EVALUATION OF mRNA/ GENE EXPRESSION OF PRO-ANGIOGENIC FACTORS – Ang 1, Ang 2 AND HGF IN PERIODONTAL HEALTH AND DISEASE

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DECLARATION BY THE CANDIDATE

I hereby declare that this dissertation titled **"EVALUATION OF mRNA/ GENE EXPRESSION OF PRO-ANGIOGENIC FACTORS – Ang 1, Ang 2 AND HGF IN PERIODONTAL HEALTH AND DISEASE"** is a bonafide and genuine research work carried out by me under the guidance of **Dr. SWARNA ALAMELU, M.D.S.,** Reader, Department of Periodontology, Ragas Dental College and Hospital, Chennai.

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This is to certify that this dissertation titled **"EVALUATION OF mRNA/ GENE EXPRESSION OF PRO-ANGIOGENIC FACTORS – Ang 1, Ang 2 AND HGF IN PERIODONTAL HEALTH AND DISEASE"** is a bonafide record of work done by **Dr. KEERTHIHA.R.S** under my guidance during the study period of 2014-2017.

This dissertation is submitted to **THE TAMILNADU Dr. MGR MEDICAL UNIVERSITY** in partial fulfilment for the degree of **MASTER OF DENTAL SURGERY, BRANCH II- PERIODONTOLOGY.** It has not been submitted (partial or full) for the award of any other degree or diploma.

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LIST OF ABBREVIATIONS

Ang 1	-	Angiopoietin 1
Ang 2	-	Angiopoietin 2
Ang 3	-	Angiopoietin 3
Ang 4	-	Angiopoietin 4
ATP	-	Adenosine Tyro Phosphate
BOP	-	Bleeding on probing
cDNA	-	Complementary Deoxyribose Nucleic Acid
CHF	-	Chronic heart failure
ct	-	Cyclic threshold
DNA	-	Deoxyribose Nucleic Acid
DR	-	Diabetic Retinopathy
EC	-	Endothelial Cells
FGF	-	Fibroblast Growth Factor
GAPDH	-	Glyceraldehyde 3-Phosphate dehydrogenase
GCF	-	Gingival Crevicular Fluid
GI	-	gingival index
HGF	-	Hepatocyte Growth Factor
IA	-	Intussusception Angiogenesis
IFN	-	Interferon
Ig	-	Immunoglobulin
IL	-	Interleukins

IP	-	Inducible Proteins
LPS	-	Lipopolysaccharide
MMPs	-	Matrix Metallo proteinases
mRNA	-	Messenger ribosome nucleic acid
PAD	-	Peripheral Arterial Disease
PBS	-	Phosphate Buffered Saline
PCR	-	Polymerase Chain Reaction
PD	-	Probing depth
PEDF	-	Platelet Epithelial Derived Factor
RNA	-	Ribosome nucleic acid
RT-PCR	-	Real time polymerization chain reaction
SD	-	Standard deviation
SF	-	Scatter factor
TGF	-	Transforming growth factor
Tie	-	Tyrosine Kinase Receptors
TNF	-	Tumor necrosis factor
uPA	-	Urokinase type Plasminogen activator
VEGF	-	Vascular Endothelial Growth Factor

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Introduction

INTRODUCTION

Periodontitis is a chronic inflammatory disease of the tooth supporting structures, resulting in the destruction of connective tissue and eventually tooth loss. Chronic inflammation is evoked as a result of the response to the microbial antigens present in the subgingival biofilm, its severity is modified by numerous host related factors such as environmental factors and genetic or epigenetic traits.[68]

The inflammation begins as gingivitis before it progresses to involve the deeper structures of the periodontium. Gingival inflammation results in increased vascularity with more capillary loops, larger vessels, inflammation-induced vasculitis and vascular permeability which allows lymphocytes and serum substances to gain access into the connective tissue and leads to a cascading immune response that results in tissue destruction.[125]

Early progression of periodontal disease shows disruption of the perivascular connective tissue followed by destruction of collagenous fibers, creating spaces within the tissue which is quickly filled up by inflammatory cells and loose connective tissue. The inflammatory cells like macrophages, monocytes and T cells at the site of tissue destruction release various cytokines and growth factors which upregulate or promote the process of angiogenesis.[64]

Angiogenesis is the growth of new blood vessels from the pre-existing vessels, which occurs in response to the presence of proangiogenic factors released from ischemic tissues, and in tissues that have increased metabolic process going on as in wound healing. The process has an important role in the etiology, progression and repair of tissues in various chronic inflammatory disease conditions. The formation of new vessels can perpetuate the chronic inflammatory process by the supply of oxygen and nutrients at the site of destruction.[66]

The process of angiogenesis involves dissolution of the basement membrane of the endothelial cells, rapid reproduction of new endothelial cells that stream out of the vessel wall in extended cords directed towards the source of the angiogenic factor.[32] The cells in each cord continue to divide and eventfully fold over into tubes, which in turn connect up with other tubes budding from another donor vessel and form a capillary loop through which blood begins to flow. Later, smooth muscles cells eventually invade into the walls.[91]

The proangiogenic factors includes the vascular endothelial growth factor, epithelial cell growth factor, basic fibroblast growth factor, angiopoietins, transforming growth factor –α, β, hepatocyte growth factor, prostaglandin E_2, interleukin-8 etc.[91] Many of these molecules are proteins that induce angiogenesis indirectly by stimulating the production of acidic or basic FGF and VEGF by macrophages and endothelial cells and direct inducers of

angiogenesis.[89] These proangiogenic factors causes new vessels to sprout from either small venules or occasionally capillaries.[36] Angiostatin, Endostatin and Pigment epithelial derived factor are the most potent inhibitors of angiogenesis.[9,17,70]

In the gingival tissue, angiogenesis plays a vital role in both the maintenance of gingival health and development of periodontal diseases. In chronic gingivitis, where alterations in tissue morphology is minimal and micro flora consist of predominantly gram-positive cocci and rods, the vessels subjacent to the lining epithelium of the gingival sulcus are only affected.[123] In periodontitis, the gingival epithelium migrates apically and deepens its extensions into the underlying connective tissue, with subsequent loss of connective tissue of the periodontium.[87] Thus in both the conditions disruption of vasculature takes place leading to accumulation of proangiogenic cytokines in the diseased site and the process of angiogenesis takes place leading to vascular remodeling and maintenance of tissue homeostasis.[49] Studies have also proved that bone formation and regeneration processes are closely linked to angiogenesis.[16,19]

The Angiopoietins have been identified in the mid1990s as a family of growth factors that are essential for blood vessel formation. There are four known members in the family so far. They are Angiopoietin1, Angiopoietin2, Angiopoietin3 and Angiopoietin4.[109] Ang-1 acts as an agonist, whereas Ang-2 is the antagonist. Ang-1 is primarily expressed by mesenchymal cell and acts

in a paracrine manner on the endothelium. Ang 1 helps in mediating vessel remodeling and maturation. Their biologic functions are endothelial cell sprouting and vessel stabilization.[6]

Ang 2 is expressed in regions of vascular remodeling under physiological and pathological conditions.[43,71] Ang-2 levels are upregulated in hypoxic conditions.[51,61,81,90] They act via an internal autocrine loop mechanism.[33] The biologic functions of Ang-2 are migration of endothelial cells and proliferation of the endothelial cells.[69] A few studies have identified and evaluated the presence and role of Ang1 and Ang2 in gingival inflammation, granulation lesions of the gingiva and periodontal disease conditions.[36] Ang1 and Ang2 have been shown to modulate angiogenesis by both proangiogenic and anti angiogenic mechanisms.[123]

Hepatocyte growth factor also known as a pleiotropic cytokine, acts on epithelial cells in several organs [55] including the endothelial cells,[13] βcells in the pancreas,[86] and epithelium in the kidney[55] and lungs.[120]

Studies have reported that periodontal fibroblasts secrete an HGF-like chemoattractant for a gingival epithelial cell line, and hypothesized that HGF may be involved in epithelial invasion following the loss of connective tissue attachment in periodontitis.[83,84] Studies by **Ohshima et al.** revealed that the HGF level in GCF correlates well with clinical parameters of periodontal

disease, and suggest that HGF may be involved in epithelial invasion through its role as a scatter factor.[82]

Although the pivotal role of angiogenesis in chronic inflammation is well established, still insights into the molecular mechanisms involved in the process are few and yet to be unraveled. So this study aims to evaluate the gene expression of few angiogenic mediators like Ang1, Ang2 and HGF in periodontal health and disease.

Aims and Objectives

AIMS AND OBJECTIVES

The objectives of the present study are:

1. To evaluate and compare the expression of angiopoietin-1, from gingival tissues in periodontal health and disease.
2. To evaluate and compare the expression of angiopoietin-2, from gingival tissues in periodontal health and disease.
3. To evaluate and compare the expression of hepatocyte growth factor, from gingival tissues in periodontal health and disease

Review of Literature

REVIEW OF LITERATURE

Periodontitis is a chronic inflammatory disease affecting the tooth supporting structures which is initiated by the emergence of a pathogenic biofilm and is characterized by non-resolving inflammation. The disease progression is thought to be episodic in nature, with shorter periods of exacerbation followed by longer periods of remission.[102]

The inflammatory response in periodontal disease results in increased vascularity with more capillary loops, larger vessels size and slowing of blood flow. Inflammation induced vasculitis and vascular permeability allows lymphocytes and serum substances to percolate into the connective tissue and leads to the events of tissue destruction.[68]

Vascular changes are important in both inflammation and repair of chronic diseases and blood flow plays a vital role in its resolution. Inflammation begins with vasodilation, increased vascular permeability, circulatory stagnation and diffusion of cells into the surrounding tissues resulting in tissue destruction.[22]

Angiogenesis refers to the growth and development of new capillary blood vessels from a pre-existing vasculature in response to external stimuli. Vasculogenesis is the development of new blood vessels which are required for many physiological processes like embryogenesis, wound healing and corpus luteus formation.[12] The process of tissue regeneration and the repair of

wounds, cyclical proliferation of the nutrient-rich endometrium in preparation for implantation of the fertilized egg, and development of the embryo and its supporting tissues, is dependent on the rapid growth of new capillary blood vessels, known as "angiogenesis".[97]

PHYSIOLOGIC ANGIOGENESIS:

Physiologic Angiogenesis is the process of formation of new blood vessels from pre-existing vascular tissues, by two different mechanisms, such as the sprouting of new vessels from pre-existing vascular tubes or by the splitting of pre-existing vessels through intussusception angiogenesis (IA).[28]

The process of angiogenesis is initiated by quiescent endothelial cells (EC), which becomes activated by angiogenic signals (Pro-angiogenic factors). These activated ECs then alter the proteolytic balance of degradation of the basement membrane and promote the dissociation of pericytes from the capillaries. These ECs then proliferate and migrate towards the “tip-cells” (angiogenic stimulus).[31] Finally tip cells fuses with cells from the neighboring sprout to generate connecting vessel loops.[16, 32, 89]

PATHOLOGICAL ANGIOGENESIS:

The loss of endothelium’s quiescent state is a common feature of pathological condition. It is characterized by the failure of the resolution phase and by the generation of a highly disorganized vascular network. Molecules

that are shared by both angiogenic sprouting and the recruitment of inflammatory cells are likely to underlie the combinational processes.[5, 35, 37, 47]

Judah Folkman coined the term "Angiogenic Switch" describing the progression and the inhibition of angiogenesis by angiogenic stimulators and inhibitors.[40,41] Pathological angiogenesis occurs in chronic diseases like Neoplasia, Vascular Malformations and Cardiovascular Disorders, Syndromes like Maffucci's Syndrome, Rendu-Osler-Weber Syndrome. It also plays a vital role in Chronic Inflammatory Diseases and Aberrant Wound Repair conditions like Diabetes, Hypertrophic scars, Non- healing fractures, Osteoradionecrosis, Psoriasis, Pyogenic granuloma, Rapidly progressing adult and juvenile periodontitis, Rheumatoid arthritis, Systemic sclerosis, Venus stasis ulcers.[77]

PROCESS OF ANGIOGENESIS:

The angiogenesis process is induced by so called stimulators, that exhibit chemotactic and mitogenic activity against epithelial cells, stimulating them to synthesize proteolytic enzymes (collagenase and plasminogen activator), thus leading to the basal membrane degradation, as well as enabling the migration of new epithelial cells, proliferation and differentiation of fibroblasts. These stimulators do also lead to formation of three-dimensional tubular structures, that will be turned into future blood vessels.[17, 56, 110, 121] The following are the various types of angiogenic stimulators that take part in both types of angiogenesis.

PRO-ANGIOGENIC MEDIATORS

Adhesion molecules

E-selectin

VE-cadherin, PECAM (CD31)

VCAM-1

Cell surface antigens

AC (CD) 133

Chemokines

Interleukin-8 (IL-8)

Monocyte chemotactic protein-1 (MCP-1)

Cyclooxgenase and related enzymes

COX-2

Nitric oxide synthase

Developmental/specification signals

Ephrins

Id1/Id3

Notch

Growth and differentiation signals

Ang 1 & Tie2

Endoglin & receptors

Acidic and basic fibroblast growth factor (a and bFGF)

Neuregulin/heregulin

Platelet derived growth factor-BB (PDGF-BB)

Scatter factor/hepatocyte growth factor/c-Met (SF/HGF)

Transforming growth factor -β1 (TGF-β1)

Tumor necrosis factor- α (TNF-α)

Vascular endothelial growth factor family/VEGFR/ Neuropilins

Hormones

Leptin

Integrins

αVβ3

αVβ5,

α5β1

Proteinases

Matrix metalloproteinases

Plasminogen activators

Plasminogen activator inhibitor type 1

ANGIOGENESIS INHIBITORS

Ang 2

Angiostatin

Canstatin, proliferin-related protein, restin

Endostatin

Interferon (IFN)-α, β, γ,

Inducible protein-10 (IP-10)

Interleukin –4 (IL-4)

Interleukin-12 (IL-12)

Interleukin-18 (IL-18)

Platelet Factor 4

Prothrobin kringle 2

Prolactin VGI

Osteopontin fragment

Mapsin

Meth-1, Meth-2

Retinoids

SPARC

TIMPs, MMP inhibitors, PEX (MMP2 C terminal fragment)

Thrombospondin-1 & -2

Vasostatin.

ANGIOGENIC FACTORS:

The vascular endothelial growth factor (VEGF) - VEGF is the most well-known and predominant member of angiogenesis. It was discovered by **Abraham and Schilling** in **1989** as endothelial cell specific mitogen.[45] They are a family of 6 structurally related proteins that regulate the growth and differentiation of the vascular system. There are 4 major isoforms of VEGF coded by a different portion of the VEGF gene.[34] Though these isoforms have identical characters, they differ in their ability to bind to the extra cellular matrix. The function of VEGF is that it stimulates vascular endothelial cell growth, survival and proliferation.[35] VEGF stimulates cellular responses by binding to tyrosine kinase receptors on cell surface, causing them to dimerise

and becomes activated through transphosphorylation. In preclinical models, VEGF has shown to facilitate survival of existing vessels, contributes to vascular abnormalities that may impede effective delivery of antitumor compounds and stimulate new growth. VEGF plays a crucial role in growth process.[5] Blocking of VEGF signals by administrating soluble VEGF receptors resulted in suppression of blood vessel invasion into the bone growth plates with significant reduction in their length.[35]

Angiopoietins- Angiopoietin is part of a family of vascular growth factors that play a vital role in embryonic and postnatal angiogenesis. They are paracrine growth factors. The Angiopoietin family includes four ligands – Angiopoietin 1, Angiopoietin 2 and Angiopoietin 3/4 and two corresponding tyrosine kinase receptors -Tie1 and Tie2.[65] Ang 1 acts in a paracrine agonistic manner inducing Tie2 phosphorylation and subsequent vessel stabilization.[23] Ang 2 acts as an autocrine antagonist of Ang 1-mediated Tie2 activation.[33] Hence, Ang 2 primes the vascular endothelium to exogenous cytokines and induces vascular destabilization at higher concentrations.. The lesser known members of the family, Ang 3 and Ang 4, are orthologues found in mouse and human, respectively.[65]They have different tissue distributions: Ang 3 is expressed in multiple mouse tissues, whereas Ang 4 is specifically present at high levels only in human lungs. Ang 4 phosphorylates Tie2, whereas Ang 3 not only fails to phosphorylate Tie2, but it even inhibits Ang 1-induced phosphorylation of Tie2 in human EC.[113] Studies have shown that Ang 3 and 4

are agonists of Tie2 receptor signaling, with Ang 3 being a specific ligand for Tie2 receptors of its own species.[64]

Fibroblast growth factor (FGF): Fibroblast growth factors are a family of growth factors, involved in angiogenesis, wound healing, embryonic development and various endocrine signaling pathways.[92] FGFs have a high affinity for heparan sulfate proteoglycans.[92] During embryonic development, FGFs have diverse roles in regulating cell proliferation, migration and differentiation.[77] In the adult organism, FGFs are homeostatic factors and function in tissue repair and response to injury. Under abnormal conditions, some FGFs can attribute to the pathogenesis of cancer.[85] There are 20 distinct FGF and four different tyrosine kinase receptors (FGFRs) .[92] FGF-1 (acidic FGF) and FGF-2 (basic FGF) are part of the first growth factors that stimulate angiogenesis.

Hepatocyte growth factor (HGF): It's a multifunctional cytokine involved in embryonic development and the repair and regeneration of various tissues /organs and their protection from injury. It is a protein secreted by fibroblasts and promotes matrix invasion of epithelial cells.[80] They exhibit mitogenic and antiapoptotic activity.[64] They enhance the mobility of different cell types, including the epithelial and vascular endothelial cells apart from the hepatocytes.[85] Under inflammatory conditions they are expressed in mesenchymal cells whereas their receptors are found in the epithelial cells, endothelial cells, and progenitor cells.[41] In endothelial cells and vascular

smooth muscle cells, HGF induces migration, proliferation and prevents cells from apoptosis.[73] Several studies have shown that HGF is involved in the development of periodontal diseases. Their expression have been studied in GCF, saliva and tissues and they have correlated with the clinical parameters.[74]

ANGIOSTATIC FACTORS:

Angiostatin is a specific endogeneous angiogenesis inhibitor produced in tumors. It inhibits primary and metastatic tumor growth by blocking tumor angiogenesis. It is derived from plasminogen spanning the first 4 kringle domains.[93] Angiostatin suppresses proliferation, migration, differentiation, and tube formation of in vitro endothelial cells and potently inhibits angiogenesis.[70] Systemic administration of angiostatin will induce mechanisms that consist of binding of the subunits of ATP synthase on the cell surface of endothelial cells which might inhibit proliferation and potently block neovascularization and growth of tumor metastasis.[116]

Endostatin was first identified from conditioned medium of a hemangioendothelioma cell line as a potent inhibitor of angiogenesis and tumor growth in vivo.[70] It is a broad-spectrum angiogenesis inhibitor and may interfere with the pro-angiogenic action of growth factors such as basic fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF).[119] Endostatin also blocks migration and proliferation of endothelial cells and increases apoptosis.[10] Endostatin interactions results in the

disruption of microfilaments and thereby facilitate to the inhibiting cell motility leading to angiostatic effect.[10,70]

Pigment epithelium derived factor: Pigment epithelium-derived factor (PEDF) was first discovered by **Joyce Tombran-Tink** and **Lincoln Johnson**.[111] They belong to the broadly expressed multifunctional member of the serine proteinase inhibitor (serpin) family. They play critical roles in neuroprotection, angiogenesis, fibrogenesis and inflammation. PEDF was originally isolated from the conditioned medium of cultured human fetal retinal pigment epithelial cells.[110] **Dawson et al**[27] demonstrated PEDF as a potent endogenous anti-angiogenic factor PEDF levels were found to decline in angiogenic tissues/organs, in conditions such as the vitreous, aqueous humors and retinas of patients with proliferative diabetic retinopathy (DR) and in tumors of cancer patients.[103, 122]

THE ANGIOGENESIS CASCADE

Angiogenesis occurs as an orderly cascade of molecular and cellular events in the wound bed:

1. Endothelial cell surface has receptors to which angiogenic growth factors bind in preexisting venules (parent vessels);
2. Growth factor-receptor binding activates signaling pathways within endothelial cells;

3. Proteolytic enzymes released by activated endothelial cells dissolve the basement membrane of surrounding parent vessels;
4. Endothelial cells proliferate and sprout outward through the basement membrane;
5. Endothelial cells migrate into the wound bed using integrins (αvβ3, αvβ5 and αvβ1) which are cell surface adhesion molecules;
6. Matrix metalloproteinases (MMPs) dissolve the surrounding tissue matrix in the path of sprouting vessels;
7. Vascular sprouts form tubular channels that connect to form vascular loops;
8. Vascular loops differentiate into afferent (arterial) and efferent (venous) limbs;
9. New blood vessels mature by recruiting mural cells (smooth muscle cells and pericytes) to stabilize the vascular architecture;
10. Blood flow begins in the mature stable vessel.

Angiogenesis in chronic diseases:

"The angiogenic switch" is a term that implies the balance between angiogenic and angiostatic factors. Disturbance of this balance leads to a number of diseases[34] that feature the over proliferation of blood vessels consisting of hypertension, cancers, psoriasis, arthritis, diabetes, obesity, asthma, atherosclerosis and other chronic inflammatory conditions.[1,57,124] Defect in angiogenesis also causes considerable heart brain ischemia,

neurodegeneration, hypertension, osteoporosis, respiratory distress, preeclampsia, endometriosis, postpartum cardiomyopathy, and ovarian hyperstimulation syndrome.[18]

Shoab et al. in 1999 stated that chronic venous stasis ulcer patients have elevated levels of VEGF in their circulation explaining the up regulation of angiogenic factors in hypoxic condition.[101]

AY Chong at al. in 2004 reported that Ang-2 levels were higher in acute Congestive Heart Failure (CHF) patients compared with chronic CHF.[24]

McCarty et al. in 2012 explained that in venous ulcers, the persistence of glomeruloid vessels may interfere with oxygen delivery and delay healing. In chronic venous stasis ulcers, high levels of proteases such as neutrophil elastase, MMPs and urokinase‐type plasminogen activator are upregulated.[75]

Weinheimer‐Haus et al. in 2014 reported that low intensity vibrations may exert beneficial effects on wound healing by enhancing angiogenesis and granulation tissue formation, and these changes are associated with an increase in pro‐angiogenic growth factors.[116]

Peripheral arterial disease (PAD) results in severe ischemia. Reduced tissue perfusion due to ischemia results in progressive tissue hypoxia, ischemia, necrosis and skin damage. According to literature, tissue hypoxia should initiate angiogenesis through an activated HIF‐1α and angiogenic

growth factors. **Konya et al 2014** reported that patients with PAD, had elevated serum levels of hepatocyte growth factor than in normal subjects thus substantiating the literature.[63]

ROLE OF ANGIOGENESIS IN PERIODONTAL DISEASE AND SURGERY:

Angiogenesis takes place both in the maintenance of gingival health and development of chronic inflammatory disease.

The development of a vascular system at the injury site enable pro inflammatory cells to be transported to the lesion, supply oxygen and nutrients and remove the cellular debris from inflamed tissues.[63] To complement this there is an increase in the potential substrate, for the production of cytokines, adhesion molecules and further progression factors for inflammation.[52]

Johnson et al in 1999 demonstrated the effect of VEGF to be a factor in initiation and progression of gingivitis to periodontitis, possibly by promoting expansion of the vascular network coincident to progression of the inflammation.[53]

Yuan et al in 2000 was the first to demonstrate the expression of Ang 1 and Ang 2 proteins in the cytoplasm of macrophages-like mesenchymal cells and smooth muscle cells by immunohistochemistry in pyogenic granuloma along with other vascular morphogenic factors.[123]

Lakey et al. in 2000 explained the importance of angiogenesis in tissue repair and explained the therapeutic implications of angiogenesis on periodontal surgery for wound healing .[64]

Uzel et al in 2001 conducted a semiquantitative analyses on phenytoin induced gingival overgrowth tissues and suggested a possible role of proangiogenic factor FGF in promoting the development of fibrotic lesions in phenytoin-induced gingival overgrowth.[112]

Byun et al. in 2007 explained that the expression patterns of VEGF and its receptors plays an important role in osteogenesis, and that osteoblasts and immature fibroblast-like cells of the distracted bone may have an autocrine growth effect during distraction osteogenesis.[14]

Suzuki et al. in 2011 stated that dental pulp stem/progenitor cells can be induced to migrate by chemotactic cytokines and act as endogenous cell sources for regeneration and mineralization.[107]

Aspriello et al. in 2011 compared the effects of enamel matrix derivative and the VEGF on gingival tissues in chronic periodontitis patients. He showed a statistically significant upregulated effect of VEGF on the gingival tissues of periodontal pockets than enamel matrix derivative component.[3]

THE ANGIOPOIETIN FAMILY:

Davis in 1996 discovered the first member of the angiopoietin family, Ang 1, by its ability to bind Tie2 extracellular domain.[26] Later, **Valenzuela et al in 1999** demonstarted that low expressions of angiopoietins were used to clone Ang 2, 3 and 4.[113] The angiopoietins share a similar overall structure with a short amino-terminal motif followed by a coiled-coil domain and carboxy-terminal fibrinogen-like domains. The Angiopoietins are secreted glycoproteins with a dimeric molecular weight of approximately 75 kDa. Ang 1 has 498 aa and is located on chromosome 8q22.[4] Ang 2 has 496 aa and is located on chromosome 8q23.[4] Both Ang 1 and Ang 2 molecules show sequence homology of about 60% in human beings.[32]

Hanahan D in 1997 proposed that the pattern of expression and regulation of Ang 1 and Ang 2 is consistent with the concept that Ang 1 provides a constitutive tonic signal to promote quiescence of the endothelium and this is modified by the more actively regulated antagonist Ang 2.[49]

. **Stratmann in 1998** explained Ang 2 expression occurs in areas of endothelial activation and angiogenesis, like in ovary and tumor vessel endothelia where it coincides with vessel destabilization during angiogenesis.[105]

Studies by **Partanen et al. in 1999** described Ang 1 as widely expressed and is present in periendothelial cells in quiescent vasculature.[88]

Gravallese in 2003 demonstrated that Ang 1 is also involved in inflammatory diseases. Synovial fibroblasts are a key player during inflammatory diseases and they are a major source of Ang 1.[47]

Fiedler in 2004 stated that Ang 2 expressed in endothelium is stored within Weibel-Palade bodies and rapidly released following stimulation with thrombin and other agonists.[39]

Witzenbichler in 2005 stated that Ang 1 acts as an anti-inflammatory cytokine and protects against endotoxic shock-induced by LPS and thereby prevent microvascular leakage.[118]

Scott in 2005 explained that Ang 1 is also upregulated during diseases by inflammation promoting cytokines, including TNF-a via the NF-kappa B signaling pathway .[100]

Colton 2010 stated that during chronic inflammation, the Ang–Tie system is involved in process expands the region of the vasculature in which vessel leakage and leukocyte emigration occurs.[20]

ANGIOPOIETIN RECEPTORS:

Tie2

Ang 1 binds and signals through the receptor tyrosine kinase Tie2.[20] This receptor and its close relative Tie1 share a similar structure with an extracellular domain of an immunoglobulin (Ig)-like motif followed by three EGF-homology domains a second Ig motif and three fibronectin type III repeats.[29] The amino acid sequence identity between Tie1 and Tie2 intracellular domains is 76%.[96]

Tie1 & Integrins:

Studies by Carlson 2001, explained that stable expression of the human α5 integrin subunit in these cells rescued adhesion to Ang 1 and promoted an increase in adhesion to Ang 2. He also suggested that Ang 1 and Ang 2 can directly support cell adhesion mediated by integrins.[15]

Dallabrida in 2005 showed that Angiopoietin 1 limits ischemia-induced cardiac injury by blocking antibodies. Further Angiopoietin 1 may prove therapeutically valuable in cardiac remodeling by supporting myocyte viability and preserving pump function.[25]

Weber in 2005 showed that the isolated receptor-binding domain of Ang 1 is capable of mediating effects on full-length Ang 1 independently of Tie2 phosphorylation, possibly through integrin ligation.[114]

Recent studies by **Saharinen in 2009** indicate that, Ang family members may signal through Tie1. Ang 1 can induce Tie1 phosphorylation in endothelial cells.[97] It is likely that either the ligand interacts with Tie1 to induce activation, or that it activates Tie1 indirectly via another Ang 1 receptor.[21] The mechanism of action of this is still unclear. Therefore, Ang 2 appears to antagonize the effects of Ang 1 on Tie1 phosphorylation. Thus, Ang 2 could act to limit Ang 1 signaling through Tie1 as it does with Tie2.[30]

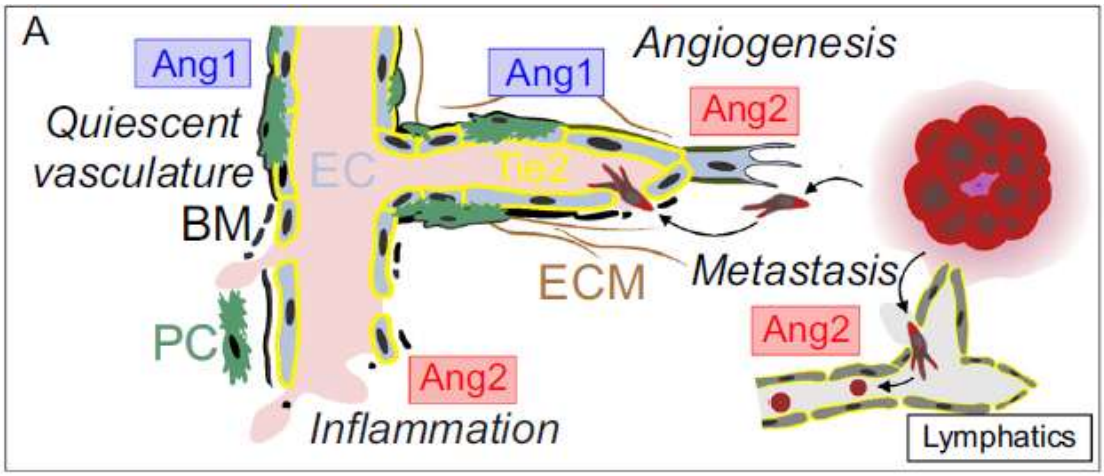
CELLULAR EFFECTS AND SIGNALLING OF ANGIOPOIETIN

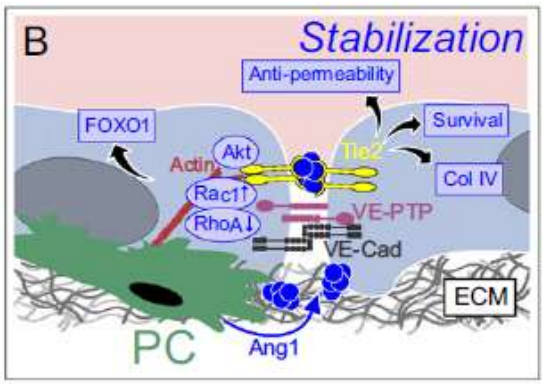
The steps involved in the cellular and signaling mechanism of Ang 1 and Ang 2 are

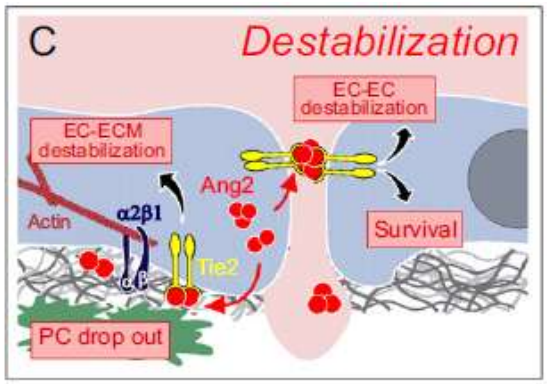
- **Apoptosis**
- **Migration**
- **Reorganization**
- **Proliferation**
- **Inflammatory gene expression**
- **Permeability**
- **Hematopoiesis**
- **Putative signaling from Tie1 and integrins.**

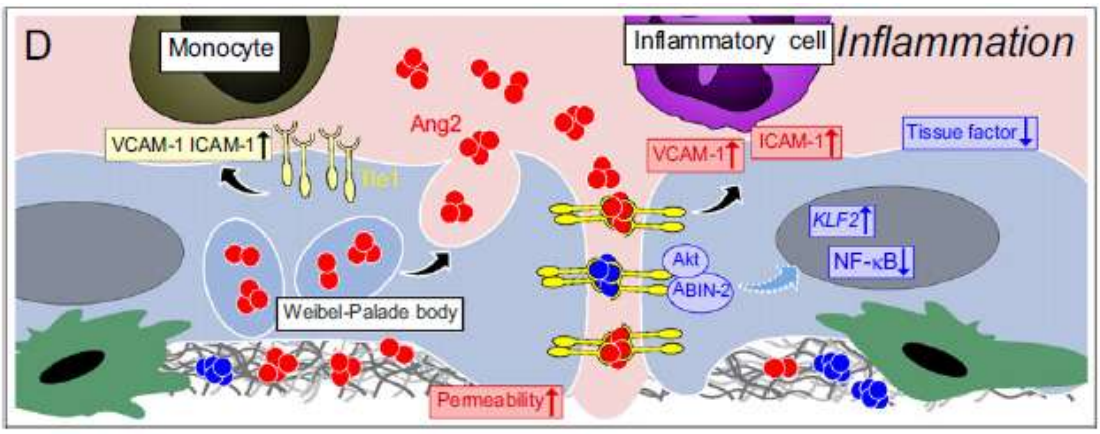
Studies by **Rossant 1995** with Tie1 gene targeted mice have shown that in vivo, Tie1 is required cell for endothelial cell survival during late embryogenesis.[94, 99] Thus it is likely that Tie1 has a role in suppression of endothelial apoptosis, possibly via the PI3K pathway.[20]

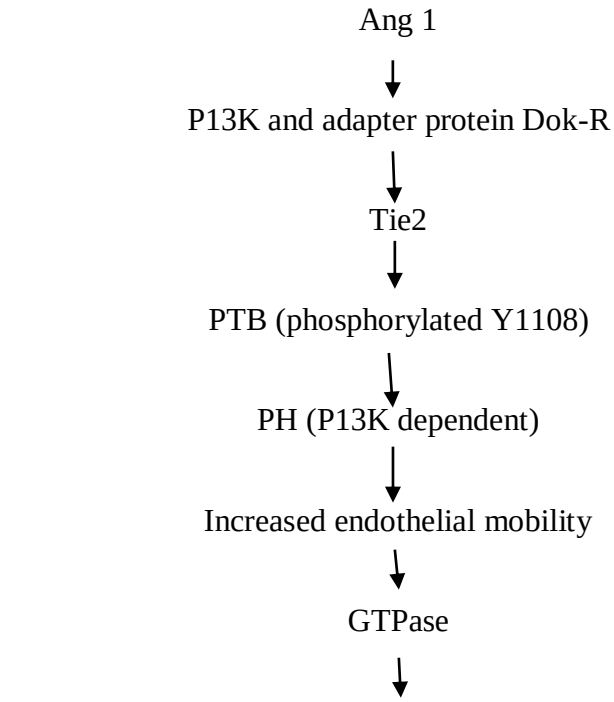
Hashiyama &Ohshiro 1996, explained the presence of Hematopoietic progenitor cells[50,8] and their consistency with hematopoiesis, the Tie2/angiopoietin-1 signaling regulates hematopoietic stem cell quiescence in the bone marrow niche.[2]


A
Angiogenesis
Ang1
Quiescent vasculature
BM
EC
Ang1
Tie2
Ang2
ECM
Metastasis
PC
Ang2
Inflammation
Ang2
Lymphatics


B
Stabilization
Anti-permeability
FOXO1
Survival
Akt
Actin
Tie2
Col IV
Rac1↑
RhoA↓
VE-PTP
VE-Cad
ECM
PC
Ang1


C
Destabilization
EC-EC destabilization
EC-ECM destabilization
Ang2
Actin
α2β1
Survival
Tie2
PC drop out


D
Monocyte
Inflammatory cell
Inflammation
VCAM-1 ICAM-1
Ang2
Tie1
VCAM-1
ICAM-1
Tissue factor
KLF2
Akt
ABIN-2
NF-κB
Weibel-Palade body
Permeability



Hansbury 2001, explained Ang 1- stimulated reorganization of endothelial tubules and invasion into matrices is not seen in endothelial cells lacking Tie2[61] but can be stimulated by a Tie2-activating antibody in the rats.[49] Consistent with such remodeling effects, Ang 1 stimulates production of proteases including plasmin and matrix metalloproteases, as well as suppressing secretion of tissue inhibitor of metalloprotease-2.[58] The inhibitors and dominant-negative constructs has indicated a number of signaling intermediates involved in Ang 1-induced re-organization of endothelium and motility, including PI3K, the adaptor protein ShcA, focal adhesion kinase and

endothelial nitric oxide synthase.[4,6,23,58] Additional growth factors may be necessary for Ang 1 to induce endothelial proliferation.

An important cellular effect of Ang 1 is its ability to improve integrity of endothelial monolayers. The ligand augments integrity of unstimulated monolayers and counteracts the increased permeability following activation with thrombin, VEGF or TNFα.[43, 98] Yet again, the signalling mechanisms mediating these effects have yet to be delineated.

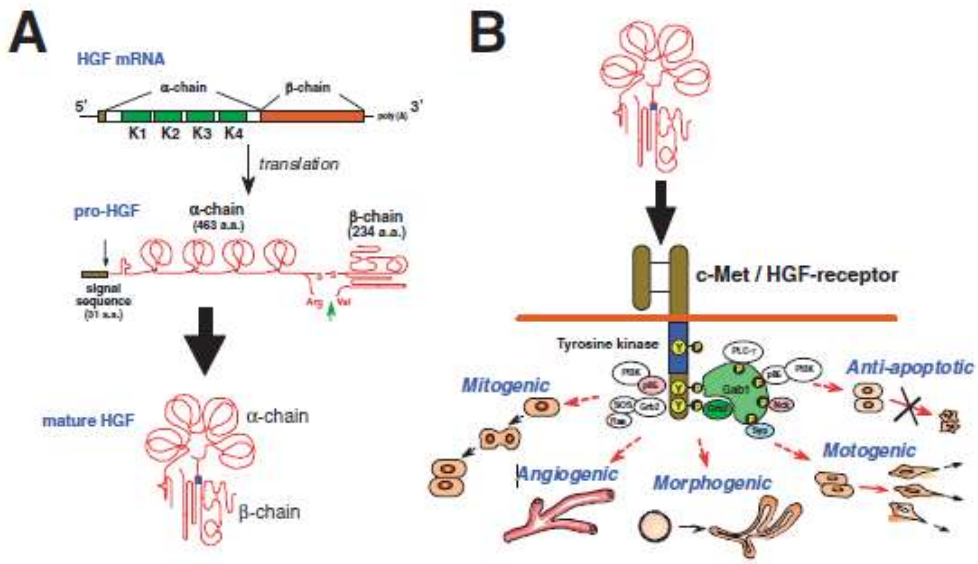
The vascular protective effects of angiopoietins have been found to promote blood vessel survival, inhibit vascular leakage and suppress vessel inflammation.[11]

Lester et al. in 2009 revealed that lower concentrations of Ang 1 within inflamed gingiva compared to healthy gingiva, suggest that Ang 1 may be a factor determining the severity of periodontal disease, he strongly explains that there is a significant negative correlation between the severity of gingival inflammation and Ang 1 concentrations in diseased sites.[66]

Hepatocyte growth factor also known as scatter factor (SF) is a heterodimeric protein secreted by cells of the mesodermal origin. HGF was first described by **Stoker and Perryman**[104] as a secretory product of fibroblast which dissociates epithelial cells, thereby increasing their motility and invasiveness. HGF causes spread of the epithelial cells and hence also known as scatter factor. **Weidner** further reveled that SF and HGF are identical.[115]

HGF affects nervous system, immune system and reticuloendothelial system. It induces a spectrum of biological activities in epithelial cells, inducing mitogenesis, stimulation of cell motility and the promotion of matrix invasion and is a potent angiogenic factor.[79] It acts as a mitogen, motogen, morphogen, anti-apoptotic factor, and scatter factor for various kinds of epithelial cells.[73] It is a protein secreted by mesenchymal cells such as fibroblasts, and promotes motility and matrix invasion of epithelial cells.[85]

HGF is produced by mesenchymal cells such as fibroblasts, macrophages, smooth muscle cells, endothelial cells and the fat storing cells in the liver. The tissues that contain HGF protein are blood cells (monocytes, leukocytes and platelets), liver, lungs, brain, bone marrow, spleen and placenta. HGF has a molecular weight of 84 kDa.[114]



Structure and biological function of HGF

Production and activation of HGF:

HGF is produced and secreted as a pro-HGF by stromal cells such as fibroblasts, macrophages, renal mesangium and so on. Secreted pro-HGF is cleaved at Arg and Val by HGF-activators, such as urokinase-type plasminogen activator (uPA).[85] Multi-faceted biological actions of HGF are mediated via the c-Met/HGF-receptor. Binding of HGF to c-Met leads to tyrosine phosphorylation, and each biological activity is elicited via the recruitment of down-stream adaptor molecules.[114]

HGF is a pluripotential regenerative cytokine – it is a key factor in the pathogenesis and progression of periodontal disease, mostly through its over-stimulation of gingival epithelial cell growth and impairment of the regeneration of collagenous structures.[73]

HGF in periodontal disease:

Studies by **Ohshima et al, 2001** showed that periodontal ligament fibroblast and gingival fibroblast secrete an HGF- like factor, and suggested that such a factor derived from periodontal fibroblasts might play a role in epithelial apical migration in periodontitis.[82]

Studies by **Otsuka K 2002**, showed that the HGF level correlated positively with PD and GI, and was significantly higher in specimens from

BOP-positive sites and those where PD exceeded 4 mm compared with those from sites that were BOP-negative or with a PD less than 3 mm.[83]

Mitsuhiro et al, 2002, suggested that synergistic expression of HGF and HGFA in gingiva may indicate a high risk for epithelial down growth followed by loss of connective tissue attachment without inflammatory cell infiltrate.[84]

In **2003, Ohshima et al** also suggested that, elevated levels of HGF in GCF may reflect disease progression, specifically loss of connective tissue attachment followed by epithelial invasion and bone resorption.[85]

Magdalena et al 2006 proved that the salivary HGF concentration in patients with periodontal disease required treatment almost 3 times higher than in healthy subjects. They further suggested that salivary HGF was a novel potential marker for early and symptomatic periodontal disease.[117]

Nagaraja, Pradeep et al 2007 proposed that HGF concentration increased proportionally with the progression of periodontal disease, and HGF concentrations showed a positive correlation with the clinical parameters, suggesting that HGF plays a key role in periodontal disease progression. Also, following non-surgical periodontal therapy, the levels of HGF decrease significantly suggesting that HGF could be useful for monitoring the response to periodontal therapy.[78]

Rudrakshi et al 2011 in a study demonstrated a significant correlation in the levels of HGF in GCF and in saliva of patients with and without chronic periodontitis. The results also indicated that the HGF levels in GCF and saliva correlated well with the clinical parameters and with the severity of the periodontal disease.[95]

Materials and Methods

MATERIALS AND METHODS

Twenty patients who reported to the Outpatient Department of Periodontics, Ragas Dental College, Chennai were recruited in the study. Patients were divided into two groups based on their periodontal health status. Informed written consent was obtained from all the patients. The patients were informed that this research work was in no way directly related to the therapy or cure of the disease. The study was undertaken following approval from the Institutional Review Board.

STUDY POPULATION / INCLUSION CRITERIA:

The subjects were divided into two groups:

Group A: Healthy subjects (Control group) who had Probing Depth <3mm with no clinical attachment loss, Bleeding on Probing <10%.

Group B: Advanced chronic periodontitis patient (Test group) who had Probing Depth ≥6 mm, Clinical attachment loss ≥5mm with bleeding on probing.

EXCLUSION CRITERIA:

- Patients with history of periodontal therapy or antibiotic therapy in the past 6 months.
- Patients with history of systemic diseases that may affect the periodontal status.
- Pregnancy and Lactation.
- Smokers.

SAMPLE COLLECTION:

Gingival sample:

Healthy tissue samples were obtained from patients who had reported to the department for crown lengthening procedure. Diseased tissue samples were obtained from patients who underwent modified Widman surgery for advanced periodontitis. Tissue samples were thoroughly washed with PBS (Phosphate buffered saline) and stored in RNA*ase* inhibitor at -20°C until further RNA extraction is carried out.

TOTAL RNA EXTRACTION

Isolation of total RNA:

Total RNA was isolated from health and diseased cells by using total RNA isolation reagent (Swift tissue RNA) kit.

REAGENTS:

1. Swift tissue RNA Kit(Buffer SL, Buffer SE)
2. β-Mercaptoethanol
3. 70% ethanol

Procedure:

600µl of buffer SL was taken in a 1.5ml tube. 6µl of β-mercaptoethanol was added and vortexed. Approximately, the volume of buffer SL and β-ME (1%) has been increased for larger volume of sample. Tissue sample of about 20-30mg was added and homogenized using tissue lyseror by plastic pestle. The homogenized mixture was centrifuged at 13,400rpm for 15mins. 350µl of the clear supernatant was pipetted out into a 1.5ml tube and equal volume of 70% ethanol (350µl) was mixed to the content for binding. 600µl of the lysate was then centrifuged at 10,000rpm for 15secs. The flow through was discarded and the SMS column was backed with the collection tube. 600µl of 70% ethanol was added to the SMS column and centrifuged at 10,000rpm for 15secs and the column was washed. The flow through was then discarded. The wash step was repeated one more time to improve the quality of RNA. The collection tube was then centrifuged for 2mins at 13,400rpm to remove the residual ethanol. It was then transferred to a new 1.5ml tube and 50-100µl of buffer SE was added on the membrane and incubated for 1min. The tubes were centrifuged at 13,400rpm for 1min. Tissue RNA was collected at the bottom of the tube. The collected RNA was stored at -20°C.

QUANTIFICATION OF RNA

The RNA pattern obtained by above mentioned method was clear as expected and was observed by spectrophotometric readings i.e., absorbance ratio at 260 and 280nm in a Nanodrop instrument (Thermoscientific Inc).

QUANTITATIVE RT-PCR ANALYSIS OF Ang 1 & Ang 2,

AND HGF EXPRESSION:

Principle

Real time-PCR selectively amplifies the first strand of cDNA that has been synthesized in vitro by RNA polymerase from mRNA templates by reverse transcription. The cDNA is first denatured by heating in the presence of a large molar of two oligonucleotide primers and the four dNTPs. The reaction mixture is then cooled to a temperature that allows the oligonucleotide primers to anneal to their target sequences, after which the annealed primers are extended with DNA polymerases. The cycle of denaturation, annealing and DNA synthesis is then repeated many times. Real-time experiments monitor and report the accumulation of PCR product, as detected by increased fluorescence, during thermal cycling. Data collection during the early exponential phase of PCR allows the software to accurately calculate initial template quantities.

First strand cDNA synthesis

The first step in RT-PCR is the reverse transcription of total RNA to a single stranded complementary DNA (cDNA) which was done using Helini cDNA Synthesis Kit (HELINI biomolecules).

Helini cDNA Synthesis Kit:

- OligodT Primer Mix
- Random hexamer enzyme mix
- cDNA reaction mix

Quantitative RT-PCR - SYBR® Premix Ex Taq™ II kit (Tli RNaseH Plus):

Kit Components:

1. SYBR Premix Ex Taq II (Tli RNaseH Plus) (2X) 1 ml x 5 -HS DNA Polymerase, dNTP mixture, Mg2+, Tli RNaseH, and SYBR Green I.
2. ROX Reference Dye (50X) 200 µl
3. ROX Reference Dye II (50X) 200 µl

Real Time-PCR was carried out on **Mastercycler realplex quantitative PCR system (Eppendorf)**. Reaction was performed using 5.0 µl of 2X reaction buffer, 1µl of 100 nM of both forward and reverse primers of gene of interest, 1 µl of forward and reverse primers of GAPDH, 3.0 µl of diluted cDNA was made upto a 10µl reaction. The thermal cycling protocol

was as follows: 95ºC for 5 minutes, followed by 40 cycles of PCR at 95ºC for 30 seconds and 58ºC for 30 seconds. All reactions were performed in triplicate along with no template control (NTC). Melt curve analysis was performed using the thermal cycling programmed at 50ºC - 95ºC for each sample to determine the presence of multiple amplicons, nonspecific products, and contaminants. The relative amount of mRNA was calculated by using the comparative Ct method.

Primer sequences:

Angiopoietin 1

F: 5’ TAACAGGAGGATGGTGGTTTG 3’

R: 3’ AACTGGGCCCTTTGAAGTAG 5’

Angiopoietin 2

F: 5’ ATCAGGACACACCACGAATG 3’

R: 3’ CATCCTCACGTCGCTGAATAA 5’

HGF

F: 5’ CACGACAGTGTTTCCCTTCT 3’

R: 3’ CTGATCCTTCAGGGCCATATAC 5’

GAPDH

F: 5’ GGTGTGAACCATGAGAAGTATGA 3’

R: 3’ GAGTCCTTCCACGATACCAAAG 5’

DATA INTERPRETATION

The values obtained in the Real time-RT PCR were interpreted using the formula of fold change,

$2^{-\Delta\Delta ct}$ = [ct of gene of interest – ct of internal control] of disease sample – [ct of gene of interest – ct of internal control] of health sample.

This form of equation may be used to compare the gene expression in two different samples (health and disease). Each sample is related to an internal control gene.

INFORMED CONSENT

DEPARTMENT OF PERIODONTICS

1) NAME:

2) AGE & SEX:

3) OCCUPATION:

4) INCOME:

5) ADDRESS:

6) CONTACT NO:

7) BIOPSY:

a) Type:

b) Nature of specimen:

8) CLINICAL DETAILS:

a) Site:

b) Colour:

c) Consistency:

d) Size:

9) PROVISIONAL DIAGNOSIS:

10) PATIENT CONSENT:

I have been informed about the surgical procedure for removal of gingival tissue while performing periodontal surgery that will not have any determined effect on my healing soft tissue. The tissue obtained will be used for research purpose only, the result which will be informed to my request. I henceforth give my consent for the above procedure.

PATIENT **SIGN**

DOCTOR SIGN **HOD SIGN**

CASE HISTORY PROFORMA

RAGAS DENTAL COLLEGE AND HOSPITALS, CHENNAI.

DEPARTMENT OF PERIODONTICS

PROFORMA

NAME: AGE: SEX: DATE:

ADDRESS: OCCUPATION:

OP.NO:

CHIEF COMPLAINT:

PAST DENTAL HISTORY:

MEDICAL HISTORY:

PERSONAL HABITS:

INTRAORAL EXAMINATION

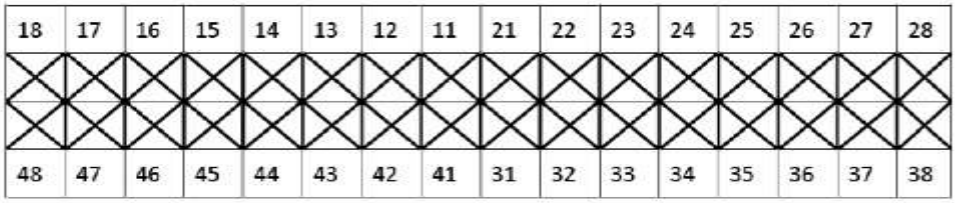
A. HARD TISSUE EXAMINATION:

B. SOFT TISSUE EXAMINATION:

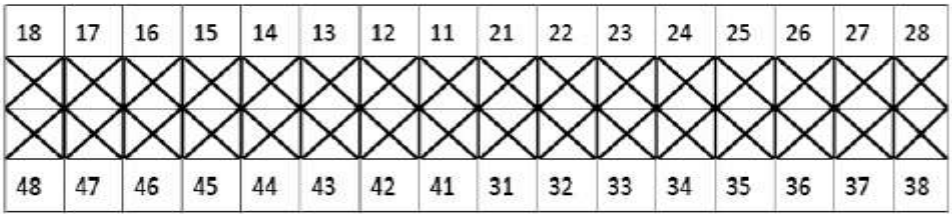
- GINGIVAL FINDINGS

- DENUDED ROOTS (MILLER'S CLASSIFICATION)

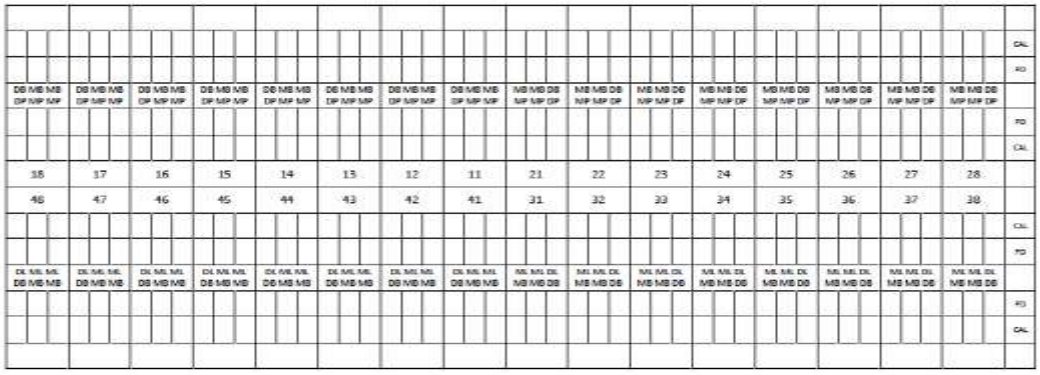
PLAQUE SCORE:



BLEEDING SCORE:



Measurement of Probing Depth and Clinical Attachment Loss



STATISTICAL ANALYSIS

All statistical analysis were performed using Statistical Package for Social Service (SPSS, Version 17 for Microsoft windows).

The data between Ang 1, Ang 2 & HGF in health and disease were normally distributed and therefore parametric tests were performed. The test chosen was independent sample student T test to evaluate the difference between the Ang 1, Ang 2 & HGF in periodontal healthy and disease groups. A two sided p value of <0.05 was considered to be statistically significant.

Photographs

PATIENT GROUPS

Fig 1: GROUP A

HEALTHY GINGIVA

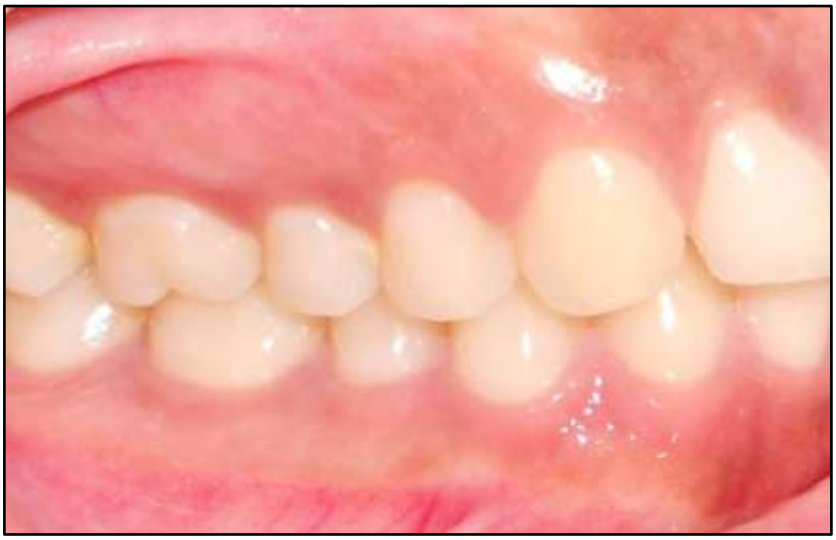


Fig 2: GROUP B

PERIODONTAL DISEASE

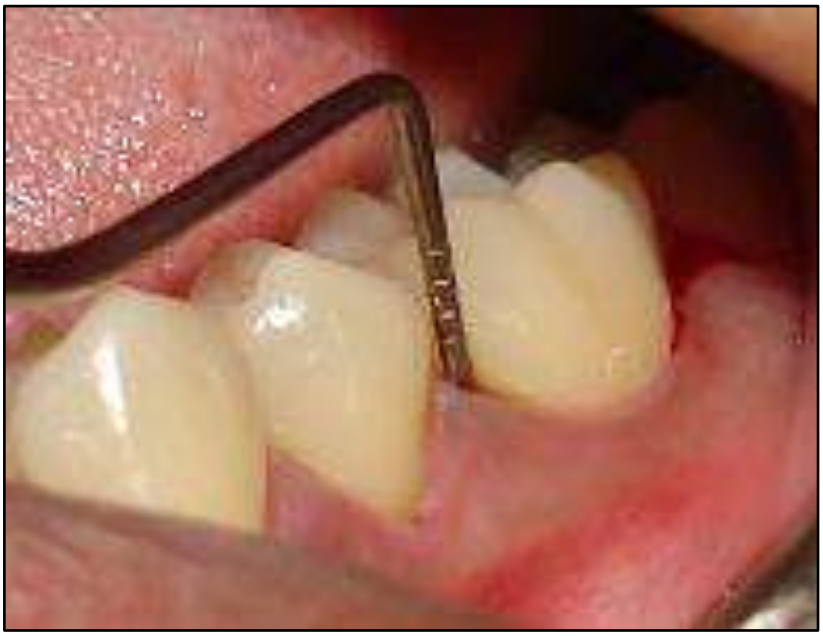


Fig 3: GINGIVAL TISSUE SAMPLES

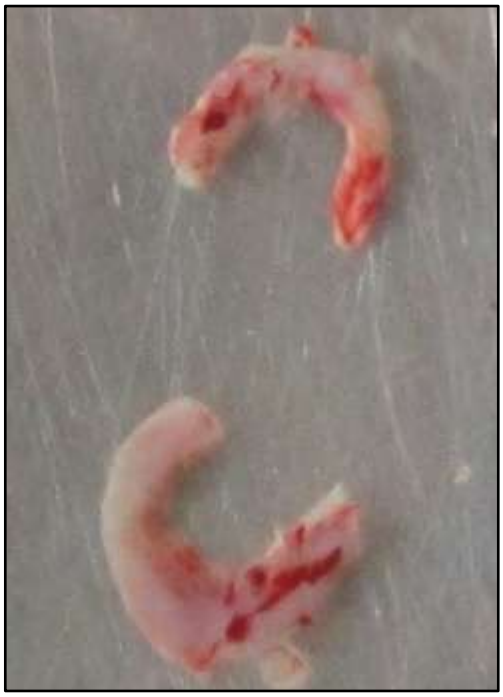


Fig 4: ARMAMENTARIUM FOR GINGIVAL SAMPLE PRESERVATION

Fig 5: TISSUE SAMPLES STORED IN RNAlater

Fig 6: SWIFT RNA ISOLATION KIT

Fig 7: MICROCENTRIFUGE

Fig 8: MICROCENTRIFUGE (Inside)

Fig 9: NANO SPECTROMETER

Fig 10: cDNA SYNTHESIS KIT

Fig 11: PRIMER KIT

Fig 12: Armamentarium for RT-PCR analysis

Mastercycler realplex^2 realtime pcr unit

Fig 13: AMPLIFICATION PLOT

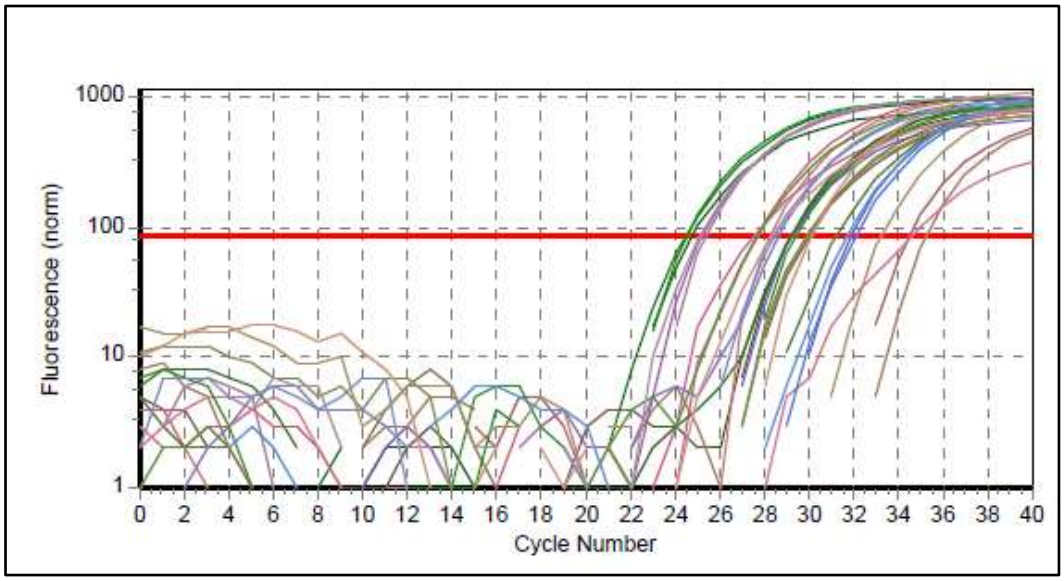
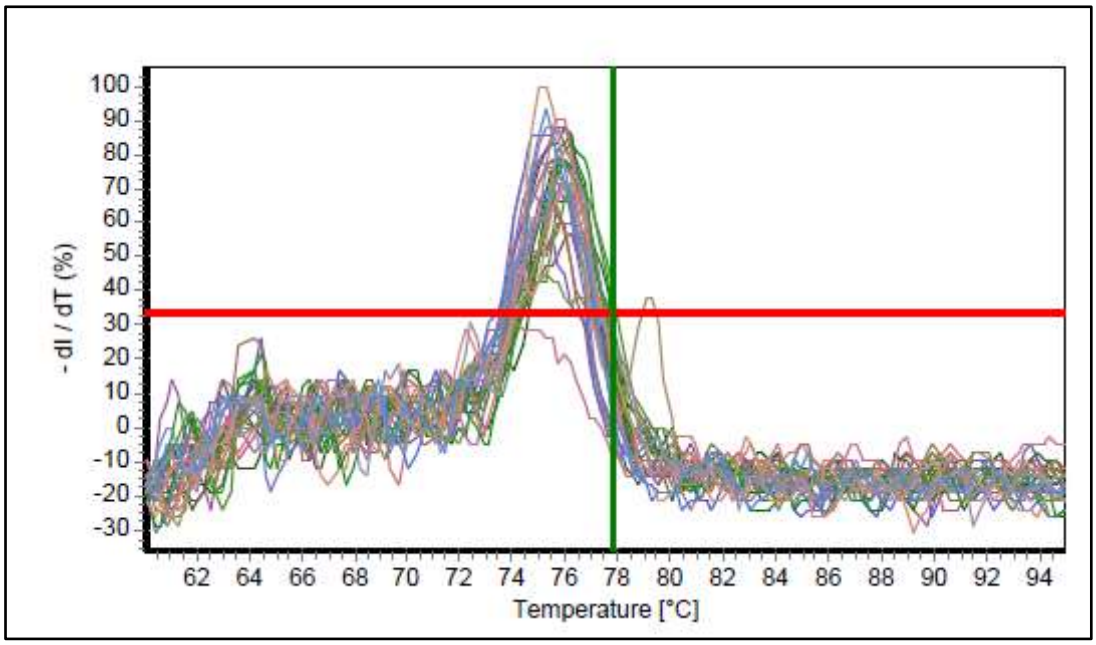


Fig 14: MELTING CURVE



Results

RESULTS

Gingival samples were obtained from 10 patients from each of periodontally healthy and diseased groups and total RNA isolated and extracted. The purity of the extracted RNA was analyzed with a NANO Gel electrophoresis system and cDNA conversion was done. The mRNA expression for **Ang 1, Ang 2** and **HGF** was evaluated by using real time RT-PCR.

The results of the present study are presented in the Tables- 1, 2, 3.

The data obtained was interpreted using the formula of fold change

$2^{-\Delta\Delta ct}$ = (ct of gene of interest – ct internal control) of disease sample – (ct of gene of interest – ct internal control) of health sample.

Ang 1:

The results have shown that the mean ct value of Ang 1 in the disease group was 32.37 and that of GAPDH was 25.60. The mean delta ct (Δct) value in the disease group was 6.725±2.68. The mean ct value of Ang 1 in health was 33.76 and that of GAPDH was 23.40. The mean Δct of Ang 1 in health was 10.40±1.39. The individual ct values of Ang 1 in disease and health over the control and the individual Δct values of the samples are summarized in Table 1a.

The fold change was derived using the above mentioned formula. The fold change of Ang 1 in disease over health was obtained as 3.68. The result showed an upregulation of Ang 1 gene in diseased samples when compared to health. The mean fold change of Ang1 over control is tabulated in Table 1b and depicted in Graph 1.

Independent sample T test was performed to compare the fold change of Ang1 in health and disease and was found to be statistically insignificant at $p = 0.129 (p > 0.05)$.The statistical analysis are tabulated in Table 1c.

Ang 2:

The results have shown that the mean ct values of Ang 2 in the disease group was 31.54 and that of GAPDH was 23.85. The mean delta ct (Δct) value in the disease group was 7.683±3.31. The mean ct value of Ang 2 in health was 30.17 and that of GAPDH was 23.58. The mean Δct of Ang 1 in health was 6.592±3.94. The individual ct values of Ang 2 in disease and health over the control and the individual Δct values of the samples are summarized in Table 2a.

The fold change of Ang 2 in disease over health was -1.09 This shows that there was a down regulation of Ang 2 in disease when compared to health. The mean fold change of Ang 2 over control is tabulated in Table 2b and depicted in Graph 2.

Statistical analysis was performed using independent sample T test to compare the fold change of Ang2 in health and disease and was found to be statistically insignificant at p-value =0.934 (p-value is >0.05).The tabulations are presented in table 2c.

HGF :

The results shows that the mean ct values of HGF in the disease group was 29.08 and that of GAPDH was 24.18. The mean delta ct (Δct) value in the disease group was 4.898±3.72. The mean ct value of HGF in health was 29.96 and that of GAPDH was 23.5. The mean Δct of HGF in health was 6.473±3.01. The individual ct value and Δct value of HGF in health and disease are summarized in Table 3a.

The fold change of HGF in disease over health was 1.574. This shows that there was a up regulation of HGF in disease when compared to health. The fold change of HGF over control is tabulated in Table 3b and depicted in Graph 3.

An independent sample T test was performed to compare the fold change of HGF in health and disease and was found to be not statistically significant with a p-value of 0.899 (p-value is >0.05). The statistical analysis are detailed in Table 3c.

Tables and Graphs

Table:1a- Relative expression of Ang 1 in health and disease

GROUP	ct Ang 1	ct GAPDH	Δct	MEAN ct
HEALTH	NA	23.50	NA	10.401
	35.09	25.50	9.58	
	34.38	25.14	9.24	
	33.30	22.50	10.8	
	NA	24.34	NA	
	35.15	23.57	11.57	
	33.19	22.05	11.14	
	31.5	21.68	9.81	
	NA	23.22	NA	
	NA	24.75	NA	
GROUP	ct Ang 1	ct GAPDH	Δct	MEAN ct
DISEASED	29.2	18.75	10.45	6.725
	32.82	31.45	1.37	
	34.66	25.29	9.37	
	32.1	25.95	6.14	
	25.99	24.51	1.47	
	33.35	24.74	8.60	
	37.93	30.05	7.88	
	NA	24.15	NA	
	34.06	26.19	7.86	
	31.28	23.89	7.39	

Table:1b- Fold change of Ang 1 in disease over health

GROUP	MEAN Δct	FOLD CHANGE
DISEASE	6.725±2.86	3.68
HEALTH	10.40±1.39	

Tables: 1c- Statistical analysis of Ang 1

S.No	**PARAMETRIC INDEPENDENT SAMPLE T TEST**	
	Table analyzed	
1	Column A Vs column B	Health Vs disease
2	P value	0.129
3	Exact or approximate p value	Gaussian approximation
4	P value summary	Not significant
5	On or two tailed p value	Two tailed

Table:2a - Relative expression of Ang 2 in health and disease

GROUP	ct Ang 2	ct GAPDH	Δct	MEAN ct
HEALTH	33.43	23.50	9.93	6.592
	35.59	25.50	10.09	
	31.20	25.14	6.06	
	25.84	22.50	3.33	
	NA	24.34	NA	
	25.10	23.57	1.52	
	31.05	22.05	9.06	
	27.24	21.68	5.55	
	NA	23.22	NA	
	31.95	24.75	7.20	
GROUP	ct Ang 2	ct GAPDH	Δct	MEAN ct
DISEASE	30.11	18.75	11.36	7.683
	NA	31.45	NA	
	32.31	25.29	7.01	
	27.94	25.95	1.98	
	33.66	24.51	9.14	
	34.09	24.74	9.35	
	NA	30.05	NA	
	NA	24.15	NA	
	NA	26.19	NA	
	31.15	23.89	7.26	

Table:2b Fold change of Ang 2 in disease over health

GROUP	MEAN Δct	FOLD CHANGE
DISEASE	7.683±3.31	-1.091
HEALTH	6.592±3.94	

Tables: 2c -Statistical analysis of Ang 2

S.No	PARAMETRIC INDEPENDENT SAMPLE T TEST	
	Table analyzed	
1	Column A Vs column B	Health Vs disease
2	P value	0.934
3	Exact or approximate p value	Gaussian approximation
4	P value summary	Not significant
5	On or two tailed p value	Two tailed

Table: 3a- Relative expression of HGF in health and disease

GROUP	ct HGF	ct GAPDH	Δct	MEAN ct
DISEASE	24.56	18.75	5.81	6.473
	NA	31.45	NA	
	29.36	25.29	4.07	
	29.92	25.95	3.96	
	34.34	24.51	9.82	
	28.66	24.74	3.92	
	NA	30.05	NA	
	25.21	24.15	1.06	
	28.76	26.19	2.57	
	31.87	23.89	7.98	
GROUP	ct HGF	ct GAPDH	Δct	MEAN ct
HEALTH	NA	23.50	NA	4.898
	28.13	25.50	2.62	
	31.42	25.14	6.28	
	26.23	22.50	3.73	
	27.73	24.34	3.38	
	30.83	23.57	7.26	
	26.12	22.05	4.07	
	33.08	21.68	11.47	
	36.20	23.22	12.98	
	NA	24.75	NA	

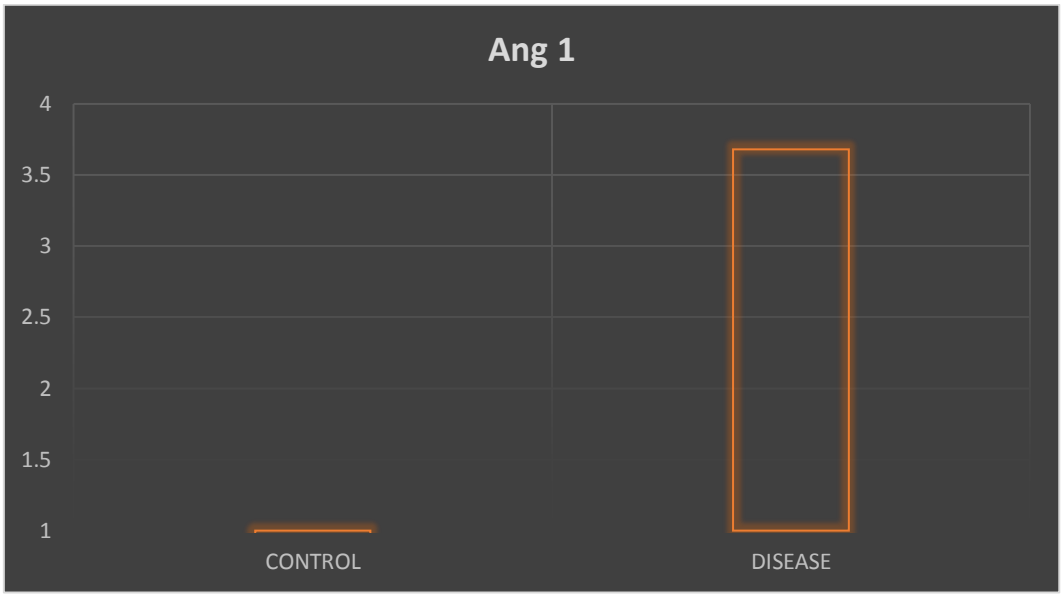
Table: 3b- Fold change of HGF in disease over health

GROUP	MEAN Δct	Fold change
DISEASE	4.898±3.72	1.574
HEALTH	6.473±3.01	

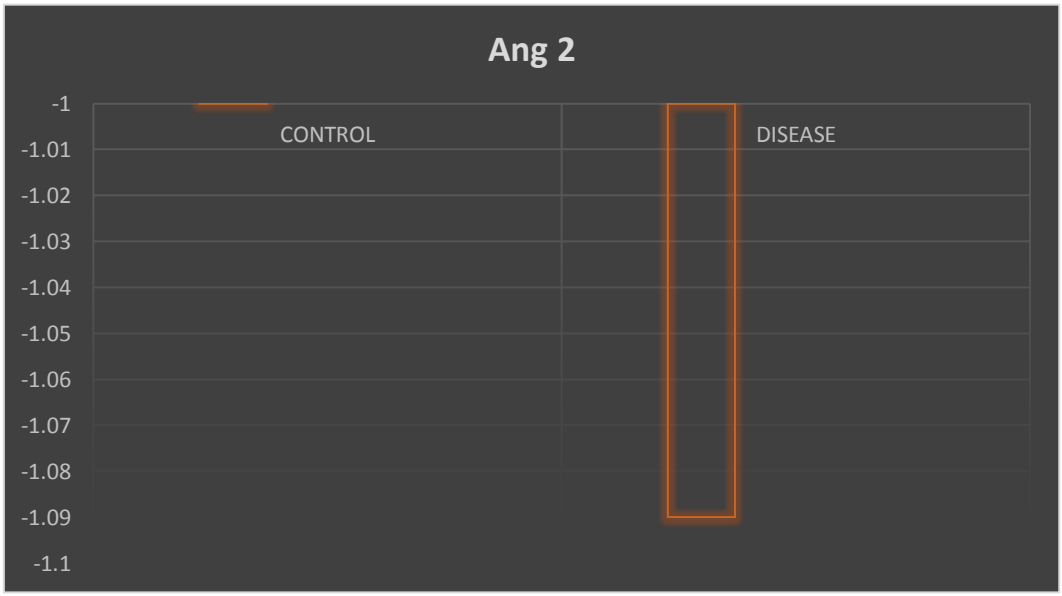
Table: 3c- Statistical analysis of HGF

S.No	PARAMETRIC INDEPENDENT SAMPLE T TEST	
	Table analyzed	
1	Column A Vs column B	Health Vs disease
2	P value	0.899
3	Exact or approximate p value	Gaussian approximation
4	P value summary	Not significant
5	On or two tailed p value	Two tailed

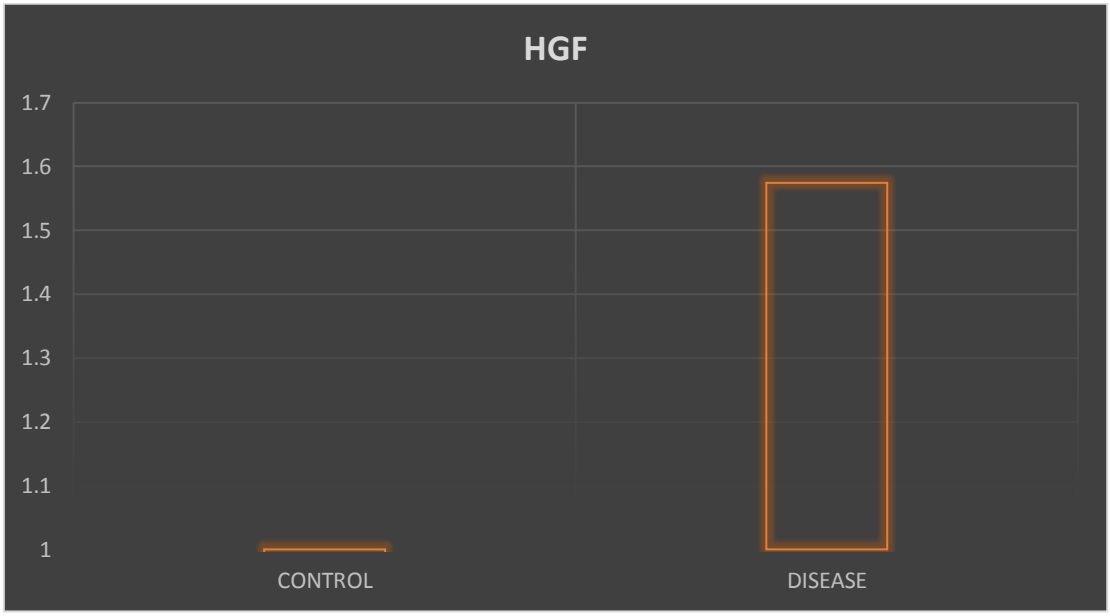
GRAPH: 1 BAR GRAPH SHOWING THE FOLD CHANGE EXPRESSION OF Ang 1 GENE IN PERIODONTAL HEALTH AND DISEASE



GRAPH: 2 BAR GRAPH SHOWING THE FOLD CHANGE EXPRESSION OF Ang 2 GENE IN PERIODONTAL HEALTH AND DISEASE



GRAPH: 3 BAR GRAPH SHOWING THE FOLD CHANGE EXPRESSION OF HGF GENE IN PERIODONTAL HEALTH AND DISEASE



Discussion

DISCUSSION

Periodontal diseases are a group of inflammatory conditions that result in the destruction of the supporting structures of the tooth. The changes in the vascularity of the periodontal connective tissues in untreated advanced periodontitis may be, in part, a consequence of altered expression of angiogenic activity by the epithelium.[22] Studies using animal models of periodontitis, tissue from gingivitis and treated human periodontitis have documented the highly vascular nature of the lesion.[125]

Angiogenesis refers to the process of creation of new blood vessels from pre-existing blood vessels. Soluble growth factors, adhesion molecules, proteases, as well as the extracellular matrix have been shown to interact spatially and temporally to coordinate the formation of new blood vessels. These factors are categorized as pro angiogenic and anti angiogenic factors, depending on its stimulatory and inhibitory functions.[91]

The proangiogenic factors include VEGF-family, FGF-family, Angiopoietin 1, Angiopoietin 2, PDGF, TGF-β, TNF-α, Angiogenin, Angiotropin, HGF, Integrins etc. The anti angiogenic factors are Angiostatin, Endostatin, PEDF etc.[110]

Studies have shown that angiogenesis plays a vital role in various chronic inflammatory conditions viz Diabetes, Rheumatoid arthritis, psoriasis, systemic sclerosis, osteoradionecrosis, periodontal diseases etc.[110]

Angiogenesis is a prominent feature in both inflammation and healing of periodontal diseases, but its role in either promoting the progression of disease or the healing of periodontal lesion is still unclear. Several studies have shown that proangiogenic factors such as VEGF and FGF are involved in the process of inflammation in chronic periodontitis.[34,48] As there is a paucity of literature on the role of the various angiogenic factors in periodontal disease conditions, this study was undertaken to evaluate if there is any evidence of Ang 1, Ang 2 and HGF in periodontal disease based on previous reports that the proangiogenic factors play a role in vascular turnover and remodeling in chronic periodontitis.[67]

Ang 1, Ang 2 and HGF were chosen in this study for the following reasons,

- Ang 1 triggers signaling pathways of Tie2 through their interaction in endothelial cell, and thereby promoting angiogenesis. Angiopoietin 2, acts as a natural antagonist for Tie2 that disrupts the process of angiogenesis. Thus Ang 1 acts as an agonist and Ang 2 its antagonist.
- The presence of elevated levels of HGF in GCF has been well established but its significance in diseased tissues has not been recorded yet. Thus this study strives to evaluate the levels of these proangiogenic factors in gingival tissues of health and disease.

The patients enrolled in this study were divided into 2 groups;

Group A (periodontally healthy) consisted of patients with no signs of periodontal disease as determined by a probing depth of ≤3mm with no clinical attachment loss and bleeding on probing. Healthy gingival tissue samples were obtained from patients who reported for crown lengthening procedures for restorative purpose, with no signs of inflammation.

Group B (periodontally diseased) consisted of patients with advanced (moderate to severe) periodontitis as per the AAP 1999 classification. Diseased tissue samples were obtained from patients undergoing Modified Widman Flap procedure.

The gingival samples were then stored in RNAlater®. It is an aqueous, non-toxic storage reagent that rapidly permeates the tissues to stabilize and protect cellular RNA and DNA in situ in unfrozen specimens. It has the advantage of preserving tissue integrity while preventing RNA degradation.[67]

Gingival tissues were homogenized, total RNA extraction done by SWIFT RNA isolation protocol and conversion to cDNA was done using standardized protocols.

Real time RT-PCR was then carried out, where the amplification of a DNA sequence is combined with a detection of thee amplified products during

each reaction cycle-in other words, in real time. It is a more sensitive and less time consuming quantitative method of measuring gene expression.

Real time RT-PCR analysis was carried out to evaluate the mRNA expression levels of Ang 1, Ang 2 and HGF in periodontal health and disease using SYBR® Premix with known primer sequences for Ang 1, Ang 2 and HGF.

Comparative ct method was used to compare the expression levels of a gene in health versus diseased samples. Relative quantification is a technique used to analyze changes in gene expression in a given sample relative to a reference sample (such as an untreated control sample). The difference in the gene expression was expressed in terms of fold change as per previous literature.[30]

The results of the present study showed the mRNA/gene expression of Ang 1 in periodontal health and disease with a fold change of 3.68 times in disease when compared to health. But this increase was not statistically significant. Likewise, Ang 2 gene was also expressed in both health and disease with a 1.09 fold decrease in disease when compared to health which was not statistically significant. The gene expression of HGF was increased in disease by 1.574 fold over health, which was statistically insignificant.

Dysregulation of angiogenesis has been often associated with many chronic inflammatory lesions of the oral cavity including the periodontium. Previous reports by Yuan et al have suggested that Ang 1, Ang 2 undergo changes in pyogenic granuloma.[123] Our results are in agreement with these studies. Etiopathogenic relevance of Ang 1, Ang 2 to the progression of periodontal disease is not well established. However, increased vascular response in the active phase of disease and neovascularization during response to phase 1 are well documented.[22,36] Vascular events that happen in the episodic phases of periodontal diseases are not yet well established.

The samples in our study have been obtained following phase 1 therapy. As a result, it may be expected that the active inflammatory phase of periodontal disease might have subsided and the healing tissue response may have been characterized by fibrosis. Previous literature has shown that Ang 1 may be liberated among other cells by the inflammatory monocytes, macrophages etc.[59] The macrophages are not only important for pro-inflammatory changes, but also for the matrix turnover that may be expected following phase 1 therapy. We therefore hypothesize that the increased levels of Ang 1 may be due to the macrophagic infiltration into the gingiva.

Ang 2 on the other hand, showed a statistically insignificant downregulation, probably as a result of counter-balancing mechanism to the increased Ang 1 levels in the gingival tissues. Ang 2 production has not been

well documented from the inflammatory cells as much as Ang 1. This could be the reason for the differential expression of Ang 1, Ang 2 observed in our samples.

Taken together, our results suggest that there is a dysregulation of Ang 1, Ang 2 proteins in periodontal disease. Although the exact pathogenic mechanisms are difficult to establish from this study, it is speculated that these changes may affect the periodontium in the following ways:

1. Neovascularization has been reported to be initiated from the renewal of microcirculation from the venous end through macrophagic liberation of Ang 1. It is therefore possible that the venous stasis observed in edematous gingival tissues could be a result of dysregulation of Ang 1and Ang 2.
2. Dysregulation of Ang 1 and Ang 2 could lead to impaired angiogenesis. This impairment could be an important reason for non-resolution of chronic inflammation in periodontal disease. The importance of vascularity in periodontal disease ensuring the supply of cells, matrix components and signaling molecules has been previously documented.[36]

HGF is a known mitogen, motogen and scatter factor. The upregulation of HGF in periodontal disease is in line with previous literature.[78,82,83,84,95,117] HGF is associated with the proliferation and apical migration of epithelial cells. The presence of c-Met receptors in the junctional epithelium, which help this process has been well documented.[73,80] The samples obtained in our study would have shown an increased level of HGF as a result of disease process due to the apical migration of junctional epithelium. The increase in the level of HGF could also possibly be due the healing response as a result of phase1 therapy as reepithelization occurs in the junctional epithelium of the healing periodontium.

The involvement of HGF in the angiogenic process is also well documented.[13,55,83,104,115] There is clear evidence for the importance of angiogenesis in the process of wound healing.[42,73,85,104] Thus, our study is in agreement with previous literature that have shown higher HGF in periodontal disease.

The clinical implications of the study are that HGF can be used as a potential therapeutic marker because of its influence on epithelial migration and angiogenesis both in the disease progression and in the phases of healing.

The limitations of this study are that a comparatively smaller sample size has been investigated. The fact that the samples were not obtained at the active phase of the disease is also a limitation of this study.

Further studies targeted at elucidating the mechanism of actions of Ang 1, Ang 2 and HGF in the active phases of the periodontal disease with a considerable larger sample size could provide a deeper insight into the involvement in periodontal disease.

Summary and Conclusion

SUMMARY AND CONCLUSION

The process of angiogenesis and its importance in chronic inflammatory disease conditions is well established. The influence of the various proangiogenic markers have been identified till date and their involvement in the disease progression periodontitis have been confirmed.

This study was carried out to evaluate the gene expression of Ang 1, Ang 2 and HGF in periodontally healthy and diseased tissue samples. 20 patients who reported to the Department of Periodontics, Ragas dental college and Hospital were enrolled in the study. The patients were divided into 2 groups based on their periodontal health status, as 10 healthy and 10 diseased groups. Healthy tissue samples were obtained from patients who underwent crown lengthening procedure. Diseased tissue samples were obtained from patients who underwent Modified Widman flap surgery. Isolation of the total RNA was done, real time reverse transcription polymerization chain reaction was carried out to evaluate the gene expression of Ang 1, Ang 2 and HGF.

The results demonstrated an upregulation of Ang 1 gene with a fold change of 3.68 and HGF gene 1.574 fold in patients with periodontal disease when compared with the periodontally healthy group, whereas there was a down regulation of Ang 2 gene with a 1.091 fold decrease in periodontal disease group, which explains the agonist and antagonist nature of Ang 1 and

Ang 2 respectively in inflammatory conditions. However further studies with a larger sample size and the collection of tissue samples during the active phase of disease is required to substantiate the role of these cells in the disease progression of periodontium.

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Annexures

ANNEXURE I

INFORMED CONSENT

DEPARTMENT OF PERIODONTICS

1) NAME:

2) AGE & SEX:

3) OCCUPATION:

4) INCOME:

5) ADDRESS:

6) CONTACT NO:

7) BIOPSY:

a) Type:

b) Nature of specimen:

8) CLINICAL DETAILS:

a) Site:

b) Colour:

c) Consistency:

d) Size:

9) PROVISIONAL DIAGNOSIS:

10) PATIENT CONSENT:

I have been informed about the surgical procedure for removal of gingival tissue while performing periodontal surgery that will not have any determined effect on my healing soft tissue. The tissue obtained will be used for research purpose only, the result which will be informed to my request. I henceforth give my consent for the above procedure.

PATIENT SIGN

DOCTOR SIGN

HOD SIGN

ANNEXURE II

RAGAS DENTAL COLLEGE & HOSPITAL
(Unit of Ragas Educational Society)
Recognized by the Dental Council of India, New Delhi
Affiliated to The Tamilnadu Dr. M.G.R. Medical University, Chennai
2/102, East Coast Road, Uthandi, Chennai - 600 119. INDIA.
Tele : (044) 24530002, 24530003-06. Principal (Dir) 24530001 Fax : (044) 24530009

TO WHOMSOEVER IT MAY CONCERN

Date: 06/01/2017

From

The Institutional Ethics Board,

Ragas Dental College and Hospital,

Uthandi,

Chennai- 600119

The dissertation topic titled **"Evaluation of mRNA/gene expression of Proangiogenic factor-Ang1,Ang2 and HGF in periodantal health and disease"** submitted by **Dr. Keerthiha. R.S.,** has been approved by the Institutional Ethics Board of Ragas dental college and hospital.

Dr. N.S.Azhagarasan,MDS,

Member secretary,

Institutional Ethics Board,

Ragas Dental College and Hospital.

PRINCIPAL
RAGAS DENTAL COLLEGE AND HOSPITAL
UTHANDI, CHENNAI-600 119.

RAGAS DENTAL COLLEGE Chennai