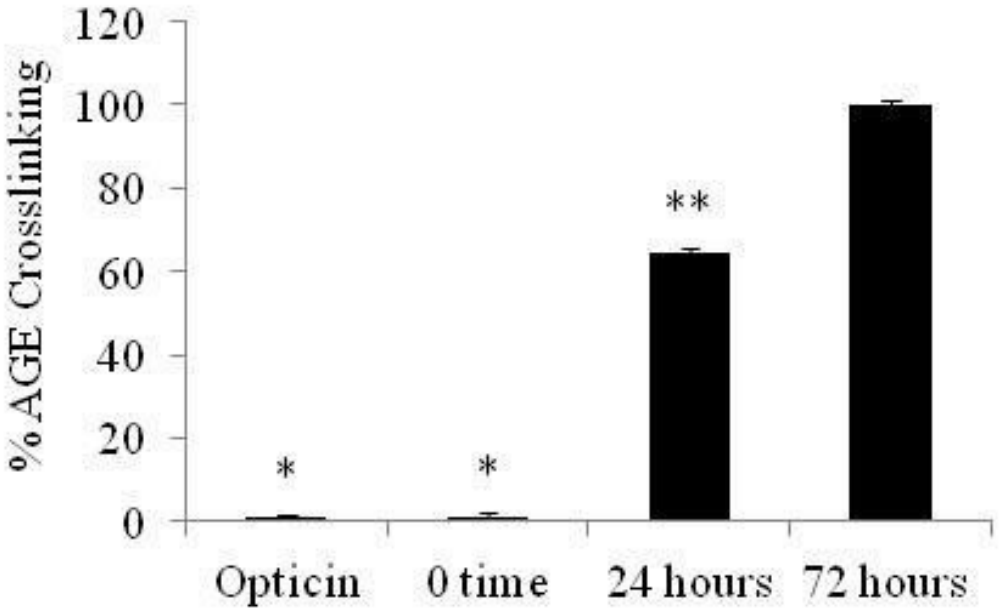


Figure 3 .8 AGE formation in the opticin-fructose system.

The effect of incubation different time courses on AGE formation in the opticinMG system. Opticin (1 mg/ml) was incubated with 0.5 M fructose in 0.1 M sodium phosphate buffer of pH 7.4 at 37 °C for 30 minutes, 24, and 72 hours. This experiment was repeated three times (n=3). The bar graph shows the mean ± S.D (*) and (**) signify a statistically significant difference ($p<0.05$, $p<0.001$).

3.1.4.3 Glycation of opticin by ribose

SDS-PAGE was used to study the effect of periods of incubation on AGE formation *in vitro*. Early glycation products were determined for varying time intervals as shown in figure 3.9, by using opticin at 1mg/ml and 0.5M ribose in 0.1 M sodium phosphate buffer (pH 7.4). Results showed a gradual increase in the molecular weight of dimers of glycated opticin which is as a characteristic of AGE formation, reaching its highest at 72 hours incubation but the levels was decreased at 72 hours. Control sample without ribose showed no glycation compared to those incubated in the presence of ribose (0 time, 24 and 72 hours.) Formation of AGEs was linearly dependent on the incubation time.

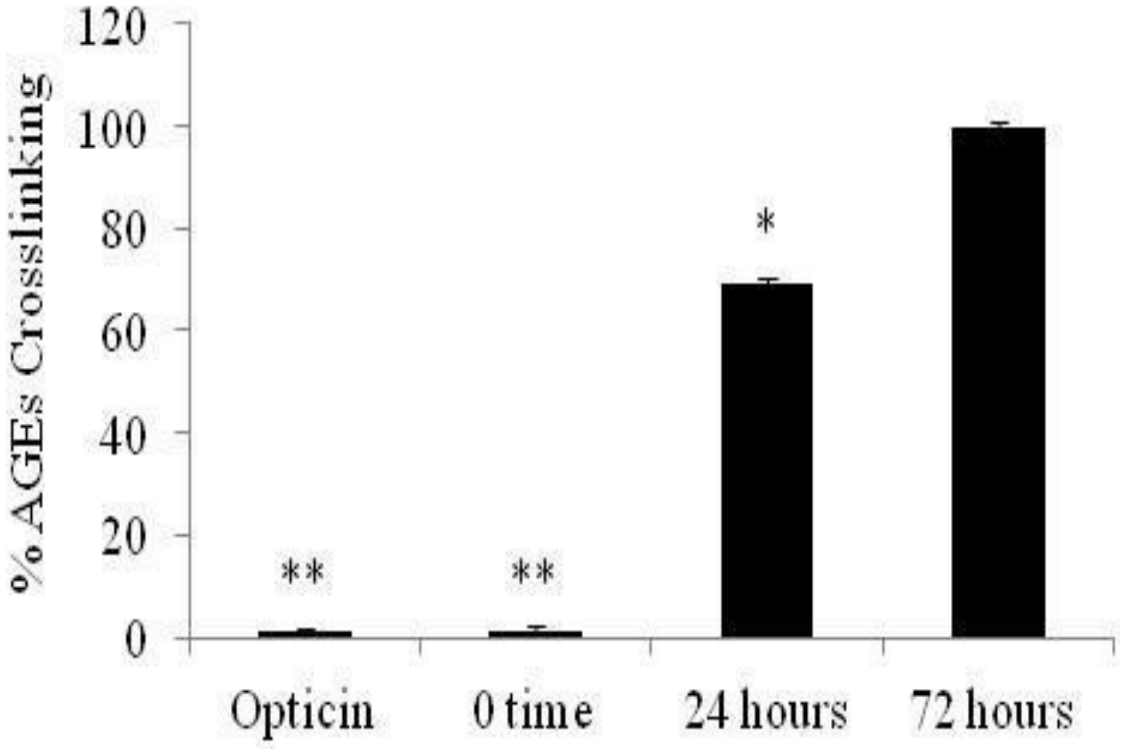


Figure 3 .9 AGE formation in the opticin- ribose system.

The effect of incubation different time courses on AGE formation in the opticinribose system. Opticin (1 mg/ml) was incubated with 0.5 Mribose in 0.1 M sodium phosphate buffer of pH 7.4 at 37 °C for 30 minutes, 24, and 72 hours. This experiment was repeated three times (n=3).). The bar graph shows the mean ± S.D (*) and (**) signify a statistically significant difference ($p<0.05$, $p<0.001$).

3.1.5 Detection of AGE using MALDI/TOF Spectra

As accuracy of molecular mass determination by SDS-PAGE is severely limited, the MALDI-TOF-MS were used for the mass determination of intact, modified proteins. (Savas, *et al*., 2011). MALDI spectra was obtained by incubating native opticin (1mg/ml) with MG (0.1M) at 37 °C for different time (0-24 hours).The molecular ion of native opticin produced as mass peak of m/z 45780 (Figure 3.10.A). This was calculated. In all of the modified opticin, at least on additional peak was observed. The mass were less increasing at time zero of incubation *m/z* (46401) (Figure 3.10.B), while the mass increase in 30 minutes (Figure 3.10.C) in to m/z (47094). After 6 hours (Figure 3.10.D), there was a further mass increase, components of *m/z* (47200) and after 24 hours the mass was m/z (47401) (Figure 3.10.E). This mass distribution was due to multiple modifications by MG. The resulting mass difference was probably caused multiple modifications with the Amadori product an early stage Maillard product, as well as post-Amadori modifications. Based on this mass shift and a theoretical sequence for this species, it should be possible to propose an approximate number of glycation sites modified assuming the mass differences for modified Cys, Arg and Lys are known.

The number of sugar molecules condensed on the reactive sites of protein can be calculated by using the mass difference (ΔM) between glycated and native proteins (Lapolla *et al*. 2000). MG can form different AGE modifications on lysine, arginine, and cysteine residues of the protein resulting in mass difference (ΔM) of 531-1531 Da between (native and glycated). The number of MG moles per mole of opticin was calculated (Figure 3.11).

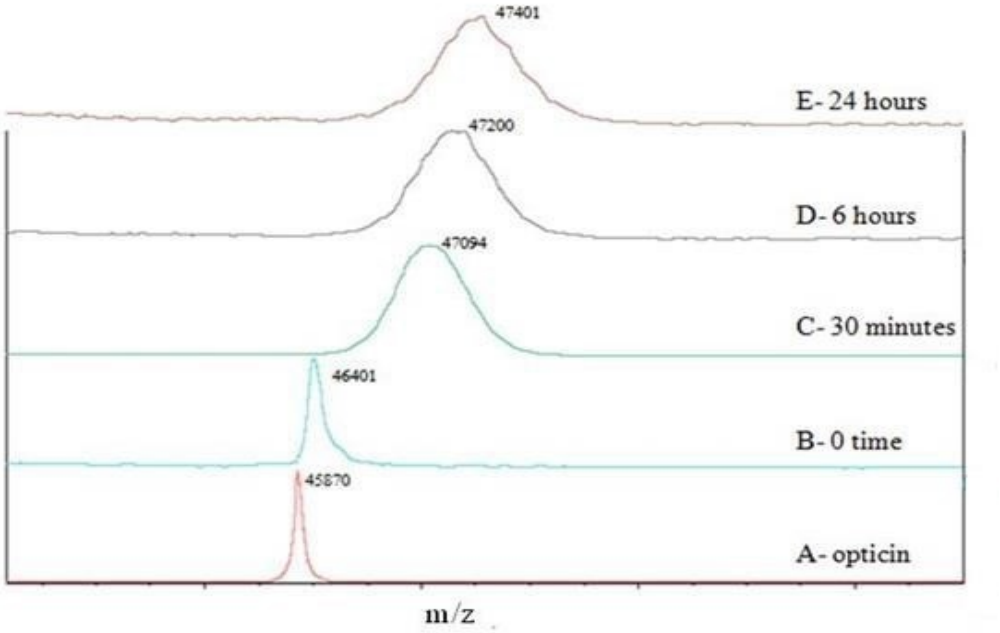


Figure 3 .10 MALDI-TOF analysis of opticin incubated in MG.

Opticin(1mg/ml) was incubated alone(A) or with MG (0.1M) for zero time (B), 30 minutes (C), 6 hours (D), and 24 hours (E) at 37°C , and then subjected to MALDI-TOF-MS as described in materials and methodted. The expirment were repeated three times, the. A representative mass spectrum from independent experiments is shown for each condition.

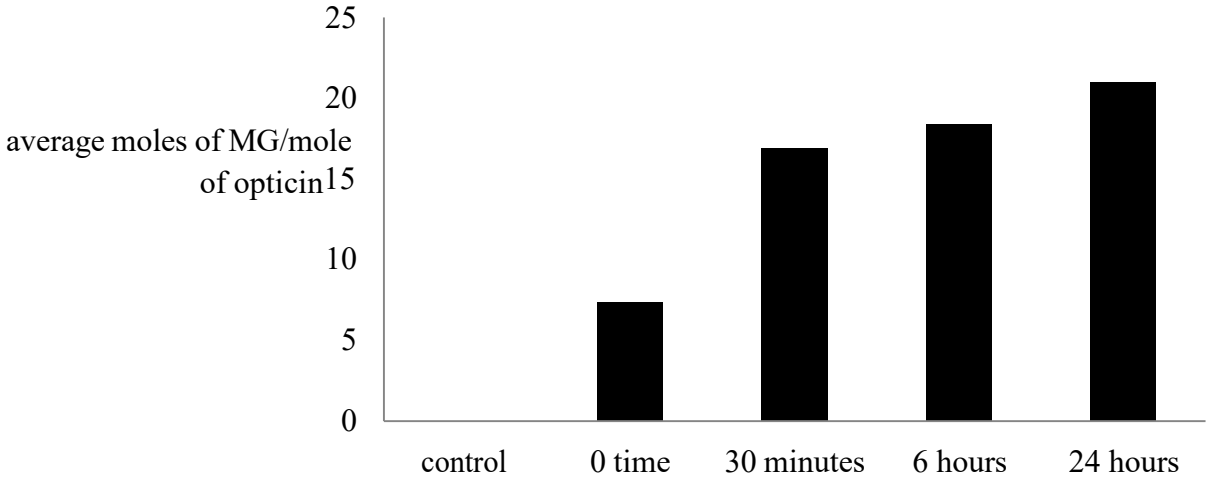


Figure 3.11 Time course study of average number of moles of MG attached to each mole of opticin.

Opticin (1mg/ml) was incubated with 0.1MG and in buffer. MALDI-TOF-MS was used for the analysis of intact protein. The difference in *m/z* values for each sample is used to calculate the number of moles attached to each mole of opticin.

Results
Section 2: *in vitro* and *in vivo* angiogenesis assays

3.2 *In vitro* angiogenesis assays

Many *in vitro* and *in vivo* angiogenesis assays used to study new therapeutic agents that either stimulate or inhibit angiogenesis*, In vitro* angiogenesis assays are used to study a particular step in the angiogenic process:

Cell proliferation study: A cell proliferation assay was used to identify the effect of compounds on BAEC growth in the presence of FGF-2

Cell Migration/wound-healing study: A cell migration assay was used to identify the inhibitory potential of compounds on EC migration when induced with FGF-2.

Cell differentiation study: (Matrigel tube-formation): This assay was used to identify the effects of GOPT on tube formation. These assays give an insight into the angiogenic cascade, and can recognize molecular mechanisms of action of molecules of interest.

FGF-2 is preferentially involved in the process of angiogenesis (Ornitz *et al*, 2001; Barrientos *et al*, 2008). FGF-2 induces ECs proliferation, migration, and angiogenesis in vitro (Basilico and Moscatelli, 1992).

3.2.1 Cell Proliferation assay

There are many different ways of performing cell proliferation assays. In this thesis a method of direct counting of total cells was carried out, as described in the Material and Methods (chapter 2). FGF-2 induced cell proliferation, migration in mediated through intracellular signal pathways involving ERK1/2 (Slevin, *et al*., 2008). The effect of native opticin on FGF-2-induced angiogenesis and antiangiogenic potential of opticin were studied in a proliferation assay (Le Goff*, et al*., 2012).

The effect of glycated opticin on FGF-2-induced angiogenesis was studied in a proliferation assay, using BAECs on *in vitro*. The inhibation of anti-angiogenic potential of GOPT in a representative experiment is depicted in Figure 3.12. The addition of opticin (native and glycated at 25µg/ml) with FGF-2 (25ng/ml) to medium containing 2 % FBS. After three days of incubation;

After three days of incubation, GOPT + FGF-2 significantly less inhibit the cell number by 1.7–fold (p= 0.011) arespectively compared with the control cells (Figure 3.1). The highest stimulation was induced by FGF-2 alone by 2.0-fold (p=0.0005) compared to control cells and a significant inhibition with opticin + FGF-2 by 1.4- fold (p = 0.002). Figure 3.12).

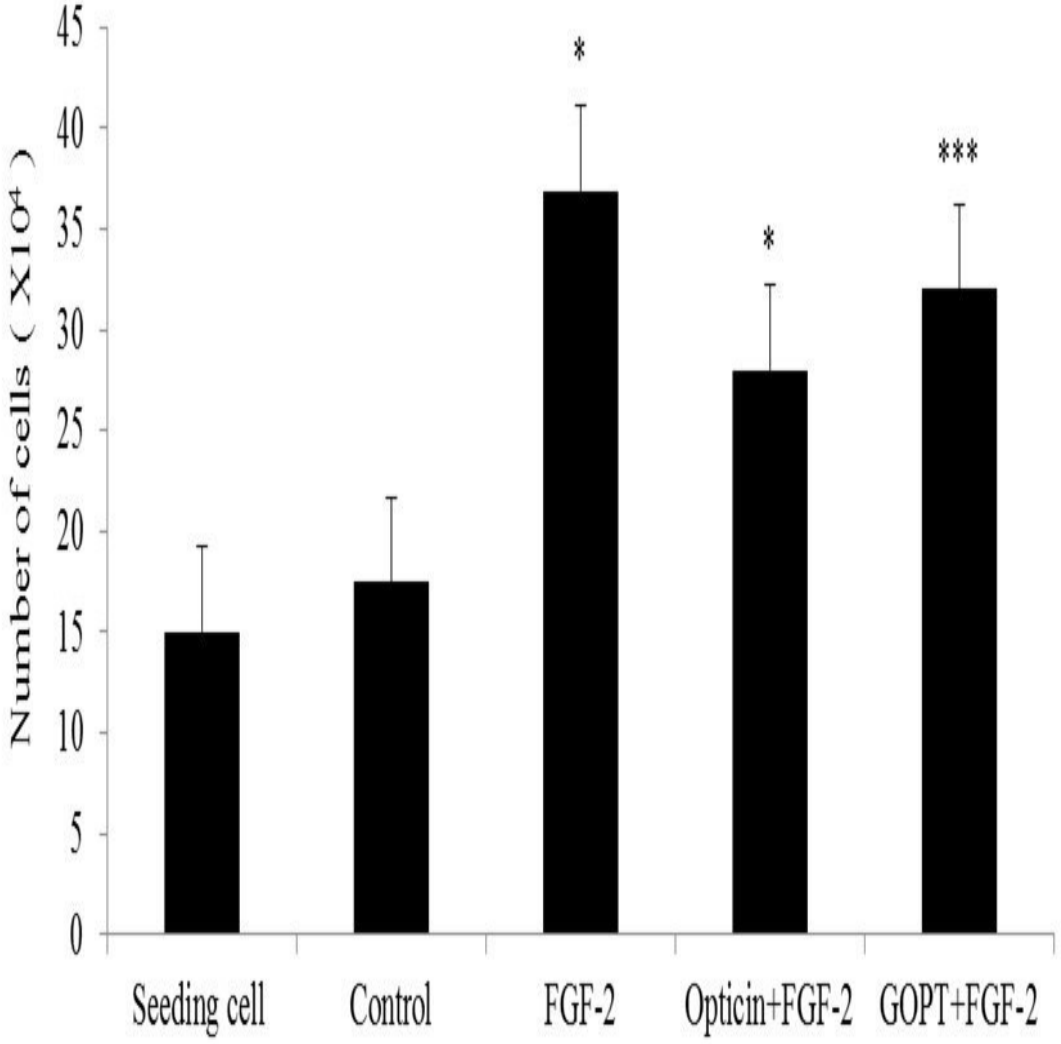


Figure 3.12 Anti-anti-angiogenic effects of GOPT combined with FGF-2 on BAEC proliferation.

BAECs ($1.5x10^5$ cell/ml) were allowed to grow and adhere in 24-well plates. The bar graph shows the stimulatory effects of opticin (native and glycated at 25µg /ml), FGF-2 (25ng /ml) and their combination on BAEC proliferation after 72 hours incubation. Cells were counted using a Coulter counter. Experiments were performed in triplicate wells and repeated three times (n=3). The bar graph shows the mean ± S.D. (*), and (***) signify a statistically significant difference ($p < 0.05$, and $p<0.001$) compared to the control. A representative example is shown.

3.2.2 Cell Migration assay a (Wound healing)

ECs are capable of migration along a gradient of angiogenic molecules such as FGF-2. Two methods to assess EC migration were used in this thesis: a wound healing assay where migrate into a denuded region created by scraping a confluent monolayer of ECs or a modified Boyden chamber chemotaxis assay, where ECs orientate and migrate towards FGF-2 through a polycarbonate filter. The effect of GOPT on FGF-2-induced angiogenesis was studied using BAEC at $1.5x10^5$ cell/ml. The confluent and uniform monolayer of BAEC was wounded with a sterile blade. (A) Control (B) with FGF-2 (25ng /ml), (C) Opticin (25µg /ml), (D) GOPT (25µg /ml), (E) the combination of (opticin/FGF-2) and (F) the combination of (GOPT/FGF-2) for 24 hours. The numbers of migrated cells in the denuded area were counted (original magnification X 40, scale bar = 100 µm). Experiments were performed in triplicate wells and repeated three times. The inhibition of anti-angiogenic potential of GOPT in a representative experiment is depicted in (Figure 3.13 and 3.14), the resut shows;

After 24 hours of incubation, GOPT + FGF-2 significantly less inhibit the number of cell migration by 2.7–fold (p= 0.002) arespectively compared with the control cells (Figure 3.13). The highest stimulation was induced by FGF-2 alone by 2.8-fold (p=0.0002) compared to control cells and a significant inhibition with opticin + FGF-2 by 1.8- fold (p = 0.001) (Figure 3.13).

In addition, distance of cell migration;

After 24 hours of incubation, GOPT + FGF-2 significantly less inhibit the distance of cell migration by 2.0 –fold (p= 0.0003) arespectively compared with the control cells (Figure 3.1). The highest stimulation was induced by FGF-2 alone by 2.2-fold (p=0.003) compared to control cells and a significant inhibition with opticin + FGF-2 by 2.2- fold (p = 0.0003) (Figure 3.13).

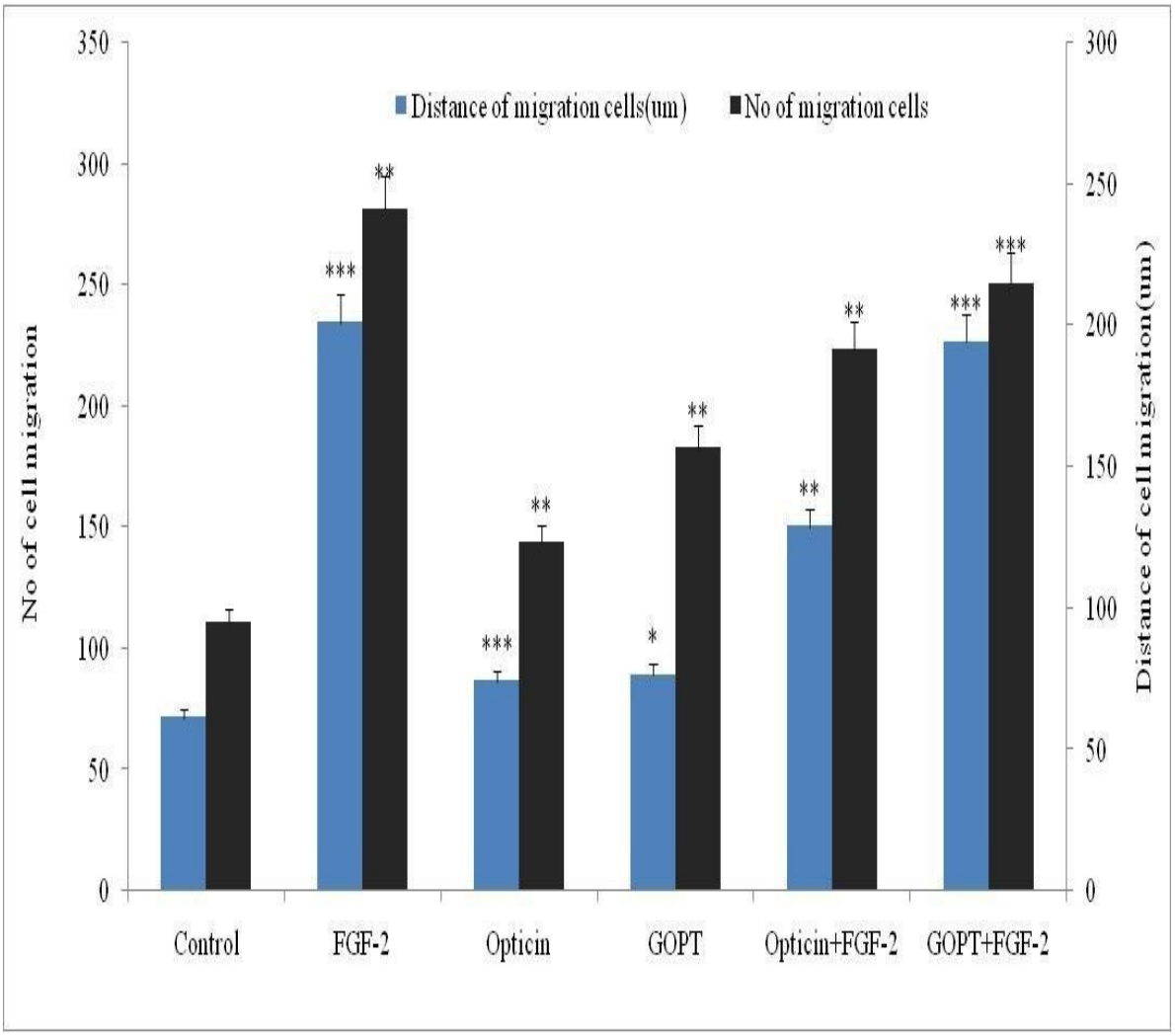


Figure 3.13 Effect of opticin1mg/ml (native and Glycated), on cell migration induced by FGF-2 on BAEC using a wound healing assay.

The confluent and uniform monolayer of BAEC was wounded with a sterile razor blade. Opticin (native and glycated), and FGF-2 were added. After incubation for 24 hours, the number of migrated cells in the denuded area was counted in five random areas and distance of migration per well (original magnification X40, Scale bar = 100 µm). The bar graph shows the stimulatory effects on FGF-2 (25ng /ml) and of GOPT (25µg /ml). Experiments were performed in triplicate wells and repeated three times (n=3). The bar graph shows the mean ± S.D. (*), (**), (***) signify a statistically significant difference ($p < 0.05$, $p < 0.01$, and $p<0.001$) compared to the control. A representative example is shown.

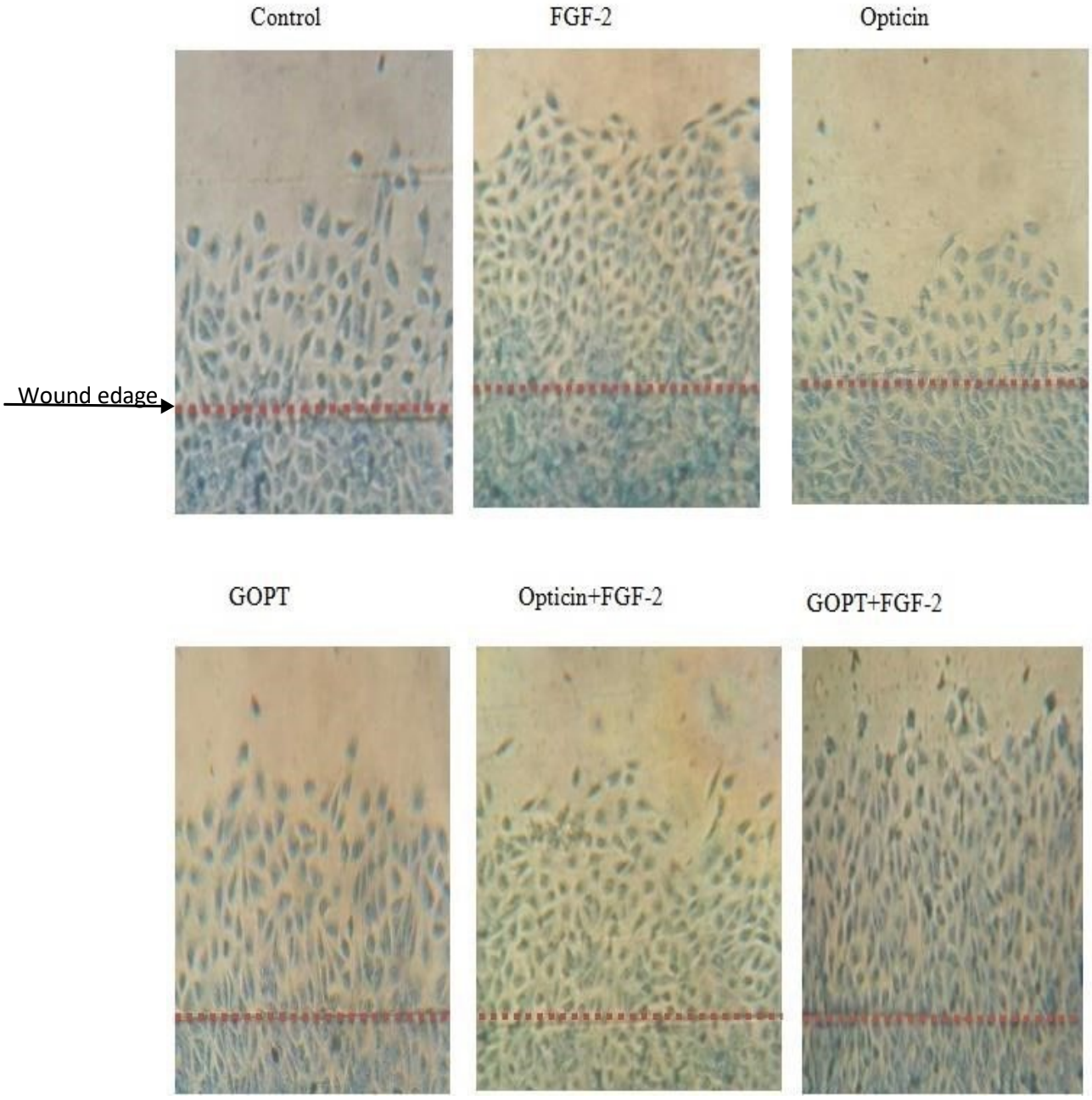


Figure 3.14 The effect of GOPT on FGF-2-induced BAEC migration.

BAECs were seeded on coverslips at a concentration of $1.5x10^5$ cell/well in DMEM containing 15% (v/v)FBS, wounded with a razor blade and incubated for 24 hour, to observe the number of ECs migrating into the denuded region. Images were taken using a phase contrast microscope (original magnification X40, scale bar = 100 µm) and a digital camera. Experiments were performed in triplicate wells and repeated at least three times. A representative example is shown.

3.2.2.1 Cell migration using the chemotaxis assay (Boyden chamber)

After 18 hours of incubation, opticin (native and glycated at 25µg/ml), FGF-2 (25ng/ml) and their combination exerted chemotactic effects on BAEC, and significantly increased the movement of cells through the filter, compared to the control. BAEC (1.5 x 10^5 cell/ml) in 100 µl of a medium were added to the upper part of a modified Boyden chamber and chemoattractant GOPT, FGF-2, and OPT + FGF-2 were added to the lower part of the Boyden chamber. Cell movement to the lower chamber was monitored under an inverted phase contrast microscope, and quantified by counting the movements through the porous membrane to the lower chamber. Results from Boyden chamber experiments indicated that:

In addition, distance of cell migration;

GOPT + FGF-2 significantly less inhibit the distance of cell migration by 1.5 - fold (p= 0.01) arespectively compared with the control cells .The highest stimulation was induced by FGF-2 alone by 1.9-fold (p=0.01) compared to control cells and a significant inhibition with opticin + FGF-2 by 1.5- fold (p = 0.01 A representative example is shown in (Figure 3.15).

The effects of GOPT and FGF-2 on BAEC chemotaxis (Boyden chamber) were determined and show increase in number of migrated cells in comparison opticin + FGF-2 cells, because GOPT had less inhibit effect on FGF-2 than opticin.

A representative example is shown in (Figure 3.15).

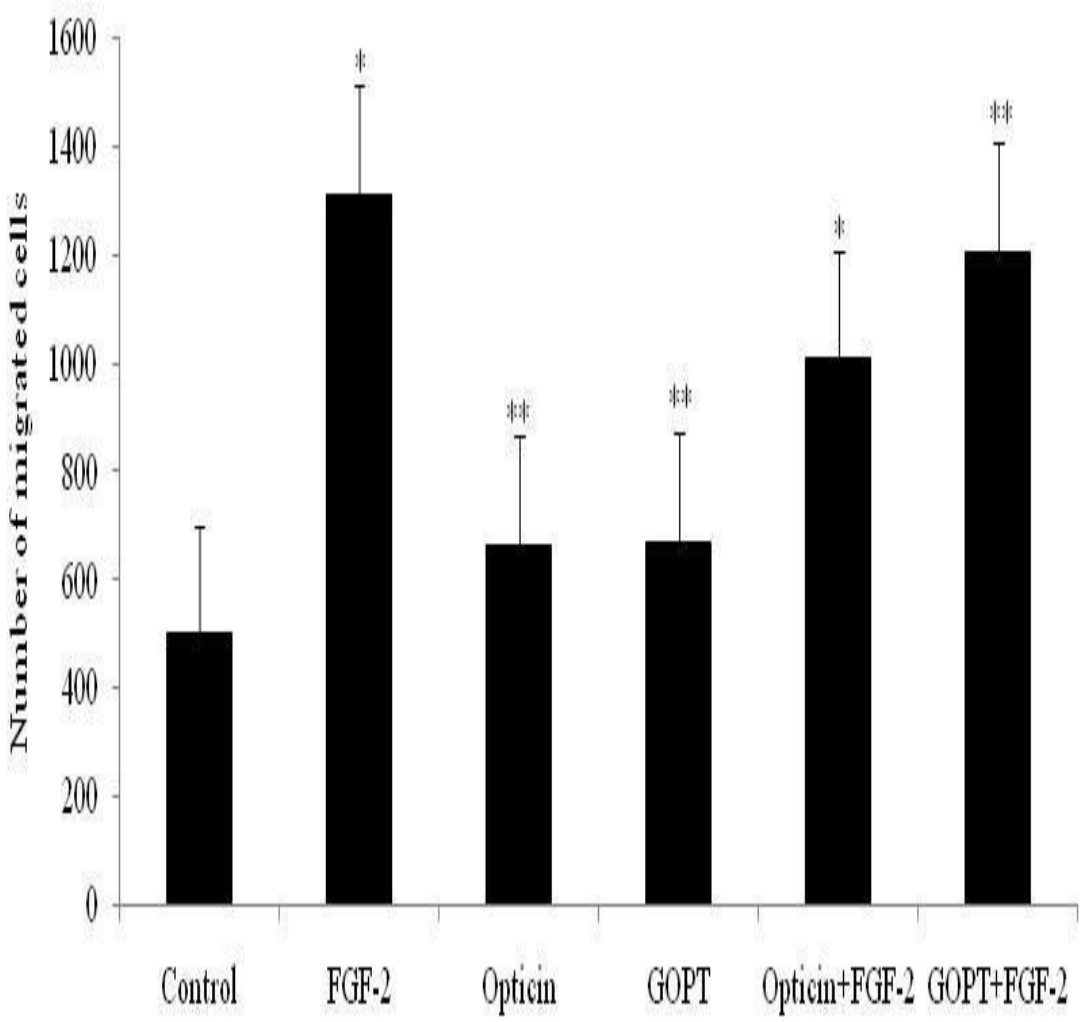


Figure 3-15 Effect of GOPT, FGF-2 and their combination on BAEC migration BAEC (1.5 x 10^5 cell/ml) in 100 µl of a medium was added to the upper portion of the Boyden chamber. Opticin (native and glycated at 25µg/ml), FGF-2 (25ng/ml) and their combination were added to the lower chamber and incubated for 18 hours. The total numbers of migrated cells were counted. Experiments were performed in duplicate wells and repeated three times (n=3). The bar graph shows the mean ± S.D. (*) and (**) signify a statistically significant difference ($p < 0.05$ and $p < 0.01$) compared with the control. A representative example is shown.

3.2.3 EC formation of tube-like structures in different matrices

Further examination of anti-anti angiogenic effect of GOPT was carried out by measuring the extent of BAEC tube formation in type-I collagen or a reconstituted basement membrane, growth factor reduced Matrigel^{TM}. EC tube formation assays in different extracellular matrices are an effective way of assessing *in vitro* angiogenesis. The assays are highly reproducible and can adapt to test various molecules alone or in combination with others.

3.2.3.1 EC tube formation in a type-I collagen gel-'sandwich' method

BAECs were seeded on coverslips at a concentration of $3.4x10^4$ cell/well in DMEM containing 1% FBS. Theinhibation of anti angiogenic possible of GOPT in a representative experiment with tube formation assay 'sandwich' methods is depicted in (Figure 3.16 and 3.17) respectively. The result shows:

GOPT + FGF-2 significantly less inhibit the distance of cell migration by 4.0 –fold (p= 0.07) arespectively compared with the control cells. The highest stimulation was induced by FGF-2 alone by 4.2-fold (p=0.001) compared to control cells and a significant inhibition with opticin + FGF-2 by 2.6 fold (p = 0.01 A representative example is shown in (Figure 3.16). There is no further significant differences were obtained when compared to the individual stimulation of either opticin by 0.96 fold (p = 0.01), GOPT by 0.96 fold (p = 0.013)

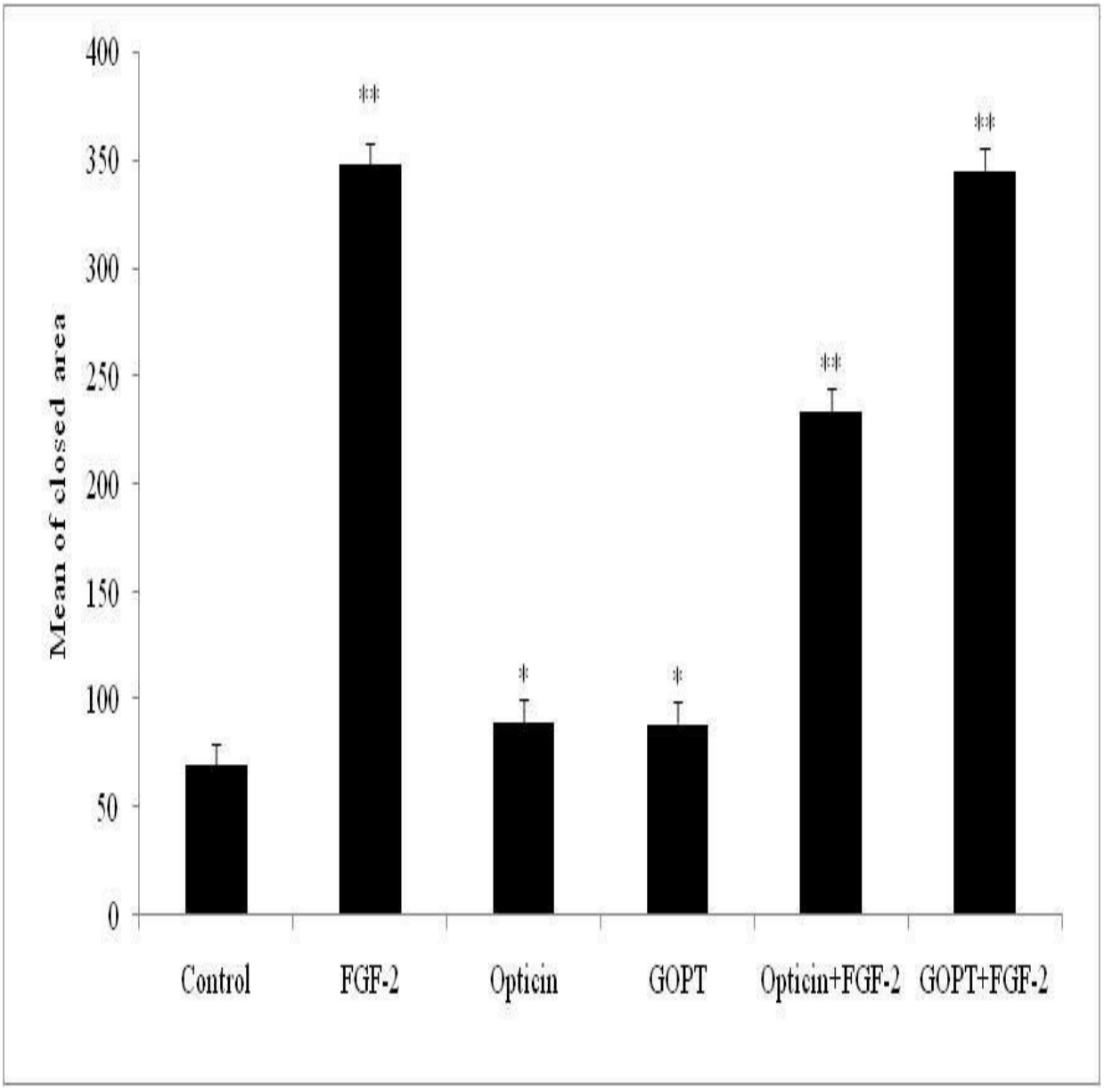


Figure 3-16 Anti-anti-angiogenic effects of GOPT and FGF-2 on BAEC tube formation.

The bar graph shows the stimulatory effects of opticin (25 µg/ml: native and glycated), FGF-2 (25ng /ml) and their combination on BAEC tube formation after 24 hours. The number of closed areas was counted. Experiments were performed in triplicate wells and repeated at least three times (n=3). The bar graph shows

the mean ± S.D. (*) and (**) signify a statistically significant difference ($p < 0.05$ and $p < 0.01$) compared with the control. A representative example is shown.

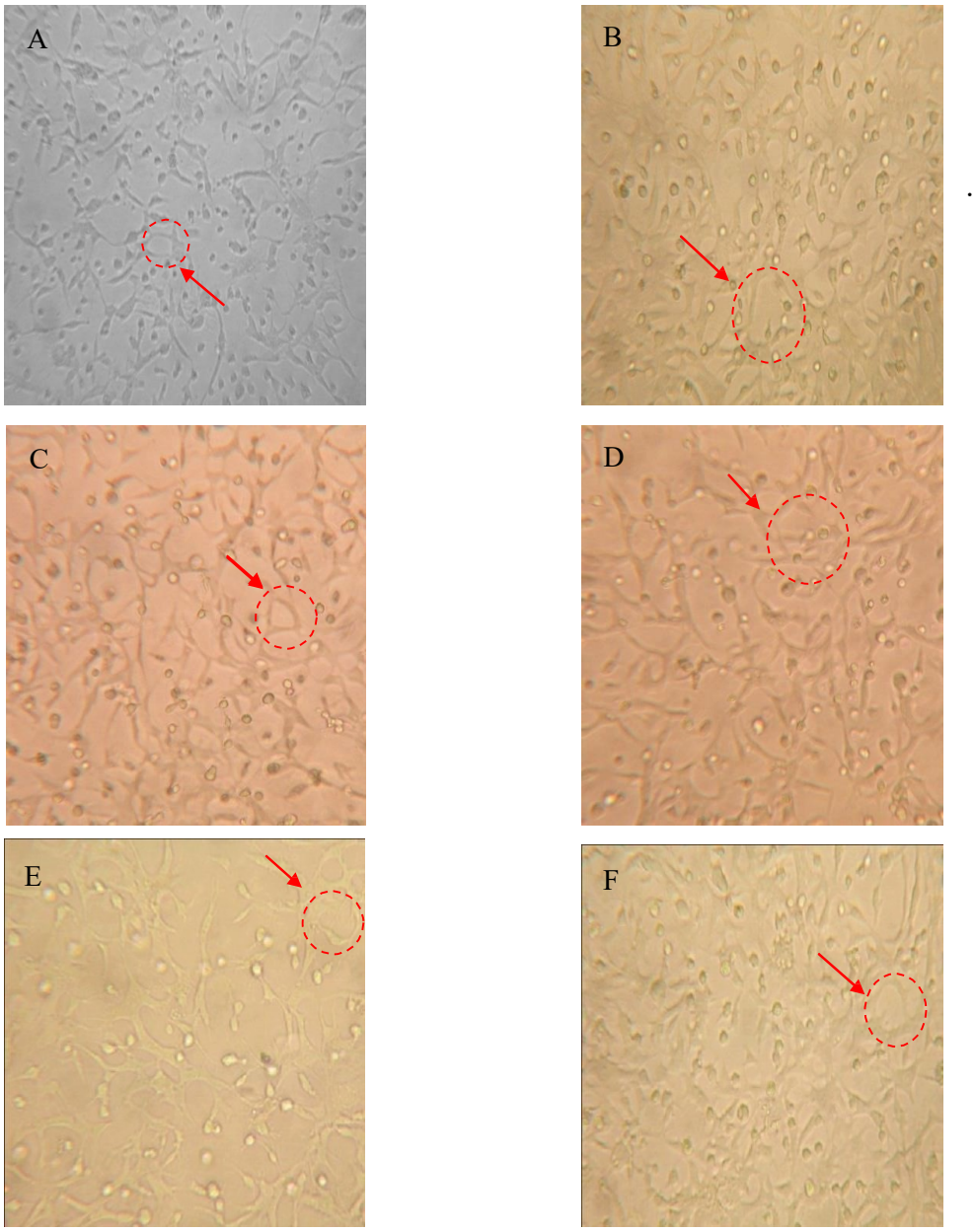


Figure 3.17 Photomicrograph showing the anti-anti-angiogenic effects of GOPT on FGF-2 on BAEC tube formation in collagen type I.

(A) Control, (B) FGF-2, (C) Opticin, (D) GOPT,(E) Opticin + FGF-2 , (F) GOPT + FGF-2 The arrow towards the circle indicates a closed area of collagen , which was used to quantify the results (original magnification X100).

3.2.3.2 EC tube formation of BAECs in a Matrigel^{TM} matrix

BAECs were seeded on coverslips at a concentration of .2.4x10^4 cell/well in DMEM containing 2% FBS. The inhibition of anti-angiogenesis potential of opticin in a representative experiment using a tube formation assay in Marigel^{MT} matrix is depicted in (Figure 3.18 and 3.19) for BAECs respectively. In tube formation assay the addition of recombinant opticin (native and glycated) at 25 µg /ml to Matrigel^{ML} and medium containing 2% FBS, and 25ng/ml, the results showen;

GOPT + FGF-2 significantly less inhibit the distance of cell migration by by 4.0 fold ($p = 0.004$) arespectively compared with the control cells. The highest stimulation was induced by FGF-2 alone by 4.0 fold ($p = 0.002$).compared to control cells and a significant inhibition with opticin + FGF-2 by 3-fold ($p = 0.001$). A representative example is shown in (Figure 3.18).

There is no further significant differences were obtained when compared to the individual stimulation of either opticin by 0.9 fold ($p = 0.006$), GOPT by 1.0 fold ($p = 0.04$) respectively

There is signficatly lower number of tube formed when FGF-2 was incubated in the precences of opticin than GOPT.

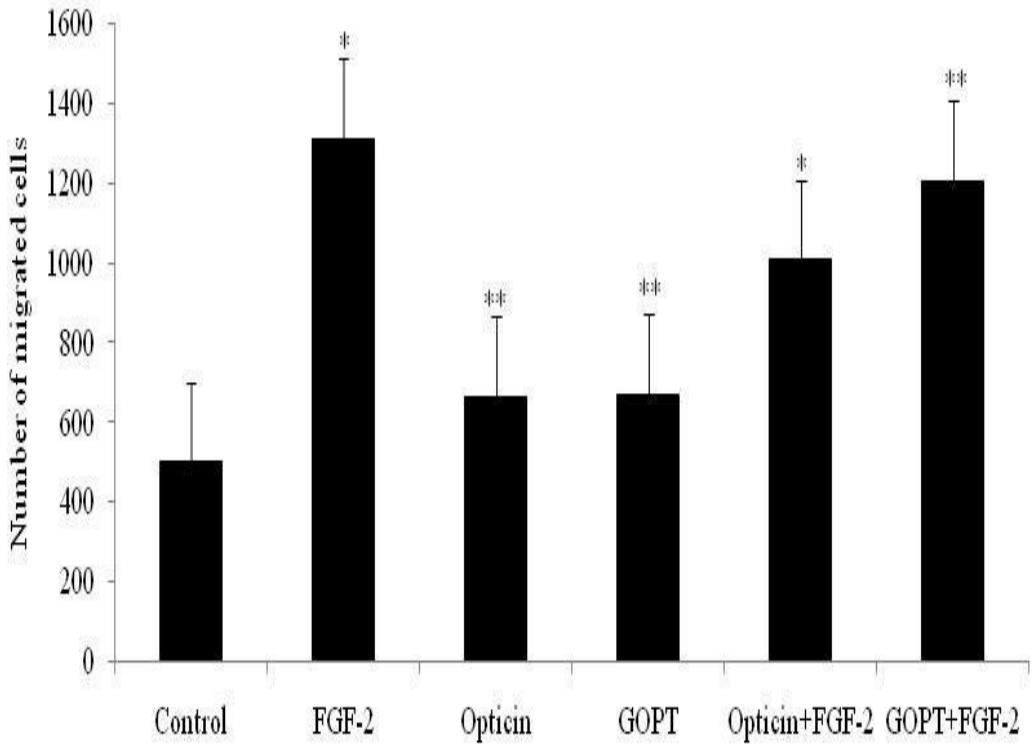


Figure 3-18 Anti-anti-angiogenic effects of GOPT and FGF-2 on BAEC tube formation.

The bar graph shows the stimulatory effects of opticin (25 µg/ml: native and glycated), FGF-2 (25ng /ml) and their combination on BAEC tube formation after 24 hours. The number of closed areas was counted. Experiments were performed in triplicate wells and repeated at least three times (n=3). The bar graph shows the mean ± S.D. (*), (**) signify a statistically significant difference ($p < 0.05$ and $p < 0.01$) compared with the control. A representative example is shown.

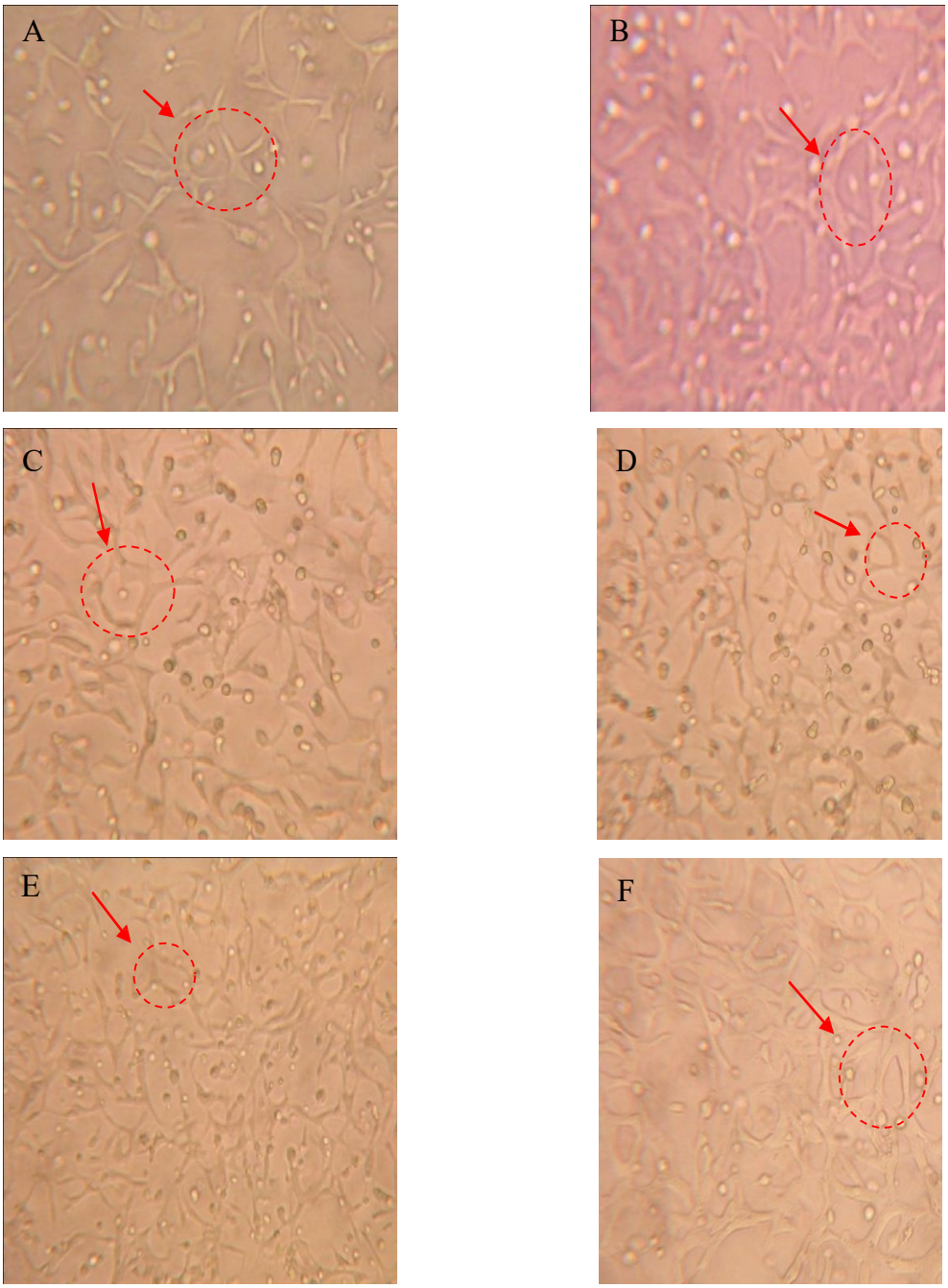


Figure 3-19 Photomicrograph showing the pro-angiogenic effects of Opticin on FGF-2 on BAEC tube formation (in Matrigel).

(A)Control, (B) FGF-2, (C) Opticin, (D) GOPT, (E) Opticin + FGF-2 , (E) GOPT + FGF-2 The arrow towards the circle indicates a closed area of a Matrigel tube, which was used to quantify the results (original magnification X100).

3.2.3.3 EC tube formation of HRECs in a Matrigel™ matrix

HRECs were seeded on coverslips at a concentration of $6.0x10^5$ cell/well in DMEM containing 2% FBS. The inhibition of anti-angiogenic potential of opticin in a representative experiment using a tube formation assay in Matrigel™ matrix is depicted in (Figure 3.20 and 3.21) for HRECs respectively. In tube formation assay the addition of recombinant opticin (native and glycated) at 25 µg/ml to Matrigel^{ML}, medium containing 2% FBS, and FGF-2 (25 ng/ml). The result shows; GOPT + FGF-2 significantly less inhibit the distance of cell migration by by 2.4 fold ($p = 0.008$) arespectively compared with the control cells (Figure 3.1). The highest stimulation was induced by FGF-2 alone by 2.6 fold ($p = 0.01$).compared to control cells and a significant inhibition with opticin + FGF-2 by 1.7-fold ($p = 0.01$). A representative example is shown in (Figure 3.20).

There is no further significant differences were obtained when compared to the individual stimulation of either opticin by 1.0 fold ($p = 0.02$), GOPT by 1.2 fold ($p = 0.06$) respectively.

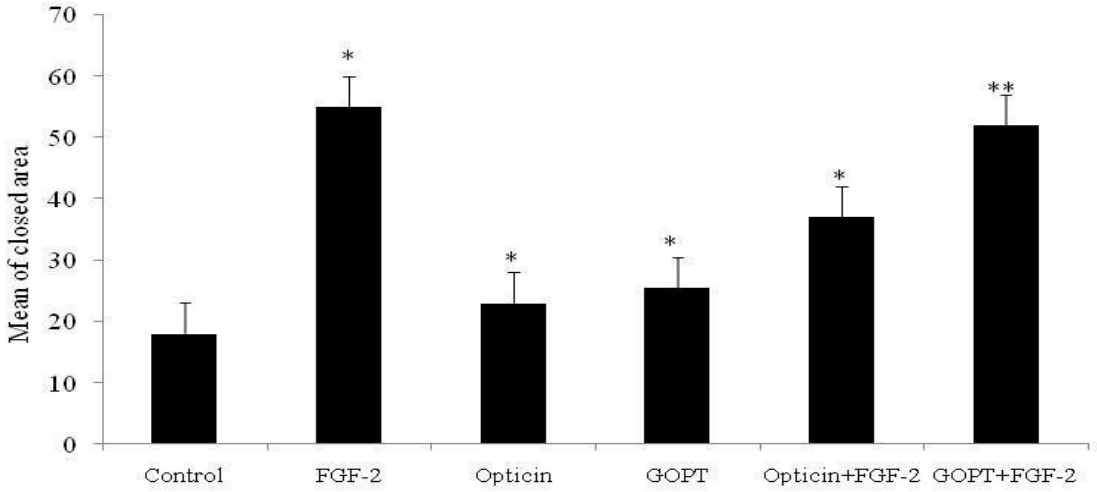


Figure 3.20 Anti-anti-angiogenic effects of GOPT and FGF-2 on HRECs tube formation.

The bar graph shows the stimulatory effects of opticin (25 µg/ml: native and glycated), FGF-2 (25ng /ml) and their combination on HREC tube formation after 24 hours. The number of closed areas was counted. Experiments were performed in triplicate wells and repeated at least three times (n=3). The bar

graph shows the mean ± S.D. (*) signify a statistically significant difference ($p < 0.05$) compared with the control. A representative example is shown.

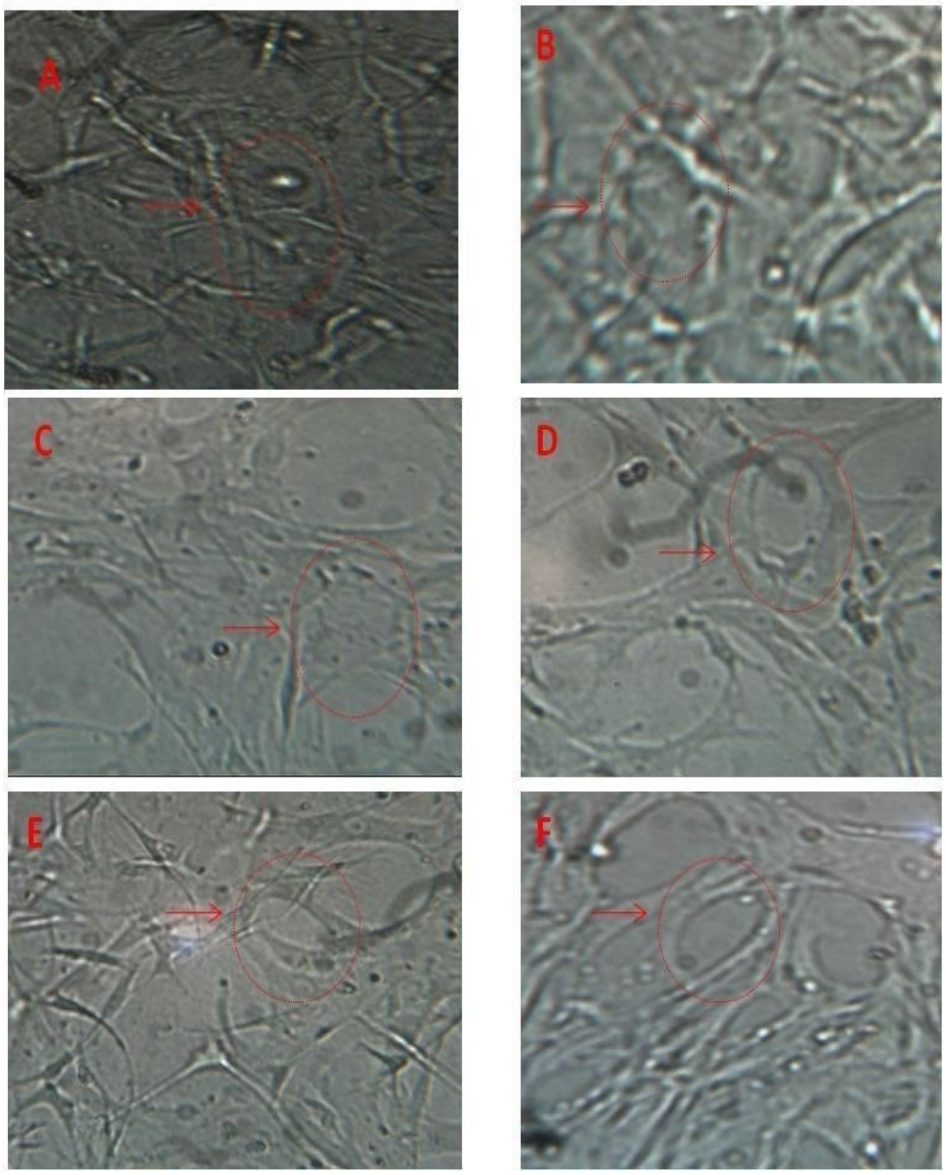


Figure 3.21 Photomicrograph showing the inhibition of antiangiogenic effects of Opticin on FGF-2 on HREC tube formation (in Matrigel).

A Control, (B) FGF-2, (C) Opticin, (D) GOPT, (E) Opticin + FGF-2, (E) GOPT + FGF-2, the slide stain with Giemsa stain. The arrow towards the circle indicates a closed area of a Matrigel tube, which was used to quantify the results (original magnification X100).

3.2.3.4 EC tube formation of HRECs in collagen type-I

HRECs were seeded on coverslips at a concentration of $1.0x10^6$ cell/ml in DMEM containing 2% FBS, opticin (native and glycated, and FGF-2 (25 ng/ml) The inhibition of anti-angiogenesis potential of opticin in a representative experiment using a tube formation assay in Matrigel^{TM} matrix is depicted in (Figure 3.22, and 3.23) for HRECs respectively. The result shows;

GOPT + FGF-2 significantly less inhibit the distance of cell migration by by 1.6 fold ($p = 0.04$) arespectively compared with the control cells. The highest stimulation was induced by FGF-2 alone by 2.0 fold ($p = 0.04$) compared to control cells and a significant inhibition with opticin + FGF-2 by 1.0 fold ($p = 0.03$). A representative example is shown in (Figure 3.22).

There is no further significant differences were obtained when compared to the individual stimulation of either opticin by 0.8 fold ($p = 0.007$), GOPT by 1.0 fold ($p = 0.03$) respectively.

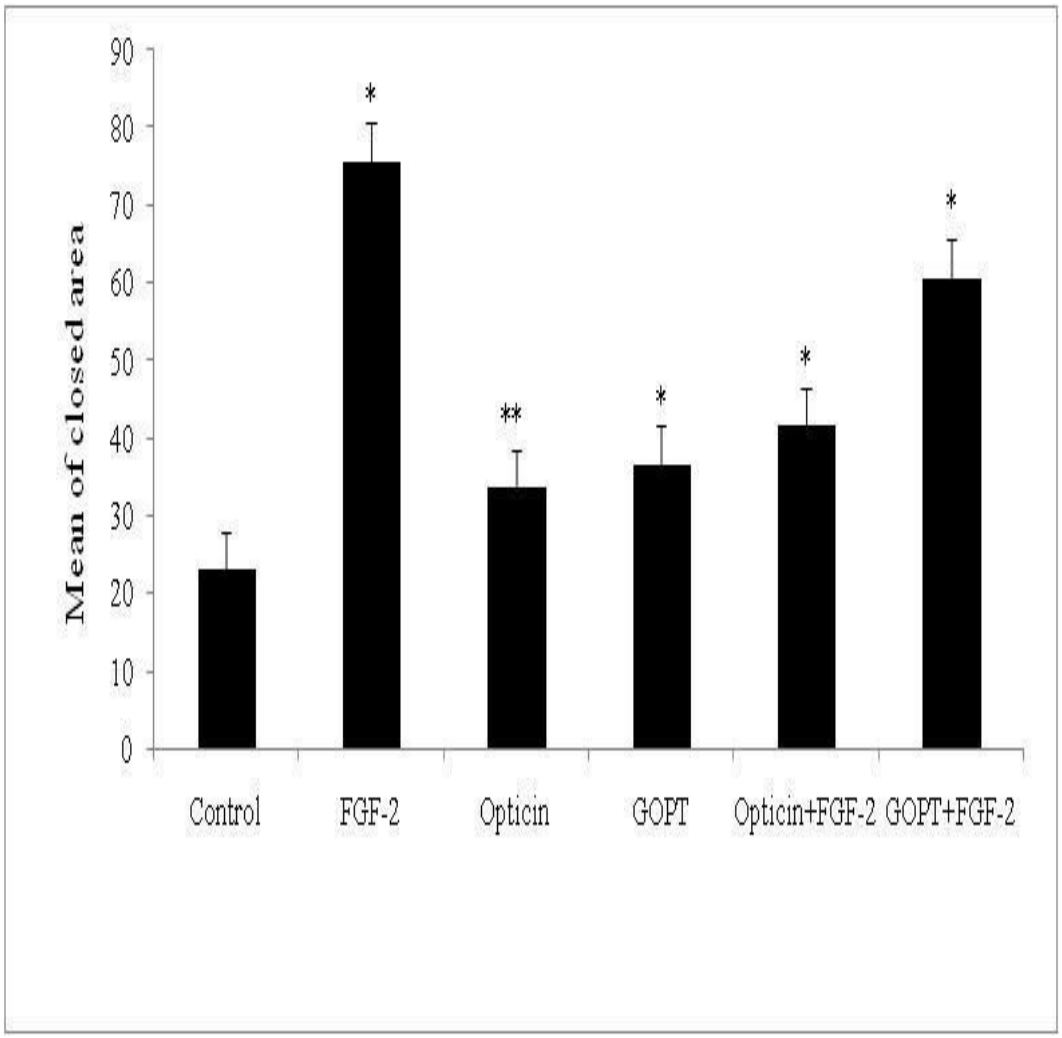


Figure 3.22. EC tube formation of HRECs in collagen type-I

The bar graph shows the stimulatory effects of opticin (25 µg/ml: native and glycated), FGF-2 (25ng /ml) and their combination on HREC tube formation after 24 hours. The number of closed areas was counted. Experiments were performed in triplicate wells and repeated at least three times (n=3). The bar graph shows the mean ± S.D. (*), and (**) signify a statistically significant difference ($p < 0.05$ and $p < 0.01$) compared with the control. A representative example is shown.

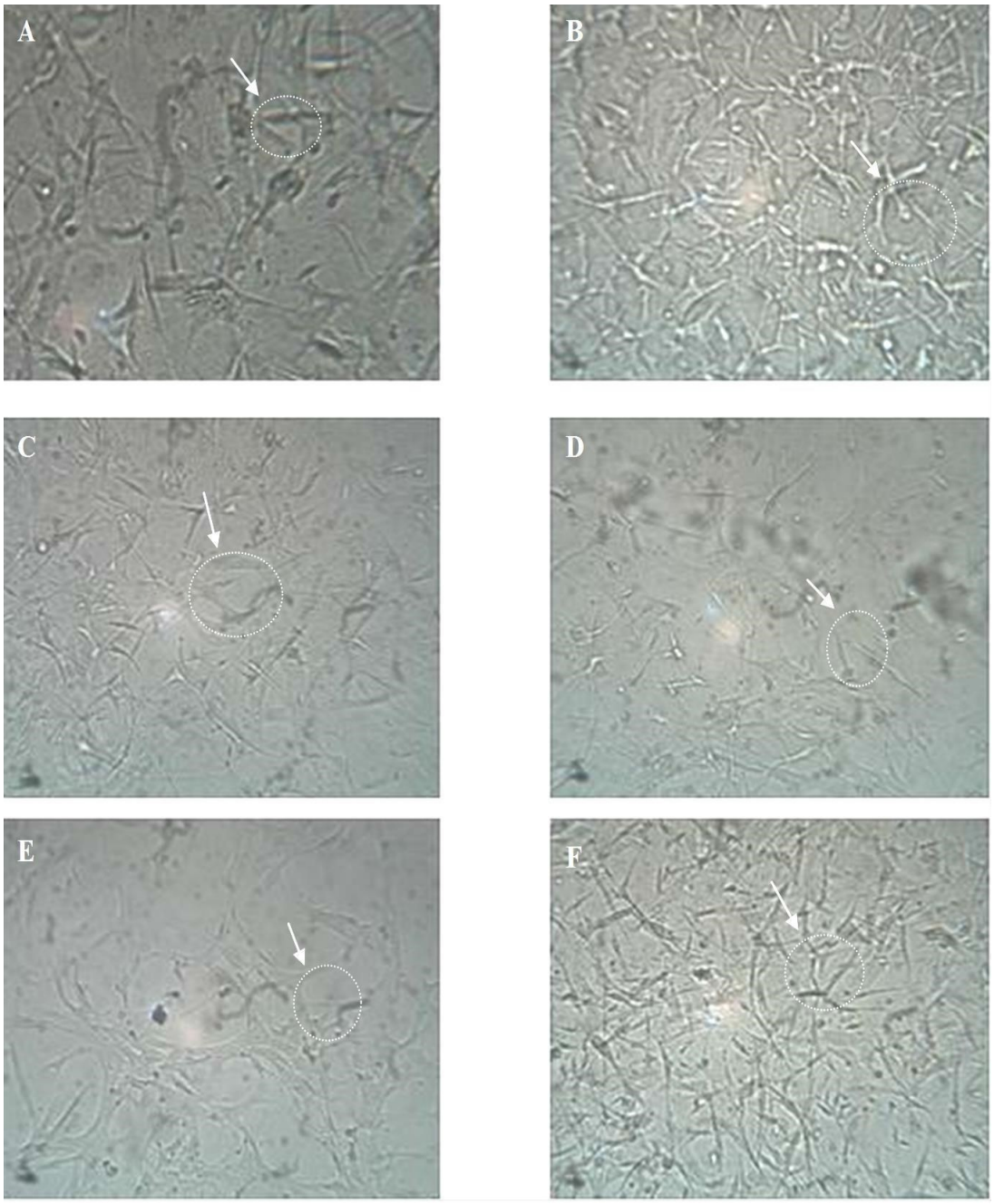


Figure 3.23 Photomicrograph showing the anti-anti-angiogenic effects of Opticin on FGF-2 on HREC tube formation in collagen type-I.

(A)Control, (B) FGF-2, (C) Opticin, (D) GOPT,(E) Opticin + FGF-2 , (E) GOPT + FGF-2. The arrow toward the circle indicates a closed area of collagen, which was used to quantify the results (original magnification X100).

3.2.4 Anti-anti-angiogenic effects of Opticin (native and glycated) and EGF on BAEC tube formation in collagen type-I.

BAECs were seeded on coverslips at a concentration of $2.4x10^4$ cell/well in DMEM containing 1% FBS, opticin (native glycated) 25ug/ml, and EGF (25 ng/ml). The reduce of anti angiogenic possible of GOPT in a representative experiment with tube formation assay 'sandwich' methods is depicted in (Figure 3.24 and 3.25) respectively.

The result shows; GOPT + EGF significantly less inhibit the distance of cell migration by 1.4-fold ($p = 1.4$) arespectively compared with the control cells (Figure 3.1). The highest stimulation was induced by EGF alone by 1.5- fold ($p = 0.003$) compared to control cells and a significant inhibition with opticin + EGF by 1.0- fold ($p = 0.01$). A representative example is shown in (Figure 3.24).

BAECs were cultured in DMEM containing 1% FBS, at concentration of $2.4x10^4$ cell/ml for 24 hours combined with collagen type-I. Experiments were carried out 3 times, each time in triplicate.

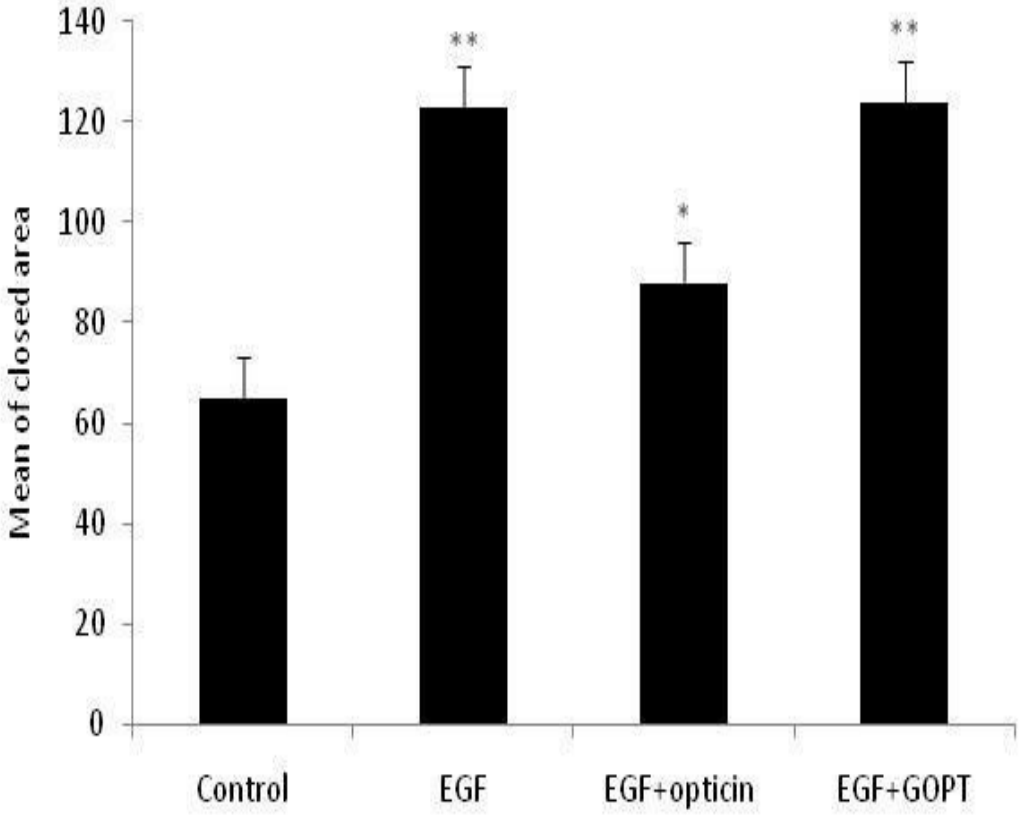


Figure 3.24 Anti-angiogenic effects of GOPT and EGF on BAECs tube formation. The bargraph shows the stimulatory effects of opticin 25 µg/ml (native and glycated), EGF (25ng /ml) and their combination on BAECs tube formation after 24 hours. The number of closed areas was counted. Experiments were performed in triplicate wells and repeated at least three times (n=3). The bar graph shows the mean ± S.D. (*), (**) signify a statistically significant difference ($p < 0.05$ and $p <$
0.01) compared with the control. A representative example is shown.

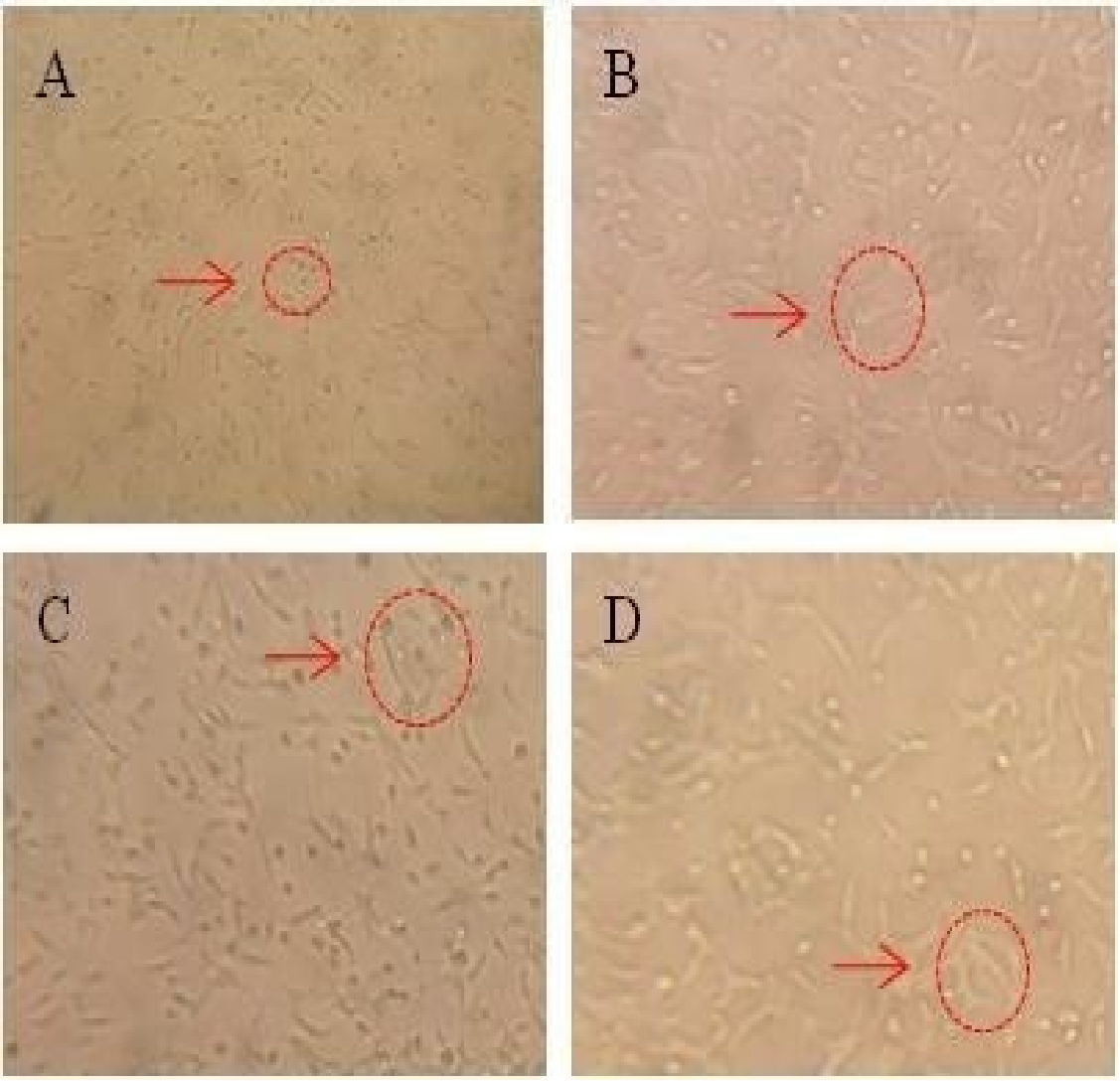


Figure 3-25 Photomicrograph showing the anti-anti-angiogenic effects of Opticin with EGF on BAEC tube formation (in collagen type I).

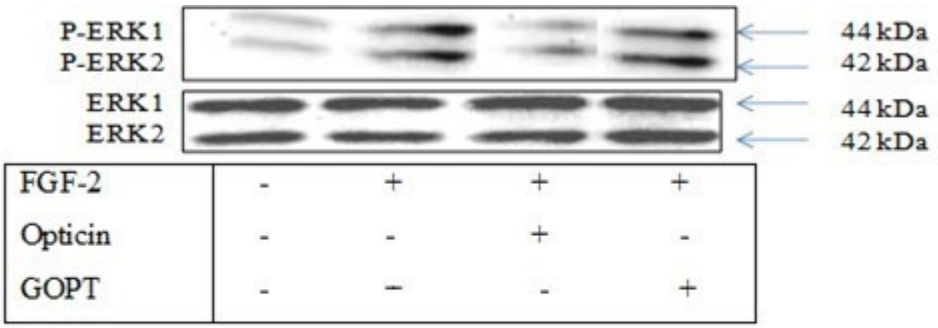
(A), Control; (B) EGF; (C) Opticin + EGF; (D) GOPT + EGF. The arrow towards the circle indicates a closed area of collagen, which was used to quantify the results (original magnification X100).

Results Section 3: Opticin modulated signalling pathways identified

3.2.5 Protein phosphorylation of ERK in BAEC treated with GOPT

ERK1/2 protein activity characterized as its phosphorylated form is required for EC proliferation, migration and in angiogenesis (Slevin *et al*, 2002). The phosphorylation of ERK was initiated by exogenous addition of FGF-2 and opticin (native glycated) for 10 min, and protein lysates were subjected to Western blotting analysis. Therefore, signalling stimulation studies were carried out to understand, the mechanism (Figure 3.26). After 10 minutes stimulation with FGF-2 and it`s combination, a significant increase (1.5-fold) in expression of phospho-ERK1/2 was observed, compared to the control cells.

As expected, incubated opticin enable inhibited P-ERK expression induced by FGF-2 (10 min); however, in contrast, glycated opticin did not notably reduce FGF2-induced P-ERK1/2 (Figure 3.26 A, B).



B.

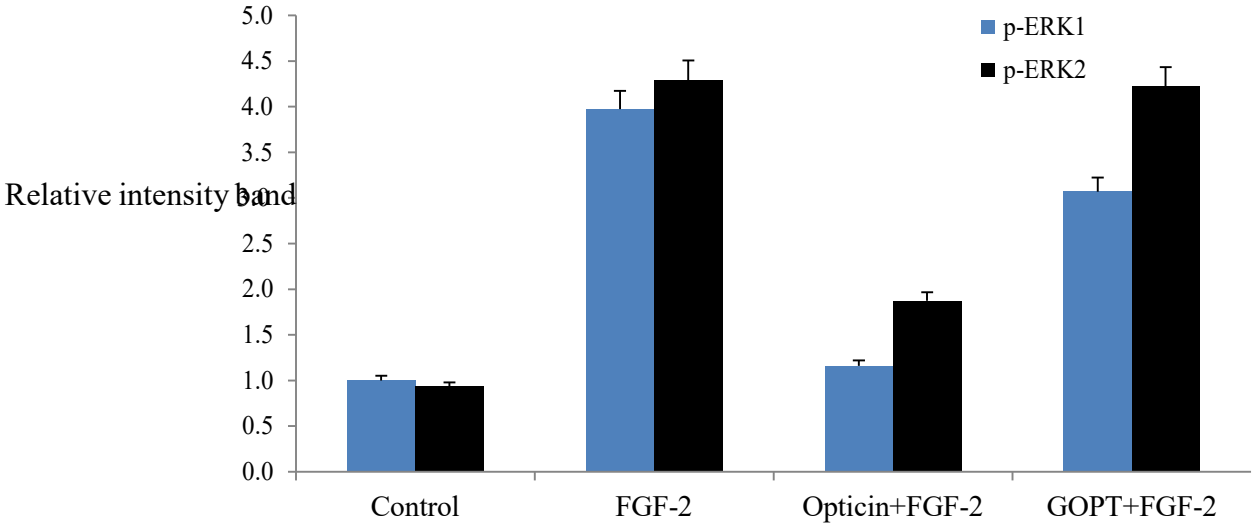


Figure 3.26 Protein phosphorylation of ERK-1 and ERK-2 in BAEC. Examination of cell lysates by Western blotting after treatments with GOPT, histograms represent relative intensity band of 25µg/ml opticin (native and glycated) compared to control cells are normalised to ERK1/2. **A**, representative Western blot gel showing the variation of phospho-ERK1/2 expression, after 10 minutes of incubation. **B**, the bar graph shows the analysis performed in function of the densitometry of the Western blot gel bands and the results are expressed as relative to total-ERK1/2 expression. To check the equal loading of proteins, total-ERK1/2 was used as loading control. Each panel was representative of at least three independent experiments.

3.2.6 Protein phosphorylation of MEK/2 in BAEC treated with GOPT

The phosphorylation of MEK1/2 was initiated by exogenous addition of FGF-2 and opticin (native glycated) for 10 min, and protein lysates were subjected to Western blotting analysis. Therefore, signalling stimulation studies were carried out to understand, the mechanism (Figure 3.27). After 10 minutes stimulation with FGF-2 and it`s combination, a significant increase (1.2-fold) in expression of phosphoMEK/2 was observed, compared to the control cells.

As expected, incubated opticin enable inhibited P-MEK expression induced by FGF-2 (10 min); however, in contrast, glycated opticin did not notably reduce FGF2-induced P-MEK/2 (Figure 3.27 A, B).

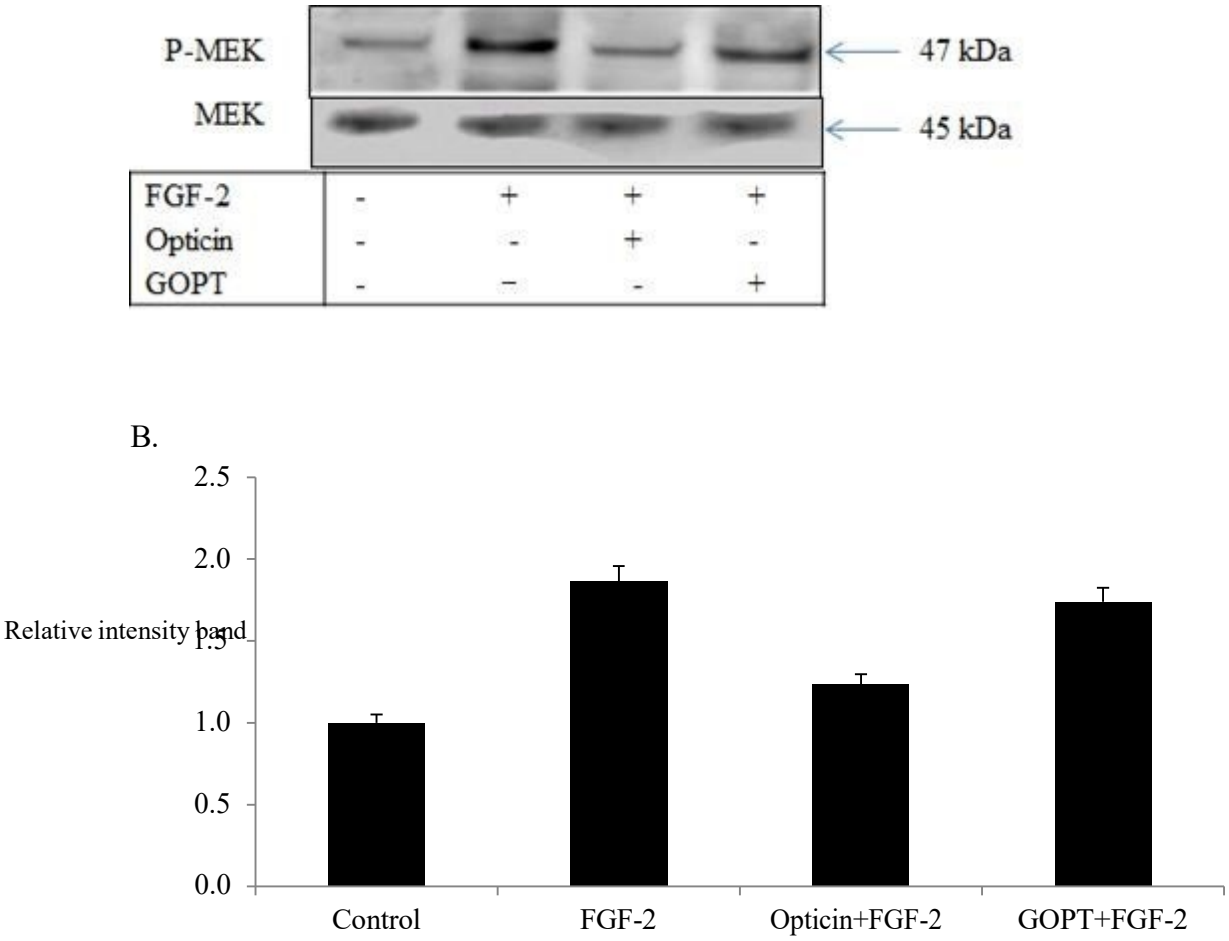


Figure 3.27 Protein phosphorylation of MEK/2 in BAEC treated with GOPT. Examination of cell lysates by Western blotting after treatments with GOPT, histograms represent relative intensity band of 25µg/ml opticin (native and glycated) compared to control cells are normalised to MEK/2. .**A**, representative Western blot gel showing the variation of phosphor-MEK/2 expression, after 10 minutes of incubation. **B**, the bar graph shows the analysis performed in function of the densitometry of the Western blot gel bands and the results are expressed as relative to total-MEK/2 expression. To check the equal loading of proteins, totalMEK/2 was used as loading control. Each panel was representative of at least three independent experiments.

3.2.7 Protein phosphorylation of AKT in BAEC treated with GOPT

The phosphorylation of AKT was initiated by exogenous addition of FGF-2 (25ng/ml) and opticin (25µg/ml); native or glycated) for 10 min, and protein lysates were subjected to Western blotting analysis. Therefore, signalling stimulation studies were carried out to understand, the mechanism (Figure 3.28). After 10 minutes stimulation with FGF-2 and it`s combination, a significant increase (1.0fold) in expression of phospho-AKT was observed, compared to the control cells. As expected, incubated opticin enable inhibited P-AKT expression induced by FGF-2 (10 min); however, in contrast, glycated opticin did not notably reduce FGF2-induced P-AKT (Figure 3.28 A, B).

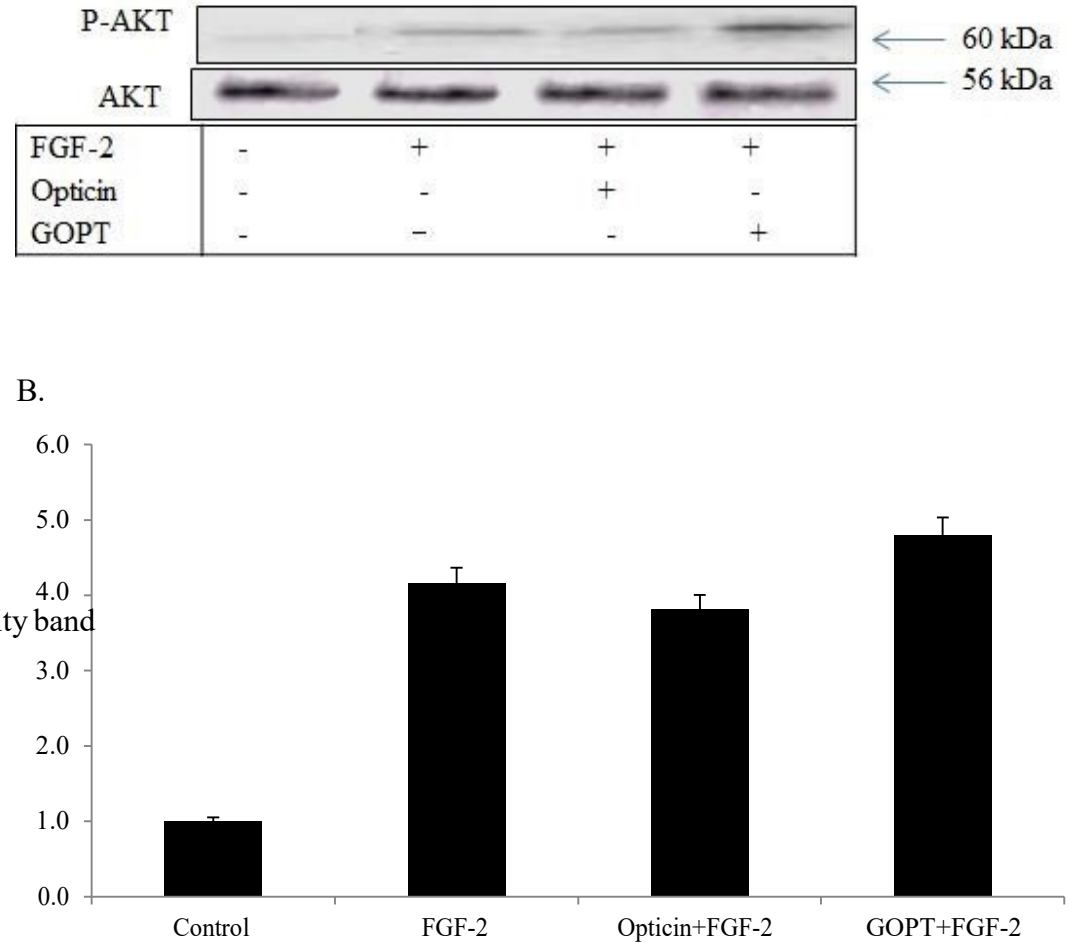
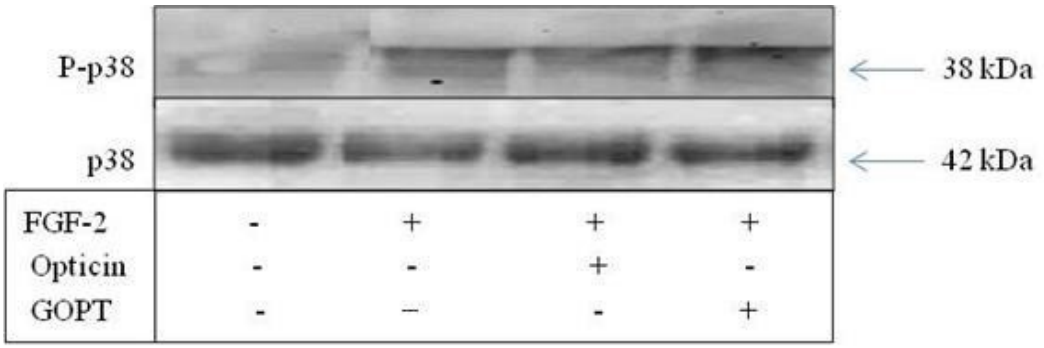


Figure 3.28 Protein phosphorylation of AKT in BAEC treated with GOPT.

Examination of cell lysates by Western blotting after treatments with GOPT, histograms represent relative intensity band of 25µg/ml opticin (native and glycated) compared to control cells are normalised to AKT when treated with FGF-2 (25 ng/ml). **A**, representative Western blot gel showing the variation of phosphor-AKT expression, after 10 minutes of incubation. **B**, the bar graph shows the analysis performed in function of the densitometry of the Western blot gel bands and the results are expressed as relative to total-AKT expression. To check the equal loading of proteins, total-AKT was used as loading control. Each panel was representative of at least three independent experiments.

3.2.8 Protein phosphorylation of p38 in BAEC treated with GOPT

The phosphorylation of p38 was initiated by exogenous addition of FGF-2 (25ng/ml) and opticin (25µg/ml); native or glycated) for 10 min, and protein lysates were subjected to Western blotting analysis. Therefore, signalling stimulation studies were carried out to understand, the mechanism (Figure 3.29). After 10 minutes stimulation with FGF-2 and it`s combination, a significant increase (0.5fold) in expression of phospho-p38 was observed, compared to the control cells. As expected, incubated opticin enable inhibited P-p38 expression induced by FGF2 (10 min); however, in contrast, glycated opticin did not notably reduce FGF-2induced P-p38 (Figure 3.29 A, B).



B.

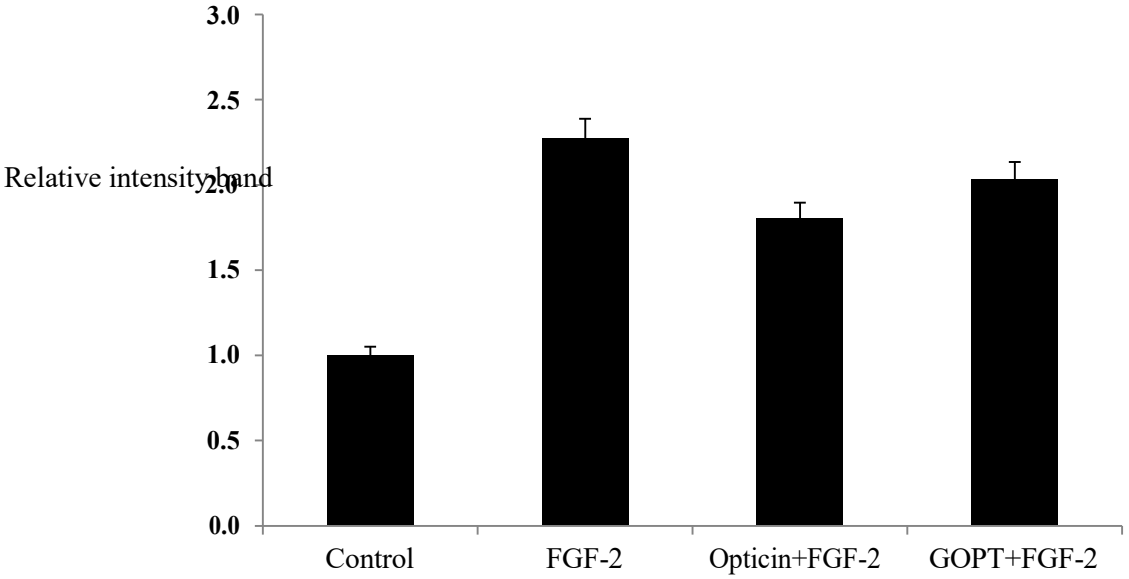
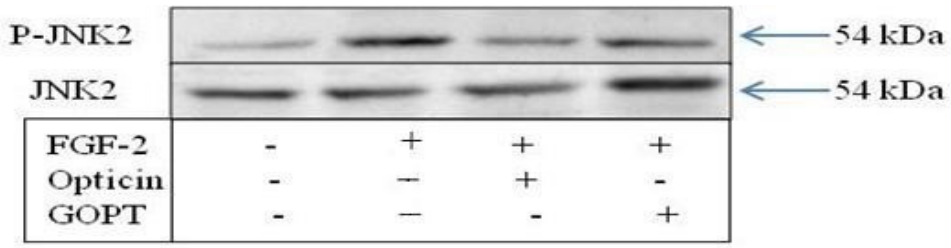


Figure 3.29 Protein phosphorylation of p38 in BAEC treated with GOPT.

Examination of cell lysates by Western blotting after treatments with GOPT, histograms represent relative intensity band of 25µg/ml opticin (native and glycated) compared to control cells are normalised to p38 when treated with FGF-2 (25 ng/ml). **A**, representative Western blot gel showing the variation of phosphor-p38 expression, after 10 minutes of incubation. **B**, the bar graph shows the analysis performed in function of the densitometry of the Western blot gel bands and the results are expressed as relative to total-p38 expression. To check the equal loading of proteins, total-p38 was used as loading control. Each panel was representative of at least three independent experiments.

3.2.9 Protein phosphorylation of JNK2 in BAEC treated with GOPT

The phosphorylation of JNK2 was initiated by exogenous addition of FGF-2 (25ng/ml) and opticin (25µg/ml); native or glycated) for 10 min, and protein lysates were subjected to Western blotting analysis. Therefore, signalling stimulation studies were carried out to understand, the mechanism (Figure 3.30). After 10 minutes stimulation with FGF-2 and it`s combination, a significant increase (2.0fold) in expression of phospho-JNK2 was observed, compared to the control cells. As expected, incubated opticin enable inhibited P-JNK2 expression induced by FGF-2 (10 min); however, in contrast, glycated opticin did not notably reduce FGF2-induced P-JNK2 (Figure 3.30 A, B).



B.

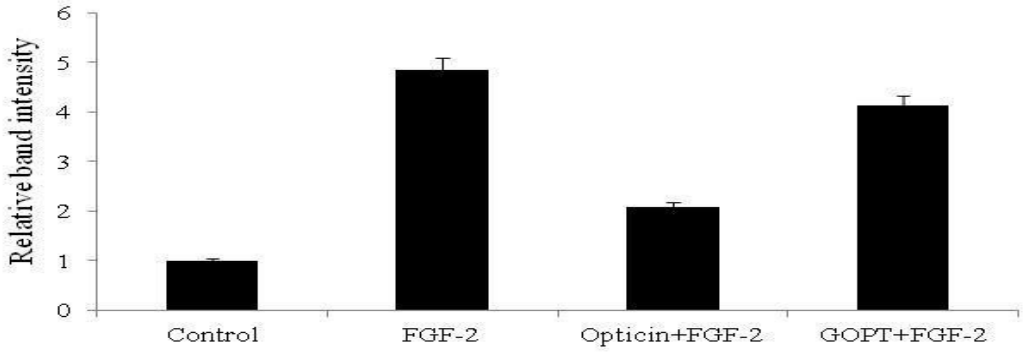


Figure 3.30.**Protein phosphorylation of JNK2 in BAEC treated with GOPT** Examination of cell lysates by Western blotting after treatments with GOPT, histograms represent relative intensity band of 25µg/ml opticin (native and glycated) compared to control cells are normalised to JNK2 when treated with FGF-2 (25 ng/ml). **A**, representative Western blot gel showing the variation of phospho-JNK2 expression, after 10 minutes of incubation. **B**, the bar graph shows the analysis performed in function of the densitometry of the Western blot gel bands and the results are expressed as relative to total-JNK2 expression. To check the equal loading of proteins, total-JNK2 was used as loading control.

Each panel was representative of at least three independent experiments.

3.2.10 Measurement of CML in diabetic mice

It is very clear from Western blots the CML expression in diabetic mice more than the non-diabetic mice. (A), in diabetic mouse eyes the CML increased by 1.5 folds than non-diabetic mouse. (Figure3.31, A&B).

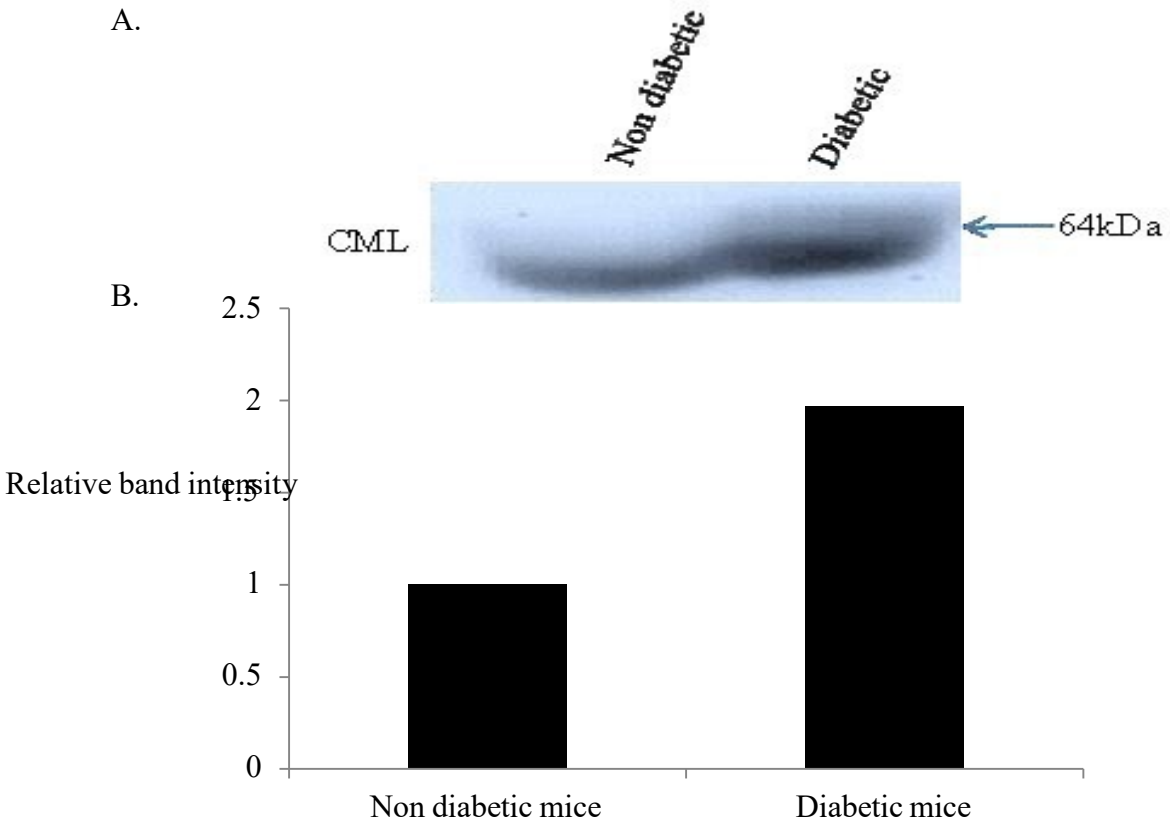


Figure 3.31 Western blots showing the expression of CML

Three mice where used or each expirment (A) A representative Western blots showing the expression of CML in treated non-diabetic and diabetic mice. (B) The bar graphs show the expression of CML in the diabetic mice compare to non diabetic mice. This figure is representative of at least three independent experiments.

3.2.11 Glycated opticin reduce binding to collagen type-I

The binding of GOPT to collagen type-I was investigated using ELISA. The experiment demonstrated that native opticin binds collagen with increasing its concentration, and bind more effectively than glycated opticin. (Figure 3.32).

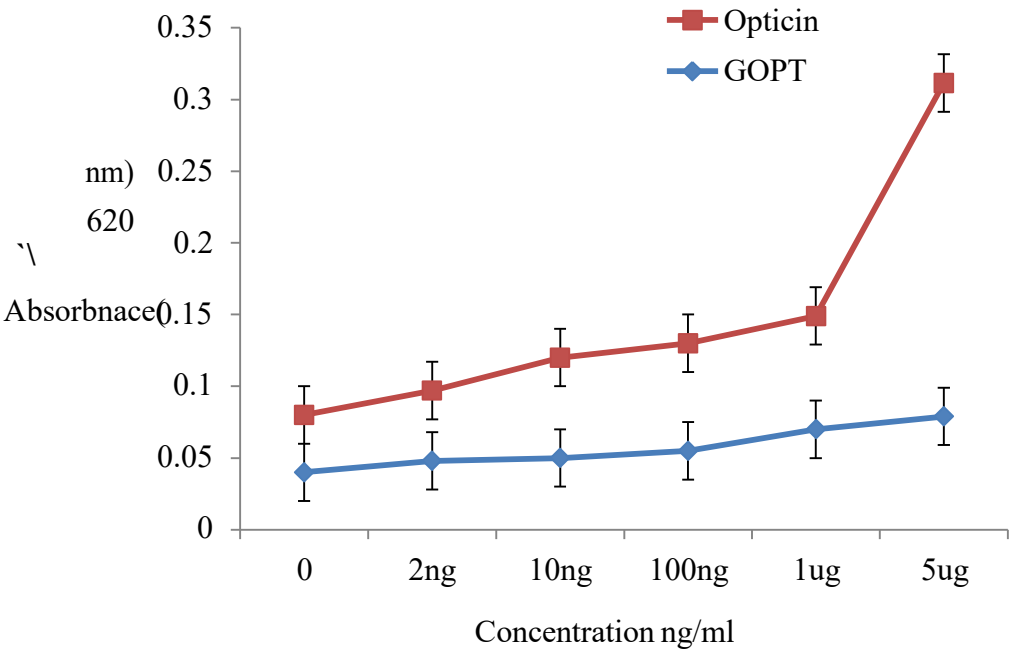


Figure 3.32 Opticin (native and glycated) binding to collagen type-I.
The diagram shows binding of GOPT and opticin following interaction with a fixed concentration of collagen type-I (50µg/ml) in PBS preincubated with increasing concentration of opticin and GOPT. The expirment was performed in triplicate.

3.2.12 Glycated opticin binding to FGFR

The opticin may not modulate the binding of FGF-2 to its receptor and not change the FGFR affinity to its ligand in contrast to GOPT may modulate the binding of FGF-2 to its receptor and change the FGFR affinity to its ligand (Figure 3.33).

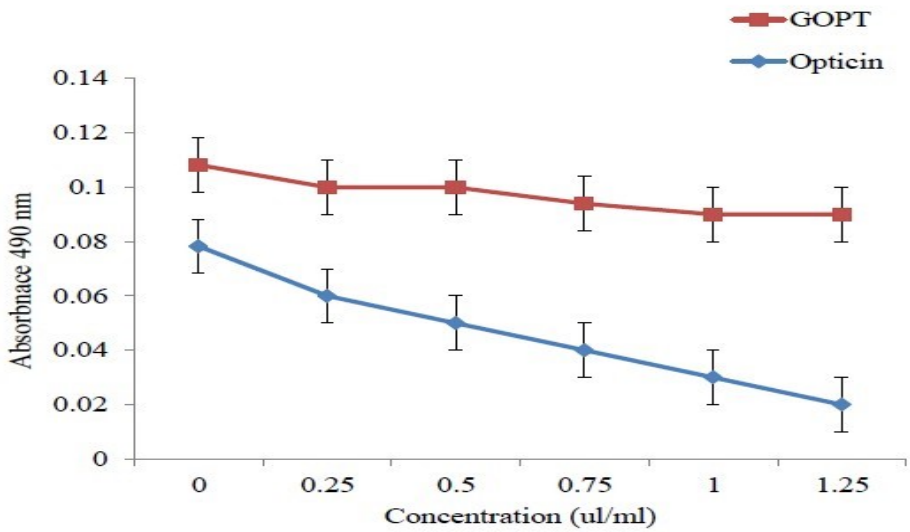


Figure 3.33 Glycated opticin binding to FGFR. The diagram shows binding of opticin (Glycated and native) following interaction with a fixed concentration of FGFR, 2µg/ml preincubated with increasing concentration of opticin and glycated opticin.with 2µg/ml FGF-2. Each data point taken from four or five replicate wells. The expirment was performed in triplicate.

Results
Section 4: Immunochemistry

3.2.13 Immunofluorescence Analysis

For examination of glycated opticin on focal adhesion, BAECs were stained with anti-paxillin, anti-α2β1, and anti-vinculin antibodies, followed by Alexa Flour® 488 nm-conjugated anti-mouse antibody. The cells were visualized by confocal microscopy. Nuclei were stained with DAPI, Paxillin, vinculin, andα2β1 stained green, while the Actin stained red.

In order to investigate the effect of GOPT +FGF-2 on vinculin integrin engagement with ECM, spreading experiments were performed in the presence of vinculin. (Figure 3.34).

In normal cells the vinculin looks like (control), if stimulated cells with FGF-2 the vinculin appears in special localization in perpherial of the cell. Whit the opticin in present of FGF-2 disturping the normal pattern of vinculin expressed. In glycated opticin in present of FGF-2, the vinculin appears on the cells become normal looking.

The disturping is a part of anti-angiogenic prosses which can slow them down from moving which has been observed in present of opticin +FGF-2. But if replaced with glycate opticin + FGF-2 it is not very elective, so continous spreading and ready to move.

The same results were obtained when cells treated with paxillin. (Figure 3.35), and α2β1 (Figure 3.36).

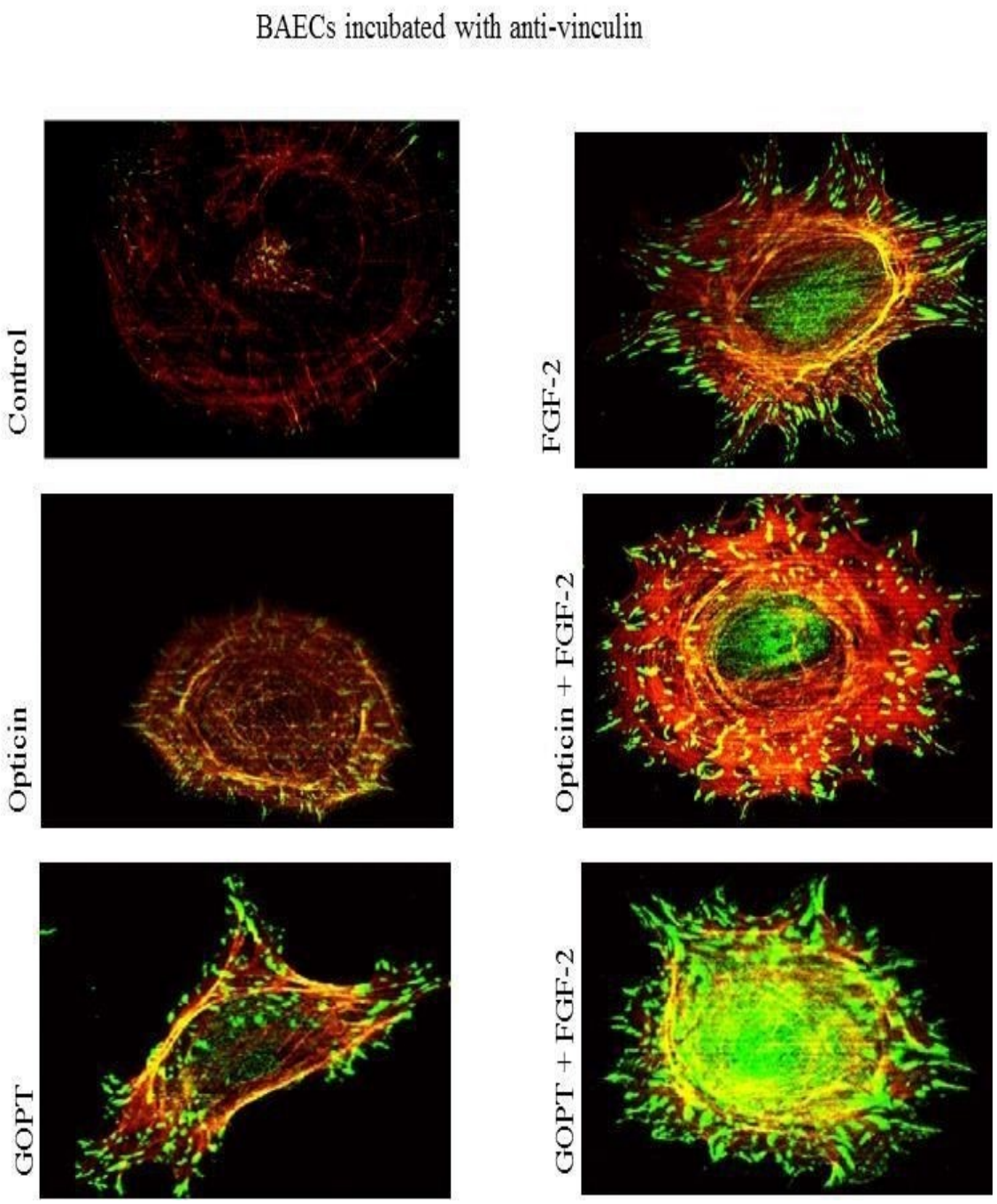


Figure 3.34 Immunofluorescence detection /Anti vinculin.

BAEC $2.4x10^4$ cells/ml were seeded on collagen-I coated coverslips for 1 h. Opticin 25 µg/ml (native and glycated) in present FGF-2 at 25ng/ml for 10 minutes prior to fixation as described in material and methods.

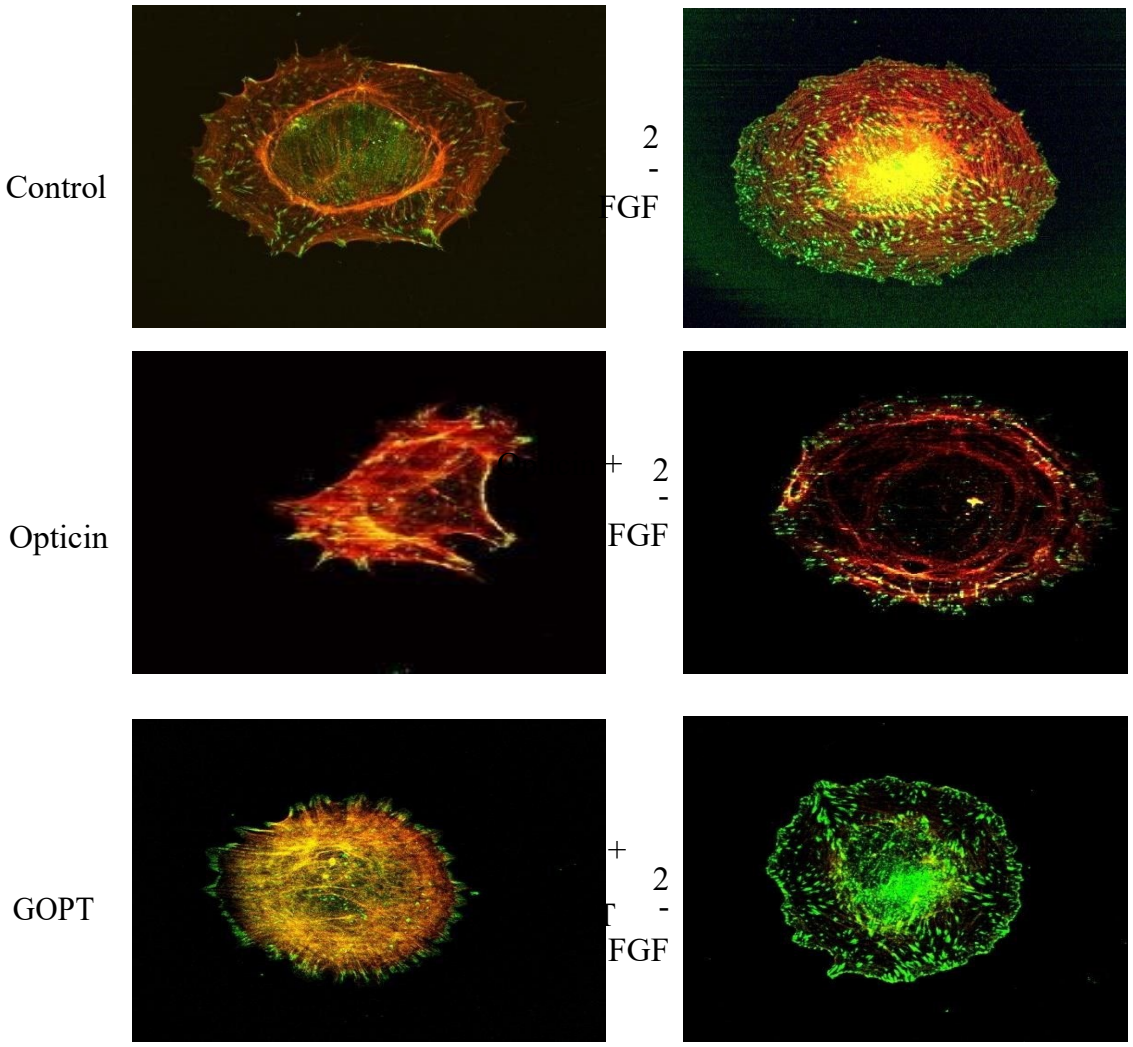


Figure 3.35 Immunofluorescence detection /Anti paxillin.

BAEC 2.4x10^4 cells/ml were seeded on collagen-I coated coverslips for 1 h. Opticin 25 µg/ml (native and glycated) in present FGF-2 at 25ng/ml for 10 minutes prior to fixation as described in material and methods.

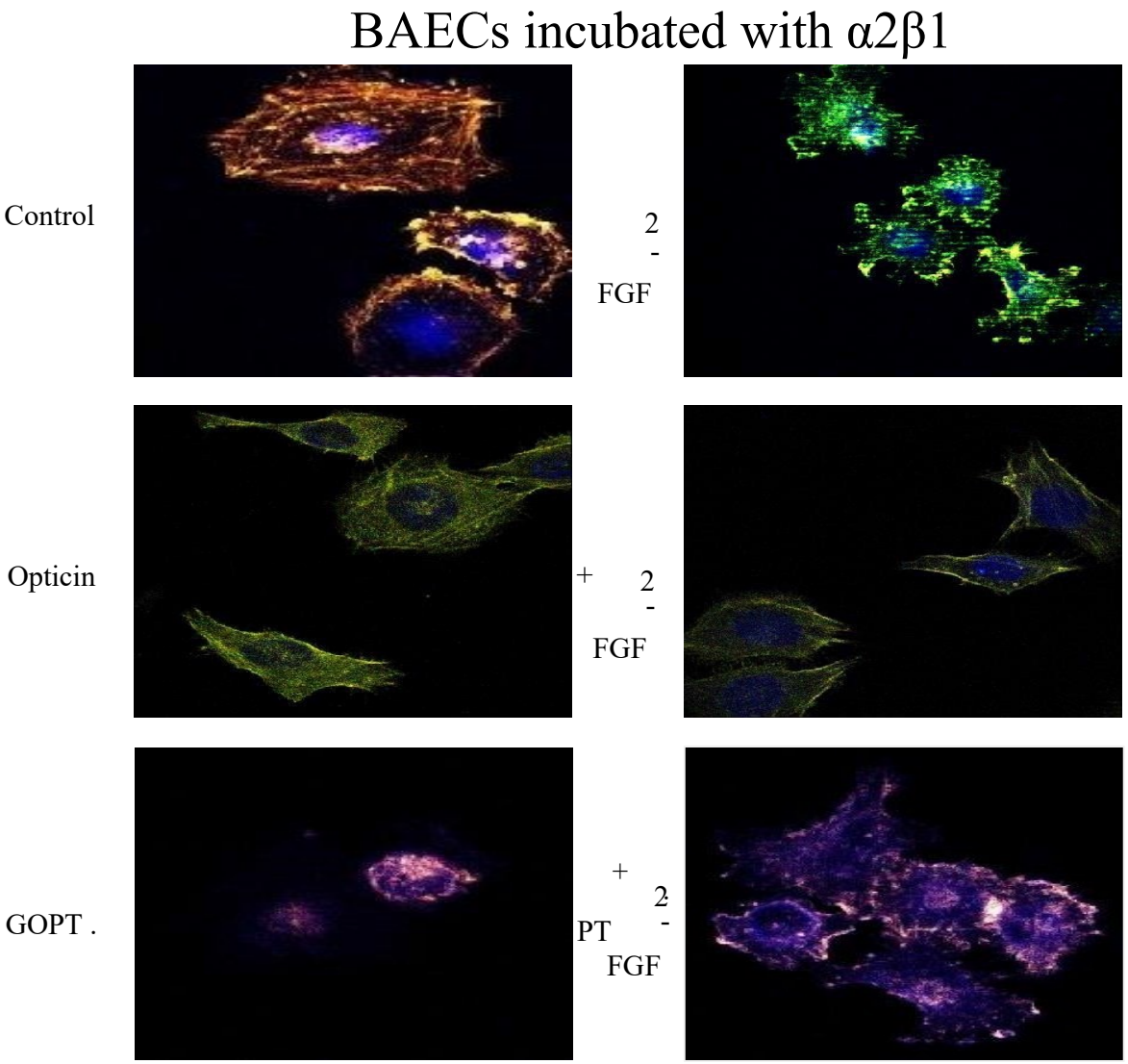


Figure 3-36 Immunofluorescence detection / α2β1.

BAEC 2.4x10^4 cells/ml were seeded on collagen-I coated coverslips for 1 hour. Opticin 25 µg/ml (native and glycated) in present FGF-2 at 25ng/ml for 10 minutes prior to fixation as described in material and methods.

3.2.14 Immunolocalization of opticin and AGEs in same section

Eyes from diabetic and non diabetic mice were sectioned by freezing microtome. Sections (4μm) were incubated with affinity-purified anti-mouse antibody (1:200), and anti-CML (2:200) followed by secondry anti-body as explained in the martial and methods. The slides visualized under laser microscope. The opticin was very clear in diabetic and non diabetic mouse, while the localization of AGEs identified (Figures 3.37-39).

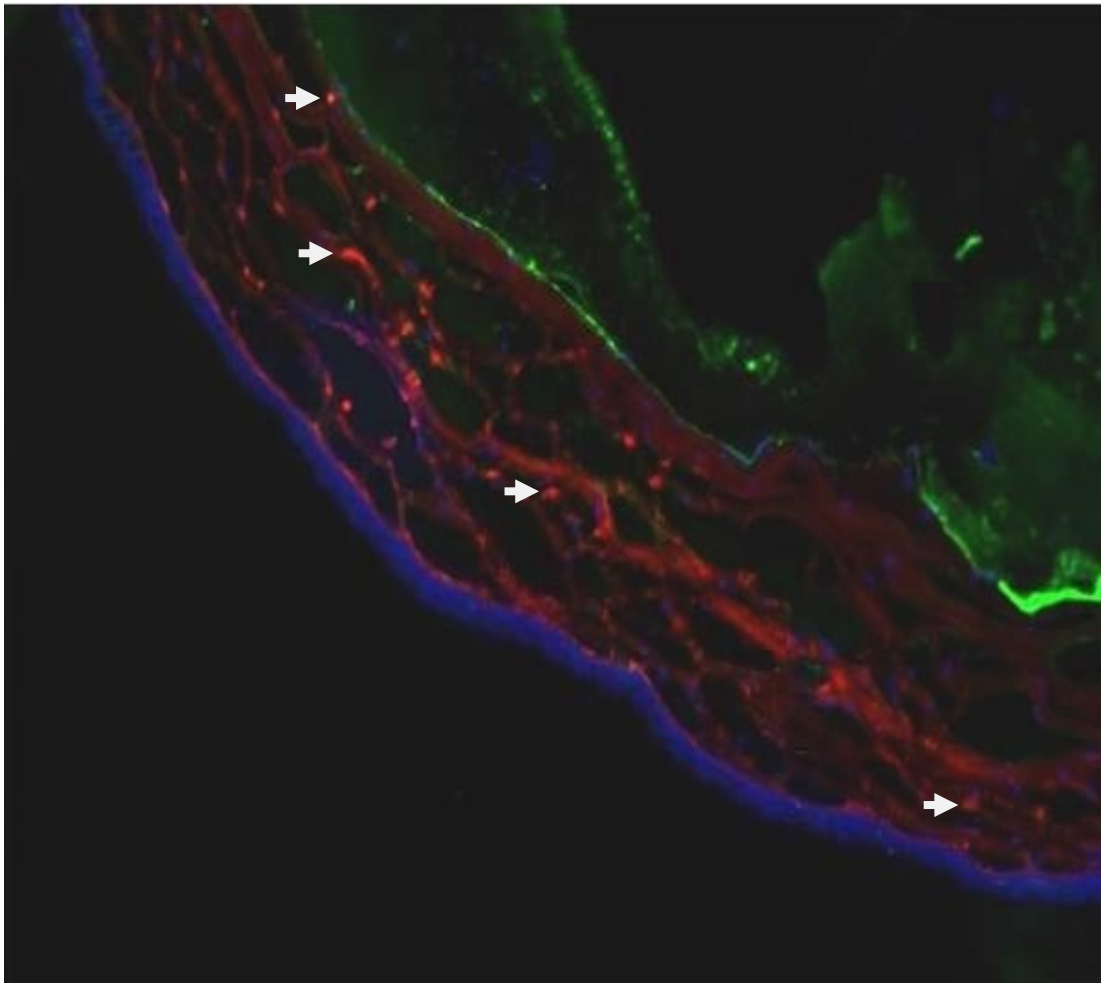


Figure 3.37 The localization of AGEs in diabetic mouse eye, the arrow shows the localization of AGEs. (Low mignification X100).

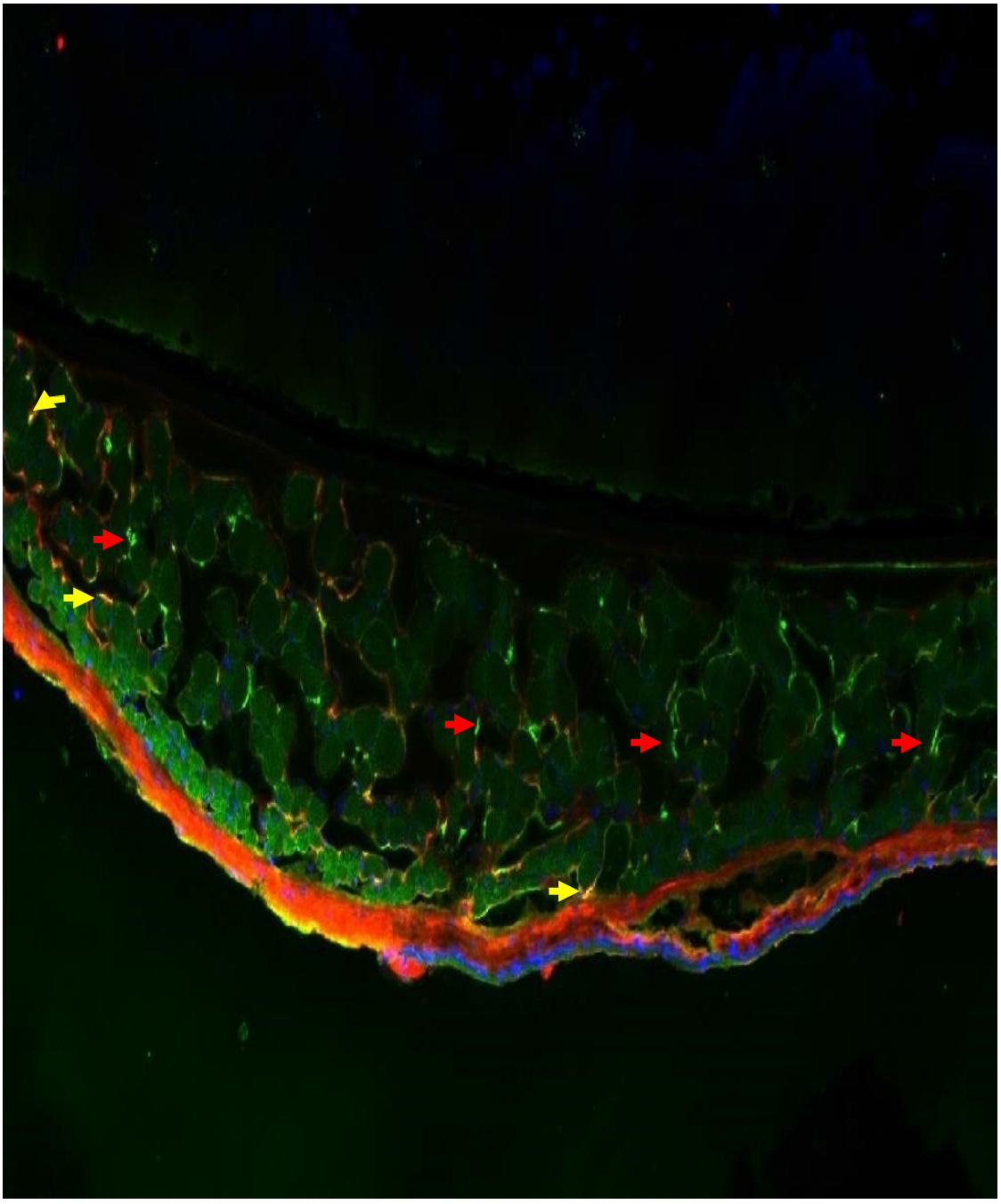


Figure 3.38 The co- localization of opticin and AGEs in diabetic mouse eyes (the yellow arrow), the arrow shows the localization of opticin (green arrow) (Low mignification X100).

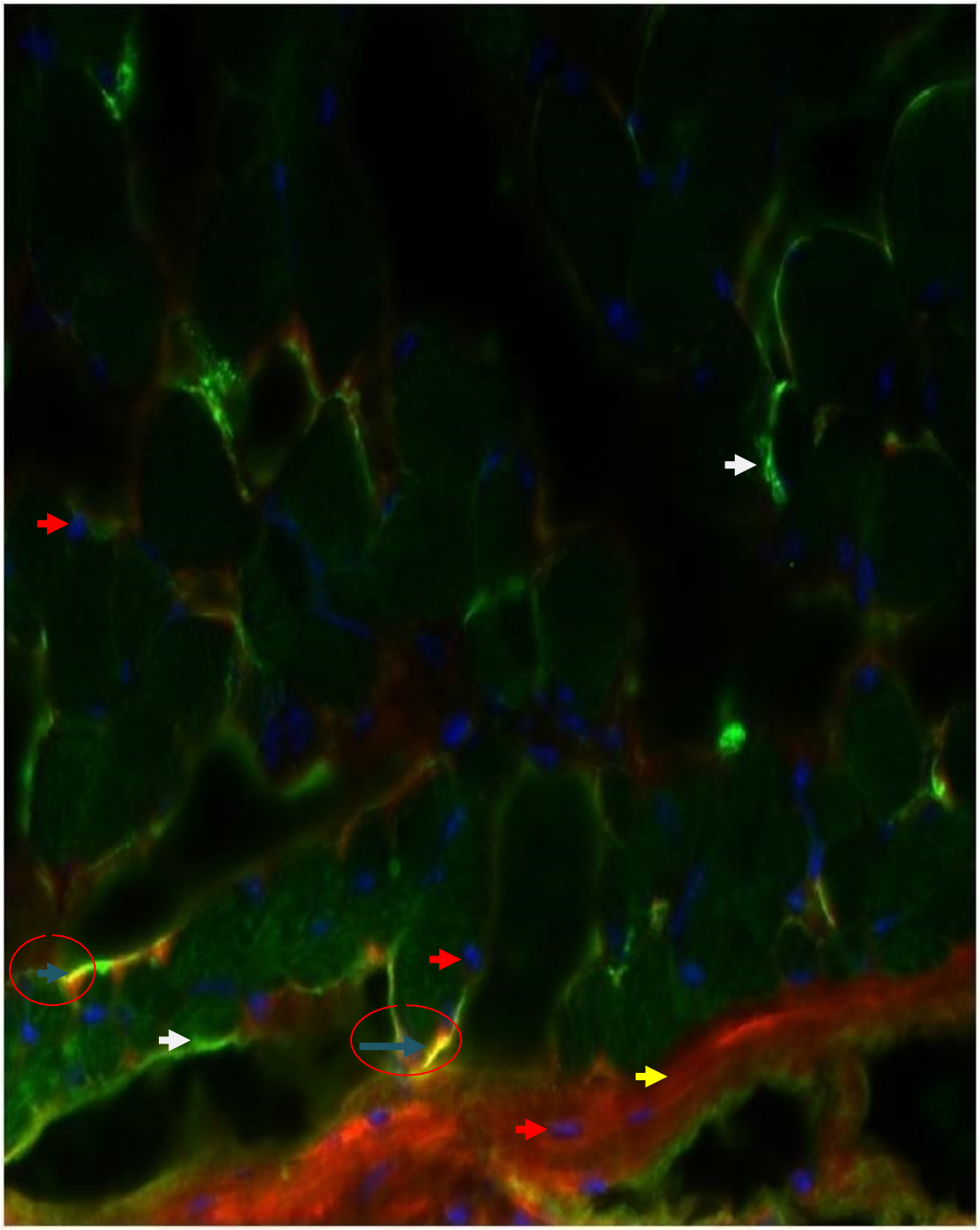


Figure 3.39 The co-localization of opticin and AGEs in diabetic mouse eyes. The blue arrow in red in red circle shows the co-localization of opticin and AGEs, white arrow opticin, orange arrows nuclei, and yellow arraw colleagen fibers.(High mignification X400).

3.2.15 Immunolocalization of opticin and AGEs separated in different sections. Eyes from diabetic and non diabetic mice were sectioned by freezing microtome. Sections (4um) were incubated with affinity-purified anti-goat antibody opticin (1:200), and anti-CML (2:200) as explained in the material and methods each antibody incubated in different section. The slides visualized under light microscope. The opticin was very clear in diabetic and non diabetic mouse, and the localization of AGEs identified (Figures 3.40-42).

Figure 3.40 The localization of AGEs in diabetic mouse eyes. The arrow showes the localization of AGEs (Low mignification X100).

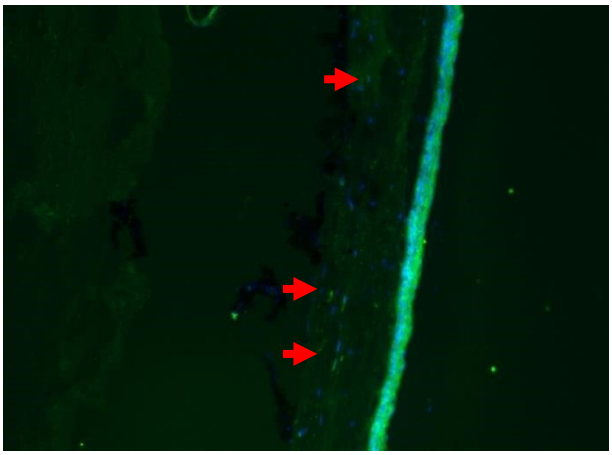


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Figure 3.41 The localization of opticin in nondiabetic mouse eyes. The arrow showes the localization of opticin (Low mignification X100).

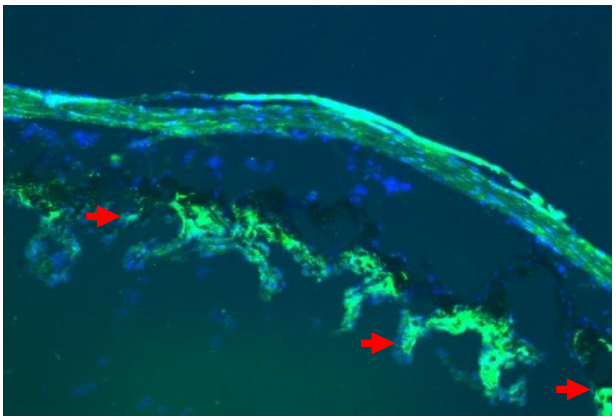
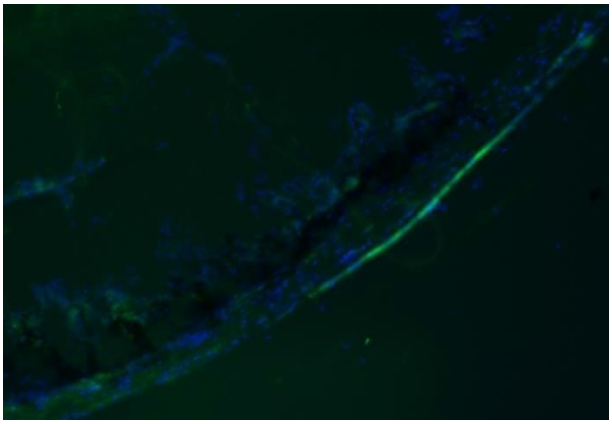


Figure 3.42 Diabetic mouse eye used as control no anti-bodies added (Low mignification X100).



Chapter 4: Discussion

4. Discussion

Opticin belongs to a family of small leucine-rich repeat proteins and proteoglycans (SLRPs). SLRPs have been shown to be able to bind various cell surface receptors, growth factors, cytokines and other ECM components resulting in the ability to influence various cellular functions. (Margaret *et al.,* 2012). Opticin is an extracellular matrix glycoprotein associated with the collagen network of the vitreous and is an important regulator of angiogenesis. Opticin is present in significant quantities in the vitreous of the eye and localizes to the cornea, iris, ciliary body, optic nerve, choroid, retina, and fetal liver. Opticin may noncovalently bind collagen fibrils and regulate fibril morphology, spacing, and organization. During ocular development, the vitreous cavity contains a network of blood vessels that regress before birth in human and shortly after birth in mice; they are subsequently replaced by secondary (mature) vitreous, a transparent and avascular gel. This vitreous remains avascular unless pathological blood vessels derived from the retina, (a process called preretinal neovascularization) invade it. Vitreous contains a network of collagen fibrils, essential to its gel structure (Bishop, 2000). These collagen fibrils provide a substrate for preretinal neovascularization by offering a scaffold for new blood vessel growth and in that way-allowing invasion into the cortical vitreous (Bishop, 2000; McLeod, 2007). This is an important cause of blindness occurring in conditions such as proliferative diabetic retinopathy (PDR) and, retinopathy of prematurity (ROP). Visual loss is caused by vitreous haemorrhage and/or tractional detachment induced by contraction of fibrous tissue associated with preretinal neovascularization (McLeod, 2007). This project investigates how glycated opticin effect angiogenesis, it is as reduce anti-angeiogenic when compared with using the opticin alone. It has being found that opticin decreased the EC network formation in collagen and Matrigel^{TM} formed following addition of FGF-2 (25 ng/ml) (Le Goff *et al.,* 2012). Fibroblast growth factor (FGF-2) and vascular endothelial cell growth factor (VEGF) are the two major angiogenic factors in the retina.

This is first time were the opticin glycaged *in vito*, however other proteins which linked to the eyes which have related to the diabetes and disaffect their functions being glycated like α-crystallin exposed to various sugars and their derivatives,

whose levels are elevated in diabetes and lead to cataract formation (Kumar *et al*., 2007). Our results show the opticin appears at 45kDa, which agree to previous work, which showed major bands of opticin at 45kDa (Le Goff and Bishop, 2007). The SDS-PAGE method was used to follow the glycation reaction. 72 hours incubations of opticin with glucose, fructose, ribose, and methylglyoxal led to the formation of cross-linked AGEs. Furthermore, the glycation of protein with methylglyoxal modifies lysine and arginine residues (Naila, 2014) very quickly, which accordingly leads to excessive protein cross-linking, which is detected via cross-linking on SDS-PAGE. These AGEs are characterised by their fluorescence, brown colour and formation of intra- and intermolecular cross-linking (Maillard, 1912; Xu *et al*., 2003). Glycation also takes place on cysteine residues (Nadeem and Debabrata, 2013). The major AGE adducts formed on proteins from glyoxal and methylglyoxal are CML and CEL, respectively (Baynes and Thorpe, 2000; Thornalley, 1996). Some AGEs, such as CML and pentosidine have become highly useful biomarkers of glycoxidative damage (Khalifah *et al*., 1999). The same sugars where used for glycation of lenticular proteins has been suggested to be responsible for the formation of a significant fraction of lens protein modifications present in the aged human lenses and in diabetic and brunescent cataractous lenses (Mikhail 2007), and the resultant modified protein may possess carbonyl groups (Chaplen 1998). MG is formed mostly inside cells but a small fraction leaks out and so glycation of both cellular and extracellular proteins by MG increases (Duran-Jimenez BD *et al*., 2009; Karachalias *et al*., 2010). Methylglyoxal was used in this study owing to the fact that it can be formed by both the fragmentation and dehydration of glucose and Amadori products. Methylglyoxal is a reactive dicarbonyl, which is believed to contribute to the development of diabetic complications either as a direct toxin or as a precursor for AGEs. It was reported that methylglyoxal induces free radical generation, with its concentration in diabetes increased (Artenie *et al*., 2004; Beisswenger *et al*., 1999; Vander Jagt and Hunsaker, 2003). Glucose and fructose are the two most important simple sugars for human consumption. They have the same molecular formula, $C_6H_{12}O_6$, but have different structures. Since glucose is the most abundant sugar in blood, to date, glycation by glucose is the most studies. However, glucose has the slowest glycation rate because the reactivity of each sugar is dependent on the percentage

of sugar in the open chain (carbonyl) structure (Bunn and Higgins, 1981). The formation of AGEs occurs at a faster rate under conditions of hyperglycemia, dyslipidemia and oxidative stress. For example, it has been demonstrated *in vitro* that the rate of the formation of intracellular AGEs may be 14-fold faster in 30 mM glucose compared to 5 mM glucose (Giardino *et al.,* 1994). In addition to direct glyation reactions, glucose autoxidation also contributes to the formation of stable AGEs (Wolff, 1987).A recent study showed that Glcmodified bovine serum albumin also inhibited growth of bovine retinal capillary pericytes but did not inhibit growth of bovine aortic endothelial cells (Kim *et al.,*2012). Fructose is a highly reactive reducing sugar. It also undergoes the Maillard reaction with proteins and amino acids, producing reactive intermediates, cross-linking of proteins, and forming brown and fluorescent polymeric materials (Stefan and Monika, 2011). The role of ribose in glycation and cross-linking of collagen has been investigated *in vitro* studies of the triggering of skin ageing (Tanaka *et al*., 1988). Luciano and colleagues (2008) prepared proteins glycated with ribose in a study of AGEs and their effects on pancreatic islet beta cells. Direct effects of AGEs on cellular viability and related insulin secretion of beta cells resulting from their exposure to glycated serum by incubation with ribose have been evaluated. Results showed reduced cellular proliferation with a corresponding increase in cell necrosis and cell apoptosis rate in comparison with untreated cells after 5 days of exposure to glycation conditions (Luciano *et al*., 2008). Compared with glucose, ribose is more active in glycation of proteins and the formation of AGEs (Khalifah *et al*., 1996).

In this study, opticin was glycated *in vitro* using different sugars. Biochemical studies including MALDI-TOF-MS analysis and SDS-PAGE under denaturing conditions. Due to excessive glycation by MG and presence of multiple heterogeneous glycation products, the individual peaks for each modification could not be observed in the intact protein. Such individual modifications were reported for MALDI-TOF-MS analysis of intact lysozyme glycated with glucose and fructose in vitro (Lapolla *et al*., 1996). Glycation depends upon the number of lysine, arginine, and cysteine residues present in a protein and their accessibility to solvent containing the glycating agent. The methylglyoxal readily reacts with opticin under our experimental conditions and result in structural

modifications. Incubation of opticin with MG resulted in the broadening of peaks and shift towards the high molecular mass. Similar peak shape has been described for lysozyme glycated with MG in an earlier report (Kislinger *et al*., 2005).

There are 26 arginine, 8 lysine and 6 cysteine residues in each chain of opticin which result in 40 glycation target sites. So theoretically, 80 glycation sites are present in a dimeric opticin molecule. Our results show that up to 40 moles of MG may be attached to opticin by incubating with 0.1M MG, 0.5M glucose, 0.5M fructose, and 0.5 M ribose. Considering the presence of 20 reactive amino acid in each chain of opticin dimer, this may result in complete saturation of glycation sites after 24h. *In vitro* glycated HSA modifications are reported ranging from a minimum of 10 moles per mole of protein to a maximum of 40 moles per moles of protein (Ahmed and Thornalley, 2002). Crosslinking may differ markedly with different proteins due to the fact the protein folding and context can be important factors controlling glycation (Bai *et al*., 1989). There is conflicting reports on the reactivates of glucose, fructose, and its process of producing AGEs.

During eye development, the primary vitreous contains the hyaloid vessels, but when primary vitreous undergo programmed regression shortly after birth in mice (and before birth in humans) where they are replaced by the transparent, virtually acellular, secondary (mature) vitreous demonstrates anti-angiogenic properties. In adult, it is possible that opticin helps to protect against abnormal angiogenesis, which could result in re-growth of vessel into the normally avascular vitreous which producing PDR. M Le Goff hypothesized that opticin might influence developmental changes in blood vessels in the vitreous cavity and retina, and pathologic processes in which blood vessels grow into the vitreous (Le Goff *et al*., 2011) A feature of diabetes is protein glycation. Structural studies indicate that opticin may become glycated if exposed to high sugar level. In this project, we have already demonstrated this to be true, and here we went to investigate the effect of glycation on the anti-angiogenic properties of opticin. The results presented showed that opticin inhibited FGF-2- induce proliferation; however, when cells were preincubated with glycated opticin the inhibition of FGF-2– induced proliferation was signify reduced. Similarly, glycation of opticin signify reduce its ability to inhibit endothelial cell migration

and tube formation. Some studies have partially explained the effect of glycation of proteins on its function, for example, glycation of FGF-2 had an antiproliferative effect on endothelial cell (Duraisamy *et al*., 2003). The mechanisms of impaired angiogenesis induced by AGEs were partly explained by Liu *et al* (2012) who demonstrated *in vitro* using endothelial cells and mouse aortas that methylglyoxal (the highly reactive AGEs precursor) reduces endothelial angiogenesis through RAGE-mediated, peroxynitrite-dependent and autophagy-induced vascular endothelial growth factor receptor 2 (VEGFR2).The growth factors no doubt act in synergy to mediate the steps of angiogenesis, including cell proliferation, migration and tube formation (Grant *et al.*, 2004) , and the major elements of vascular growth not perturbed by glycated opticin. At the onset of PDR it seems that while the concentration of the pro-angiogenic molecules (e.g. FGF-3, VEGF, IGF-1,and TNF) increases, the ability of these containing anti-angiogenic molecules (e.g. angiostatin, PEDF, TSP, andopticin) to prevent angiogenesis is not sufficient, practically for example opticin if glycated doesn`t function any more. So its function may be impaired and could be crucial determinant enabling debilitating haemorrhagic vascularisation (Simó *et al*., 2002). The results presented here show that BAECs and HRECs did respond to FGF-2 in cell migration in an expected manner. The two cell types produced very similar results, indicating that both macrovessel and microvessel ECs were responding to FGF-2 when compared to control cells.

AGEs and their intermediates have been implicated in pathophysiological dysfunction associated with the vascular complications of diabetes mellitus, such as retinopathy (Brownlee, 1995). Mitogen-activated protein kinase (MAPK) pathways are evolutionarily conserved kinase modules that link extracellular signals to the machinery that controls fundamental cellular processes such as growth, proliferation, differentiation, migration and apoptosis (Figure 4.1). When FGF-2 stimulates the phosphorylation of ERK it leads to activation of early response genes that have prime importance in various biological phenomena (Slevin *et al*, 2000) including cell invasion, migration and proliferation (Kaladdhar *et al.,* 2003) MEK1/2 and ERK1/2 also promotes proliferation in endothelial cells (Pages *et al*., 2000; Pages *et al*., 1993). FGF-2-induced increase in JNK which is pro-angiogenic (Uchida *et al*., 2008) and also

p38 pro-apoptotic (Ferrari *et al*., 2006). FGF-2 has a dual role in the control of endothelial cell apoptosis. On one hand, FGF-2 up-regulates VEGF expression, which is required for TGF-1 induction of apoptosis; on the other hand, it protects endothelial cells from apoptosis through a VEGF-independent mechanism. The p38 isoforms have opposing effects on the same substrate (Rousseau, 2007). In addition, the amount of p38 MAPK activity should determine the networks of substrates being phosphorylated, which in turn would impinge on the cellular response, there is evidence that strong p38 MAPK activation is likely to engage apoptosis, whereas lower levels of p38 MAPK activity tend to be associated with cell survival (Dolado, 2008).

The experiments showed in this thesis opticin decreased FGF-2-induced phosphorylation of, ERK1/2, MEK1/2 (MAPK kinase), JNK and p38 (MAPK) MAPKs phosphorylation is known to play crucial roles in cell migration and tubular morphogenesis (Mathew *et al*., 2007). It is important to understand signal mechanisms when opticin glycated. In order to understand the mechanism why glycated opticin affected its inhibit-anti-angiogenic capability the glycated opticin were incubated with FGF-2. Western blots demonstrate that pre-incubation of BAEC with opticin reduced the ability of FGF-2 increase the MAPK signal pathways (e.g. JNK, ERK, and p38) which link to angiogenesis. However, in the presence of glycated opticin there was significantly weaker inhibition of FGF-2 induce signal, so there is increase in phosphorylation expression compared to the cells treated with opticin. These results suggest the glycation of opticin reduce its ability to inhibit intracellular signalling via FGF-2 and/or its receptors.

The vitreous is normally able to resist EC invasion and the succeeding consequences of neovascularization, which may occur under pathological conditions (Neely, 1998; Campocchiaro, 2000). The failure and prevention of vessel invasion may be due partly to the influence of angiogenic inhibitors (Lutty *el al*., 1985), as several studies have assigned anti-angiogenic properties to the vitreous.

As opticin is shown to combine with collagen fibrils (Rcardon *et al*., 2000) it is proposed that opticin would not diffuse out of the matrix, therefore not affecting

activated ECs in the state of tube formation, but only affecting ECs once they began the invasion process.

Initiation of focal complexes via integrin or growth factor signalling results in dynamic changes in cytoskeletal architecture, a hallmark of cell motility (Kim *et al.*, 2011).

The presence of opticin in the vitreous along with findings observed here suggest that not only is opticin of structural importance (Bishop 2000; Hindson *el al,* 2005) but also may contribute to inhibition in the vitreous along with other vascular molecules including TGF-P (Eisenstein and Grant-Bertacchini, 1991) PEDF (Dawson *el al*., 1999) TSP-I (Sheibani *el al*., 2000) and chondromodulin-l (Funaki *el al*., 2001).

Decorin binds various ECM components, also inhibited EC migration and formation in type-I collagen gels, when ECs were grown on decorin coated surfaces (Davies *el al*., 2001).Many studies reported that decorin has antiangiogenic activity under certain circumstances Konerirajapuram *el al* (2005) showed decorin can inhibit VEGF, FGF-2, and serum-induced HUVEC migration and tube formation on Matrigel in a similar morphological representation as opticin. Decorin interact with α2β1 integrin via its glycosaminoglycan chain, and it is proposed that decorin expression in angiogenic ECs promotes angiogenesis in a collagen type-I-rich environment by signalling through insulin-like growth factor 1 receptor and influencing α2β1 integrin activity (Fiedler *et al*., 2008). Endogenous inhibitors of angiogenesis that have been previously identified in the vitreous include PEDF and TSP-I and cleaved fragments of larger molecules including angiostatin and endosiatin (O'Reilly *et al*., 1994; Dawson *et al*., 1999; Sheibani *et al*., 2000; Tombran-Tink *et al*., 2003; Maatta *et al*., 2007).

Many recent studies have elucidated that each developmental/cellular event is often regulated by a combination of multiple signalling pathways, and a significant number of extracellular proteins act as extracellular signalling coordinators. These proteins are known as matricellular proteins (Dellett *et al.*, 2012). FGFs implement their biological actions by activating cell surface fibroblast growth factor receptors (FGFRs).

The class III SLRP opticin is an extracellular matrix glycoprotein inhibits processes involved in angiogenesis through several mechanisms. It inhibits the pro-angiogenic actions of the growth factors (Le Goff *et al*., 2004). However, opticin also inhibits angiogenesis through other mechanisms, as it is effective at inhibiting the stimulatory actions of growth factors that it does not bind including FGF-1 and VEGF12ι. Other mechanisms through which opticin inhibits angiogenesis are through direct growth factor receptor interactions with VEGFR-1 and FGFR-2, thereby reducing biological activity mediated by these receptors and by interacting with integrins involved in angiogenesis including αVβ3 and α5βl thereby affecting integrin-ligand binding and/or outside-to-inside signalling and indirectly suppressing signalling through growth factor receptors. Opticin instead has a different mode of action, i.e. it binds to the ECM rather than the integrins themselves (Le Goff *et al*., 2012).

Le Goff provides evidence that opticin binds collagen and thereby inhibits α1β1 and α2β1 integrin binding to the collagen. Opticin thereby decreases the strength of EC adhesion to collagen, and the weakened adhesion is insufficient to promote angiogenesis (Le Goff *et al*., 2012).Opticin inhibits the interaction between FGF2 and FGF-Rl (A) and VEGF165 to VEGF-R2 (B) in a dose-dependent manner. Therefore in both cases the binding of opticin to the growth factor inhibits the interaction of the growth factor with its receptor. The reports have exposed that the increased formation of AGEs in the vitreous may be implicated in the development of diabetic retinopathy via inducing the production of bFGF (by retinal Müller cells (Le Goff *et al*., 2012).

Binding to RAGE activates a variety of signalling pathways leading to increased oxidative stress and synthesis of local growth factors, cytokines and adhesion molecules (Le Goff *et al*., 2012). Therefore, here we wanted to investigate if glycation of opticin affected its ability to bind directly with FGF-2 and interfere with FGF-2 integrin, thus potently affecting its ability to inhibit angiogenesis.

The results of the ELISA assays in our experiments suggest that two possibilities either opticin binds to FGF-2 prevents its binding to receptors, or opticin binds to FGFR preventing FGF-2 binding. Further investigation should carry on finding which these true or with the combination of both.

Glycated opticin may modulate the binding of FGF-2 to its receptor and change the FGFR affinity to its ligand. We notice when glycated the opticin reduce its ability to bind to collagen. The importance of collagen type-I lies on the in initiate the early stages of angiogenesis. (Vernon and Sage 1995), via outside-in signalling through α1β1 and/or α2β1 integrins (Senger *et al*., 2002; Valdramidou *et al*., 2008). Therefore seems that one of the mechanisms through which glycated opticin, opticin losses its ability as anti-angiogenic by modulation of binding to collagen, that haven observed when we used the binding assy.

Furthermore it has been shown that collagen type-I can initiate the early stages of angiogenesis via outside-in signalling through α1β1 and/or α2β1 integrins, which lead to change cell shape, contractility, and polymerization and arrangement of cytoskel et al actin into stress fibres (Davis and Senger 2005; Hoang *et al.,*2004; Senger *et al*., 2002).

Several studies have highlighted the importance of strong interactions between these integrins and their extracellular ligands in pathological angiogenesis (Davis and Senger 2005; Senger *et al*., 2002).

Bishop 2012 shows that opticin, by binding to collagen type-I, and II, hinders strong EC interactions with both α1β1and α2β1 integrins, resulting in a failure of formation of focal adhesions and actin stress fibres.

Many of the known ECM inhibitors of angiogenesis exert their effects by perturbing integrin binding and signalling pathways, including endorepellin, arresten, tumstatin, endostatin, and canstatin (Mongiat *et al*., 2003; Sudhakar, 2003).

Proteoglycans are existent in mammalian tissues, both on cell surfaces and in the extracellular matrix, where they play fundamental roles in development, homeostasis, and disease (Sarrazin *et al*., 2011; Iozzo, 2011).

The ocular distribution of the SRLP core proteins has been studied in some animal species. For example, decorin was detected throughout the neurosensory retina of the mouse and rat (Ali *et al*., 2011; Inatani *et al*., 1999) Its importance in retinal development has been demonstrated in a recent study of the avian

embryo, where inhibition of decorin function led to loss of polarization in retinal progenitor cells, along with many other abnormalities (Zagris *et al*., 2011).

Biglycan distribution has been examined in the mouse eye, where it was located throughout the retina in both embryological and adult stages (Ali *et al*., 1992).

Opticin is thought to be secreted by the ciliary body into the vitreous cavity where it co-localizes with the fine network of collagen fibrils that maintains the gel state of the vitreous and the inner-limiting lamina, a basement membrane on the inner surface of the retina (Le Goff *et al*., 2012; Bishop 2000; Ramesh, 2004) Glycation can induce abnormal cross-links between vitreous collagen fibrils leading to dissociation from hyaluronic acid and resultant destabilisation of gel structure. AGEs accumulate in the vitreous of aged people and diabetic patients. Even though nonglycational physiological and biochemical processes also contribute to vitreous degeneration, it seems likely that AGEs may play a significant role in diabetic and aging vitreous dysfunction. In terms of Maillard products and diabetic retinopathy, clinical studies have demonstrated that the levels of AGEs in the serum, skin or cornea (Sell *et al*., 1992; Sato *et al*., 2001) correlate with the onset or grade of diabetic retinopathy. AGEs are significantly increased in diabetic retinopathy patients. AGEs are localized in retinal vessels and neuroglia of diabetic patients where they exert a range of deleterious effects on cell function (Gardiner *et al*., 2003; Hammes *et al* 1999; Stitt *et al*., 1997). *In vivo* and *in vitro* studies suggest that elevated AGE level occurring in diabetes may be an important factor in retinopathy initiation and progression .

Our result shows the distributions of the opticin mostly in front of the eye and small amount in retina and vitreous. Furthermore, the diabetic mouse has more AGEs than the non-diabetic mouse, and the co-localization of AGEs with opticin was very clear in diabetic mouse compared with non-diabetic mouse, that suggested that opticin was glycated, can also being suggested by Western blot results.

This accumulation of AGEs in retina may play a causative role in the corneal epithelial disorders of diabetic patients.

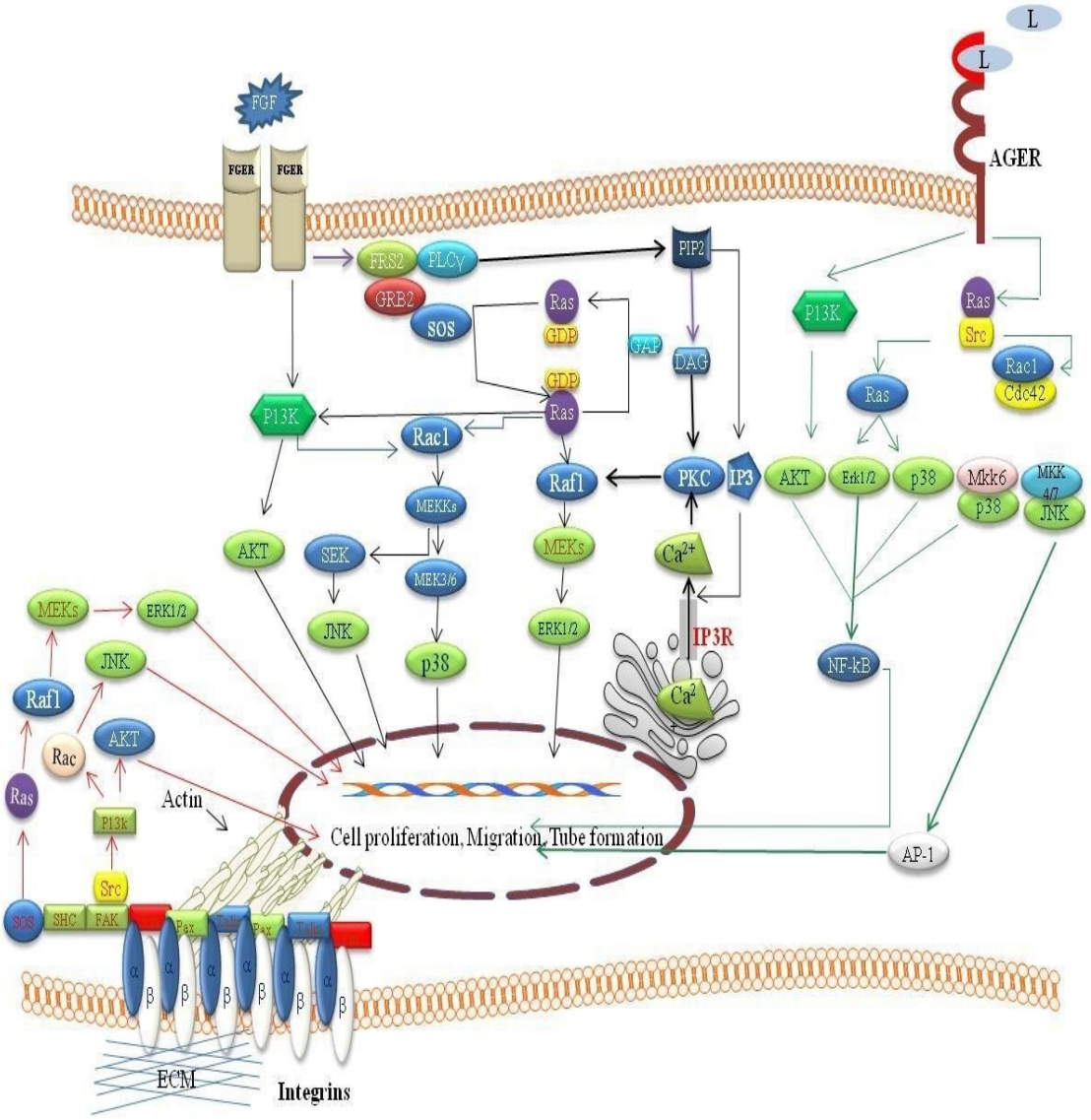


Figure 4 .1 Signal pathways; Extracellular receptors are integral transmembrane proteins and make up most receptors. Signal transduction occurs as a result of a ligand binding to the outside.Ligand to cell membrane receptor FGFs signal to the nucleus by binding to FGFR and activate multiple signal transduction pathways, including those involving Ras, MAPKs ,ERKs, Src, p38, MAPKs, JNK, and PKC .Three signaling pathways activated by Integrin receptors are cytoskeletal organization, cell proliferation and cell survival pathways. Integrins do not themselves possess a kinase domain or enzymatic activity but rely on specific ECM ligands The signaling events mediated by RAGE are complex. RAGE signaling depending on the intensity and duration of RAGE ligation, specific signaling modules such as ERK1/2, p38 MAPK, and NF-κB.

Conclusion.

Opticin can be glycated *in vitro* by exposed it to different reducing sugars. Our results show that up to 40 moles of MG may be attached to opticin by incubating with 0.1M MG, 0.5M glucose, 0.5M fructose, and 0.5 M ribose. Considering the presence of 20 reactive amino acid in each chain of opticin dimer, this may result in complete saturation of glycation sites after 24h. It is clear that our findings strongly suggest an important role for GOPT in modulation of angiogenesis. Here we demonstrat that when opticin was glycated with methylglyoxal produce formation of a glycated molecule that significantly lost its ability to reduce endothelial proliferation, cell migration and tube formation this might be one mechanism through which the angiogenic switch is altered in pro-angiogenic proliferative retinopathy. Whilst native opticin inhibited FGF-2 induced cell growth, glycated opticin had a much weaker effect. Native opticin also inhibited EGF-induced cell growth whilst glycated opticin had a weaker effect. Glycated opticin may modulate the binding of FGF-2 to its receptor and change the FGFR affinity to its ligand. Western blots demonstrate that pre-incubation of BAEC with opticin reduced the ability of FGF-2 increasing the MAPK signal pathways (e.g. JNK, ERK, and p38) which linked to angiogenesis. However in the presence of glycated opticin there was increase in phosphorylation expression of ERK, JNK, p38, and MEK, compared to the cells treated with opticin. These results suggest the glycation of opticin reduce its ability to inhibit intracellular signalling via FGF-2 and/or its receptors. We notice when glycated the opticin had reduced it ability to bind to collagen. After exposure of BAECs on collagen type-I to opticin

(native and glycated), vinculin, α2β1, and paxillin-containing focal adhesions and actin stress fibres in glycated opticin not more appeared than native opticin. This could be evidence when opticin glycated cause less binds to collagen and thereby reduce inhibits α2β1 integrin binding to the collagen that effect the opticin to promote angiogenesis which may play a causative role in the corneal epithelial disorders of diabetic patients.

Future work

- ☞ Studies should be directed at confirming these finding in a model of glycation and it is important of opticin in modulation of vascular regression vascular development.
- ☞ To examine signalling pathways in more depth.
- ☞ To investigate signalling pathways affected by glycation of opticin. ☞ To consider pre-clinical trials of glycation to prevent of opticin to inhibit PDR in the eye.
- ☞ Study GOPT on *in vivo* by using CAMB assay.
- ☞ To study if there is any relation of GOPT with cancer *in vitro.*
- ☞ How to treat GOPT with inhibitors.

Chapter 5: References

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