MECHANISM OF INDUCTION OF VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF) IN OSTEOARTHRITIS

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MECHANISM OF INDUCTION OF VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF) IN OSTEOARTHRITIS

Presented by Jing Chen

A candidate for the degree of Master of Science

And hereby certify that in their opinion it is worthy of acceptance.

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ABSTRACT

Vascular endothelial growth factor (VEGF) is an endothelial cell mitogen and an angiogenic factor. Angiogenesis regulated by VEGF is a critical event in the pathogenesis of Osteoarthritis (OA). Over-expression of VEGF in arthritis plays an important role in the progression of the disease. This study was directed at understanding the regulation of VEGF in OA. In the pathogenic condition of OA, a novel transcription factor called Serum Amyloid A-activating factor (SAF-1) is abundantly present and is involved in the regulation of several genes in the diseased joint tissue. To assess whether SAF-1 plays any role in the VEGF expression in arthritic joint, transient transfection, and CAT assay in articular chondrocyte cells were preformed. The results showed that SAF-1 increases VEGF promoter linked reporter gene expression in a dose dependent manner. Deletion of SAF binding sites in the promoter region of VEGF completely abolishes cytokine-induced VEGF promoter activity. Electrophoretic Mobility Shift Assay demonstrated that SAF-1 directly binds to VEGF promoter, and NF-κB interrupts the binding of SAF-1 to

VEGF promoter. These results suggest that SAF-1 play a crucial role in promoting VEGF expression in OA.

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LIST OF ABBREEVIATIONS

AP-1: Activator Protein-1

C/EBP: CCAAT Enhancer Binding Protein

CAT: Chloramphenicol Acetyl Transferase

ECM: Extracellular Matrix

EDTA: Ethylene diamine tetra acetate

EMSA: Electrophoretic Mobility Shift Assay

HIF: Hypoxia- inducible factor

IFN: Interferon

IL: Interleukin

JNK: Jun-N-terminal Kinase

MMP: Matrix Metalloproteinase

NF-κB: Nuclear Factor-κB

OA: Osteoarthritis

PKA: Protein Kinase A

PKC: Protein Kinase C

RA: rheumatoid arthritis

SAA: Serum Amyloid A

SAF-1: Serum Amyloid A Activating Factor-1

Sp-1: Nuclear Stimulating Protein-1

TGF-β: Tissue Growth Factor-β

TLC: Thin Layer Chromatography

TNF: Tumor Necrosis Factor

VEGF: vascular endothelial growth factor

VEGFR: vascular endothelial growth factor receptor

Chapter 1

INTRODUCTION

Osteoarthritis (OA) is a chronic, painful and disabling condition that affects the synovial joints. It is the common joint disease and age is the strongest risk factor for OA. Cartilage degradation, osteophyte formation, synovial inflammation, and angiogenesis are some key characteristics of OA. Cartilage degradation is a hallmark feature of the pathogenesis of OA. Cartilage components are extracellular matrix which is rich in collagens and proteoglycans, and chondrocyte cells which are embed of in the matrix. OA is defined as a gradual loss of articular cartilage, combined with thickening of the subchondral bone, bone outgrowths at joint margins with nonspecific synovial inflammation.

Matrix metalloproteinase (MMP) is linked with various pathological diseases that involve connective tissue destruction and breakdown of the extracellular matrix. Overexpression of MMPs by IL-1β and TNF-α is also critical for the cartilage degradation. Serum amyloid A-activating factor (SAF-1), a novel transcription factor, and the AP-1 family proteins cooperatively regulate cytokine-mediated induction of MMP-1 and MMP-9 in the resident cells of the joint capsule. In the MMP9 promoter, SAF-1 and AP-1 DNA-binding elements are present in close proximity. SAF-1 and AP-1 function in a mutually beneficial manner acting as essential co-activators to drive cytokine-mediated transcriptional activation of MMP9.

Many inflammatory agents, including LPS, IL-1 and IL-6, activate SAF-1 to induce SAA over-expression. SAF-1 transgenic mice were generated for understanding the role of this transcriptional factor in the process of inflammation. Challenged with

Borrelia burgdorferi, SAF-1 transgenic mice developed severe Lyme disease compared to its non-transgenic littermates(Ray et al 2004), as observed by increasing cartilage degradation, high rate of synovial inflammation, angiogenesis and macrophage infiltration. It is consistent with the role of SAF-1, as a regulator of several MMPs (Ray et al , 2003, 2005). VEGF is a potent stimulator of angiogenesis and plays an important role in the pathogenesis of inflammation and osteoarthritis (OA) in human (Haywood 2003). Interestingly, the expression of SAF-1 and VEGF are higher and correlated well in the synovial joint of SAF-1 transgenic mice compared to non-transgenic mice during *B. burgdorferi* infection, suggesting that SAF-1 may regulate VEGF expression.

Angiogenesis and inflammation are important processes in the pathogenesis of OA. Cartilage in OA exhibits reduced resistance to vascular invasion. More blood vessels are found superficially in the articular cartilage with severe OA. Due to the importance of VEGF in the pathogenesis of OA, there has been a considerable interest to understand the molecular mechanisms that are responsible for VEGF overexpression in the arthritis tissues. The present study focuses on the elucidation of role of an inflammation-responsive transcription factor, SAF-1, in the overexpression of VEGF in OA.

Chapter 2

LITTERATURE REVIEW

Discovery of VEGF

Tumor growth is accompanied by increased vascularity. In the early 20th century, Ide et al postulated the existence of a tumor-derived blood vessel growth-stimulating factor responsible for inducing new vessel formation, which can deliver nutrition to the growing tumors (Ide AG, Baker NH et al. 1939). In 1968, the first evidence was provided that tumor angiogenesis was mediated by diffusible factor(s) produced by the tumor cells (Greenblatt and Shubi 1968); (Ehrmann and Knoth 1968).

In the 1970s, Dvorak found fibrin deposited in tumor, which required local vascular permeability to plasma proteins, and extravascular activation of the clotting system. Fibrin results from the clotting of fibrinogen, a plasma protein that is normally retained within the vasculature (Dvorak 2006). Dvorak found that tumor supernatant contains a factor called vascular permeability factor (VPF). In 1983, Senger (Senger, Galli et al. 1983) purified VPF, a 34-43 kDa dimeric protein, from a guinea pig tumor cell line. Since VPF was responsible for the accumulation of ascites fluid in many human tumors, this protein could be a target for tumor therapy (Senger, Galli et al. 1983). Subsequently, the evidence showed that VPF was an endothelial cell mitogen in vitro and an angiogenic factor in vivo (Connolly, Heuvelman et al. 1989).

Independently, at the same time, Ferrara isolated a diffusible endothelial cell-specific mitogen from the bovine pituitary folliculi-stellate cells (Ferrara and Henzel 1989). It is a cationic, heat stable dimeric protein with a molecular mass of 45 kDa, composed of two subunits of identical molecular mass (23kDa), and has a unique NH2-

terminal amino acid sequence. It was called vascular endothelial growth factor (VEGF). cDNA cloning of the VEGF (Leung, Cachianes et al. 1989) and VPF(Keck, Hauser et al. 1989) was reported, and demonstrated that VEGF and VPF are encoded by the same gene and produced from alternative splicing of messenger RNA (Ferrara, Houck et al. 1991; Houck, Ferrara et al. 1991).

VEGF is a secreted angiogenic mitogen, in contrast to other endothelial cell mitogens such as acidic fibroblast growth factor (aFGF) or basic fibroblast growth factor (bFGF), and platelet-derived endothelial cell growth factor(PDGF) (Leung, Cachianes et al. 1989). FGFs do not increase vascular endothelial permeability and VEGF has potent, diffusible and specific activity for vascular endothelial cells, which led to the hypothesis that VEGF may play an important role in the regulation of blood vessels under physiological and pathological conditions.

VEGF Isoforms

The human VEGF gene has been assigned to chromosome 6p21.3 (Vincenti, Cassano et al. 1996). DNA sequencing suggests the existence of several molecular species of VEGF (Leung, Cachianes et al. 1989). The gene for human VEGF is organized into eight exons, and the mRNA alternative splicing events generate five transcripts encoding VEGF proteins containing 121, 145, 165, 189, and 206 amino acids (Maruotti, Cantatore et al. 2006), respectively named, VEGF121, VEGF145, VEGF165, VEGF189, and VEGF206 (Figure 1). The domain encoded by exons 1-5 contains information for the recognition of the VEGF receptors Flt-1 (VEGF receptor-1) and

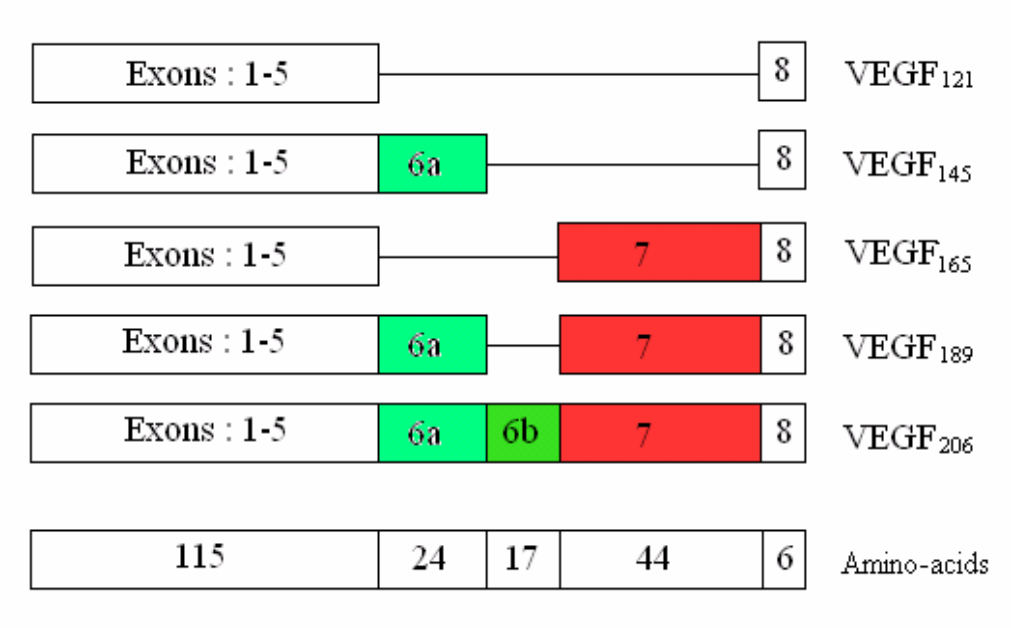

Figure 1. Several isoforms of VEGF are generated by alternative splicing of VEGF mRNA.

FDR/Flk-1 (VEGF receptor-2) (Keyt, Nguyen et al. 1996) and is present in all VEGF isoforms. The amino acids encoded by exon 8 are also present in all the VEGF splice variants (Poltorak, Cohen et al. 1997). The VEGF isoforms are distinguished by the presence or the absence of the peptides encoded by exon 6 and 7 of the VEGF gene. VEGF121 is 121 amino acids long encoded by exon 1-5 and 8, but lack both exon 6 and 7. VEGF 145 contains exons 1-6 and 8 (Poltorak, Cohen et al. 1997). When compared with VEGF121, VEGF 165 contains exon 7-encoded peptide, whereas VEGF189 contains both exon 6- and exon 7-encoded peptide. The fifth isoform VEGF206 contains both exon 6- and exon 7-encoded peptide plus an additional 17 amino acids between exon 6 and 7(Houck, Ferrara et al. 1991). There is no intron among exon 6, the additional 17 amino acids, and exon 7.

VEGF is a heparin-binding homodimeric glycoprotein (Ferrara and Henzel 1989). VEGF165 is the major VEGF isoform (Houck, Leung et al. 1992). Different isoform of VEGF displays different heparin-binding properties that determine whether the different glycoproteins are secreted or remain cell-associated. For example,VEGF121, VEGF145 and VEGF165 are secreted efficiently from the producing cells (Poltorak, Cohen et al. 1997), whereas VEGF189 and VEGF206 remain bound to the cell surface and extracellular matrix (Ferrara, Houck et al. 1991).

VEGF receptors (VEGFRs)

VEGF binding sites or VEGF receptors were first identified on the cell surface of vascular endothelial cells in vitro (Plouet and Moukadiri 1990; Vaisman, Gospodarowicz et al. 1990) and in vivo (Jakeman, Winer et al. 1992; Jakeman, Armanini et al. 1993).

Subsequently, VEGFRs were shown to exist also on bone marrow-derived cells such as monocytes (Shen, Clauss et al. 1993). VEGF binds two highly related receptor tyrosine kinases (RTKs), the fms-like tyrosine kinase-1 (Flt-1 /VEGFR-1), and fetal liver kinase-1 (Flk-1/KDR/VEGFR-2). Ligand binding results in phosphorylation and activation of the receptors and multiple cellular substrates, and induces a variety of cellular responses (Ullrich et al, 1990). RTKs are key regulators of developmental processes due to their important role in the regulation of growth and differentiation. There is a high level of protein tyrosine phosphorylation in the early embryo, which decreases in the later stages of embryogenesis and is relatively low in adult animal tissues (Maher and Pasquale, 1988).

Both Flt-1 and Flk-1/KDR have seven Ig-like domains in the extracellular domain, a single-transmembrane region, and a consensus tyrosine kinase sequence that is interrupted by a kinase insert domain (Shibuya, Yamaguchi et al. 1990; Matthews, Jordan et al. 1991; Terman, Carrion et al. 1991). Flt-1 encodes for a 180 kDa glycoprotein (Seetharam, Gotoh et al. 1995). Expression of Flt complementary DNA in COS cells conferred specific, high-affinity binding of VEGF (de Vries, Escobedo et al. 1992). Flk-1 transcripts were abundant in proliferating endothelial cells of vascular sprouts, branching vessels of embryonic, and early postnatal brain (Millauer, Wizigmann-Voos et al. 1993). Millauer et al identified that the angiogenic mitogen, VEGF, is the high affinity ligand of Flk-1 and correlation of the temporal and spatial expression pattern of Flk-1 and VEGF suggests a major role of this ligand-receptor signaling system in vasculogenesis and angiogenesis (Millauer, Wizigmann-Voos et al. 1993). Both Flt-1 and Flk/KDR were

phosphorylated in vitro in response to VEGF. However, Flk-1/KDR was much more efficiently than Flt-1 (Waltenberger, Claesson-Welsh et al. 1994).

Function of VEGF

The most well-known function of VEGF is its ability to promote growth of vascular endothelial cells in arteries, veins, and lymphatics (Ferrara and Davis-Smyth 1997). VEGF-promotes angiogenesis has been demonstrated in tridimensional in vitro models (Pepper, Ferrara et al. 1992). VEGF promoted angiogenesis in lymphatic endothelial cells in a manner very similar to endothelial cells in vascular system (Pepper, Wasi et al. 1994).

Another important function of VEGF is to increase the vascular permeability in inflammation and other pathological conditions (Senger, Galli et al. 1983; Dvorak, Brown et al. 1995), since VEGF induces endothelial fenestration in some vascular beds (Roberts and Palade 1995). An increase in microvascular permeability is a crucial step in angiogenesis related with tumors and wounds (Dvorak 1986, Dvorak, Harvey et al 1987).

VEGF is also a major survival factor for endothelial cells both in vivo and in vitro (Alon, Hemo et al. 1995; Gerber, McMurtrey et al. 1998). In vitro, VEGF prevents endothelial cell apoptosis induced by serum starvation through phosphatidylinositol 3-kinase (PI3 kinase)/Akt signal transduction pathway (Gerber, McMurtrey et al. 1998; Fujio and Walsh 1999). VEGF induces expression of antiapoptotic proteins Bcl-2 and Al (Gerber, Dixit et al. 1998), XIAP and survivin (Tran, Rak et al. 1999) in human umbilical vein endothelial (HUVE) cells.

VEGF affects and controls hematopoietic stem cell (HSC) survival (Gerber, Malik et al. 2002). VEGF also increases production of B cells and the generation of

immature myeloid cells (Hattori, Dias et al. 2001). VEGF may regulate the coagulant properties of endothelium and monocytes, and may promote monocyte migration into the tumor bed (Clauss, Gerlach et al. 1990). VEGF regulates natural killer (NK) cells adhesion to tumor endothelium (Melder, Koenig et al. 1996).

VEGF has an inhibitory effect on the maturation of antigen-presenting cells such as dendritic cells. VEGF may facilitate tumor growth also by allowing the tumor to avoid the induction of an immune response (Gabrilovich, Chen et al. 1996).

VEGF increases the expression of vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and E-selectin through activating phospholipase C (PLCγ), protein kinase C (PKC), and nuclear factor-κB (NF-κB) signal transduction pathway in human umbilical vein endothelial cells by it's binding to the Flk-1/KDR receptor (figure 2) (Kim, Moon et al. 2001).

VEGF induces expression of the serine proteases urokinase-type and tissue-type plasminogen activators (PA) and also PA inhibitor 1 (PAI-1) in cultured bovine microvascular endothelial cells (Pepper, Ferrara et al. 1991). Moreover, VEGF increases expression of the metalloproteinase interstitial collagenase in human umbilical vein endothelial cells (Unemori, Ferrara et al. 1992).

There were also other works which showed that VEGF has mitogenic effects on certain epithelial cells, pancreatic duct cells (Guerrin, Moukadiri et al. 1995; Oberg-Welsh, Sandler et al. 1997).

Regulation of VEGF Gene Expression

1. Hypoxia-inducible factor (HIF)

Oxygen tension plays a key role in regulating the expression of a variety of genes (Safran and Kaelin 2003). VEGF mRNA expression is induced by exposure to low pressure of oxygen in a variety of pathophysiological circumstances (Dor, Porat et al. 2001) Semenza 2003).

Hypoxia–inducible factor 1 (HIF) is a transcriptional activator that functions as a major regulator of oxygen homeostasis and responses to hypoxia. HIF-1 target genes encode proteins that increase oxygen delivery and mediate adaptive responses to oxygen deprivation. HIF-1 activity is regulated by the cellular oxygen concentration and by the major growth factor-stimulated signal transduction pathways (Semenza 2002). HIF-1 is a basic, heterodimeric, helix-loop-helix protein composed of an inducibly-expressed HIF-1a subunit, 120-kDa, and a constitutively-expressed HIF-1b subunit, 91-, 93-, and 94-kDa (Wang, Jiang et al. 1995). HIF-1 binding site (HBS) and its downstream HIF-1 ancillary sequence (HAS) within the hypoxia response element (HRE) are indispensable to the transcriptional activation of the VEGF gene by hypoxia and NO (Kimura, Weisz et al. 2001).

2. Transcriptional regulation of VEGF gene by transcription factors.

Transcription factors bind to the promoter which contains specific DNA sequence to activate the transcription of a gene directly. The transcriptional regulation of the VEGF gene plays a preeminent role in the control of VEGF expression. Inducible and cell-type specific expression of VEGF is determined by the unique promoter elements where specific transcription factors bind. DNA sequence of VEGF gene reveals some of these promoter elements (Figure 3).

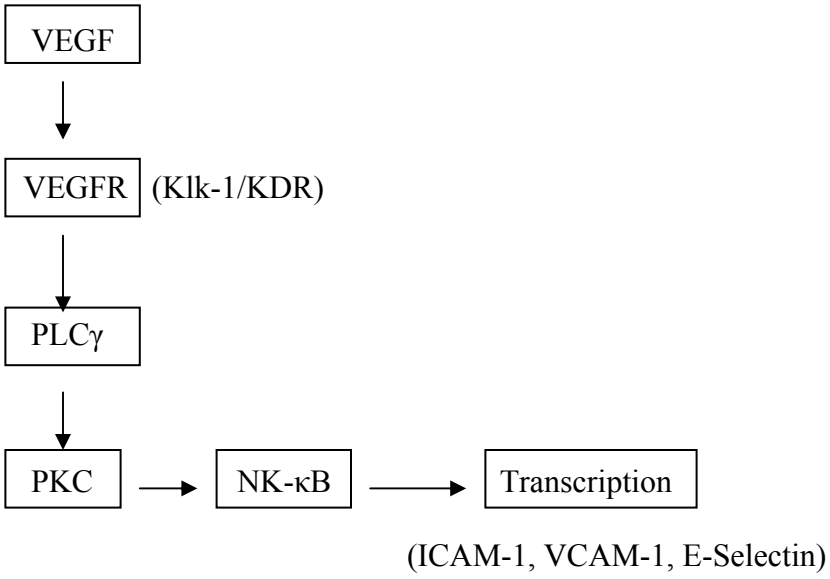

Figure 2. One of the pathways in VEGF-stimulated expression of ICAM-1, VCAM-1, and E-selectin in endothelial cells. After the binding of VEGF to the VEGF receptor, PLCγ was activated. NK-κB was activated through activation of PLCγ-sphingosine kinase-PKC cascade. This cascade is the main pathway that induces the transcription of ICAM-1, VCAM-1, and E-selectin.

Transcription factor NF-κB plays a critical role in inflammation and immunity. It is an important factor that controls gene expression of various proinflammatory cytokines, cell adhesion molecules, and chemokines, bacterial toxins (Barnes and Karin M. 1997; Chen, Castranova et al. 1999). NF-κB has contradictory properties, which either promotes harmful inflammation or leads the regeneration required to repair inflamed tissues (Youssef and Steinman, 2006). NF-κB family consists of five subunits: RelA (or P65), RelB, c-Rel, NF-κB1 (or p50) and NF-κB2 (or p52). In the key regulatory mechanism, the phosphorylated inhibitory proteins IκB are degradated, and NF-κB are subsequently released from IκB complex, and become activated. Hetero- or homodimers of NF-κB subunits are translocated into the nucleus to activate the target gene (Figure 4). NF-κB activity is divided into the NF-κB1 canonical pathway and the NF-κB2 alternative pathway. The NF-κB is also involved in the upregulation of VEGF and its activity is high in breast cancer cell line which is associated with high expression of VEGF mRNA (Shibata, Nagaya et al. 2002). Recently, a study found that VEGF is induced by HIF through the PI-3K/Akt-NF-kB signaling pathway in lung epithelial cell line in mice (Tong et al, 2006).

Transcription factor AP-1 (activator protein-1) is composed of Jun, Fos or ATF (activating transcription factor) subunits. Hypoxia highly induces the binding ability of AP-1 to DNA as well as the transcriptional activation of gene (Hirota, Matsui et al. 1997). The whole complex of activating factors, such as HIF-1, AP-1, NF-κB contributes to activation and expression of the VEGF gene in hypoxic conditions (Figure 5).

Cloned Human VEGF promoter sequence

-1158

AGACGTTCCTTAGTGCTGGCGGGTAGGTTTGAATCATCACGCAGGCCCTGGCCTCCACC

CGCCCCCACCAGCCCCCTGGCCTCAGTTCCCTGGCAACATCTGGGGTTGGGGGGGCAGC

AGGAACAAGGGCCTCTGTCTGCCCAGCTGCCTCCCCCTTTGGGTTTTGCCAGACTCCAC

AGTGCATACGTGGGCTCCAACAGGTCCTCTTCCCTCCCAGTCACTGACTAACCCCGGAA

CCACACAGCTTCCCGTTCTCAGCTCCACAAACTTGGTGCCAAATTCTTCTCCCCTGGGA

AGCATCCCTGGACACTTCCCAAAGGACCCCAGTCACTCCAGCCTGTTGGCTGCCGCTCA

CTTTGATGTCTGCAGGCCAGATGAGGGCTCCAGATGGCACATTGTCAGAGGGACACACT

GTGGCCCCTGTGCCCAGCCCTGGGCTCTCTGTACATGAAGCAACTCCAGTCCCAAATAT

GTAGCTGTTTGGGAGGTCAGAAATAGGGGGTCCAGGAGCAAACTCCCCCCACCCCCTTT

CCAAAGCCCATTCCCTCTTTAGCCAGAGCCGGGGTGTGCAGACGGCAGTCACTAGGGGG

CGCTCGGCCACCACAGGGAAGCTGGGTGAATGGAGCGAGCAGCGTCTTCGAGAGTGAGG

ACGTGTGTGTCTGTGTGGGTGAGTGAGTGTGTGCGTGTGGGGTTGAGGGTGTTGGAGCG

GGGAGAAGGCCAGGGGTCACTCCAGGATTCCAATAGATCTGTGTGTCCCTCTCCCCACC

CGTCCCTGTCCGGCTCTCCGCCTTCCCCTGCCCCCTTCAATATTCCTAGCAAAGAGGGA

ACGGCTCTCAGGCCCTGTCCGCACGTAACCTCACTTTCCTGCTCCCTCCTCGCCAATGC

CCCGCGGGCGCGTGTCTCTGGACAGAGTTTCCGGGGGCGGATGGGTAATTTTCAGGCTG

TGAACCTTGGTGGGGGTCGAGCTTCCCCTTCATTGCGGCGGGCTGCGGGCCAGGCTTCA

CTGGGCGTCCGCAGAGCCCGGGCCCGAGCCGCGTGTGGAGGGGCTGAGGCTCGCCTGTC

CCCGCCCCCCGGGGCGGGCCGGGGGCGGGGTCCCGGCGGGGCGGAGCCATGCGCCCCCC

CCTTTTTTTTTTAAAAGTCGGCTGGTAGCGGGGAGGATCGCG

Figure 3. Human VEGF promoter sequence. The arrow indicates transcription start site. DNA sequence up to nucleotide position -1158 is shown.

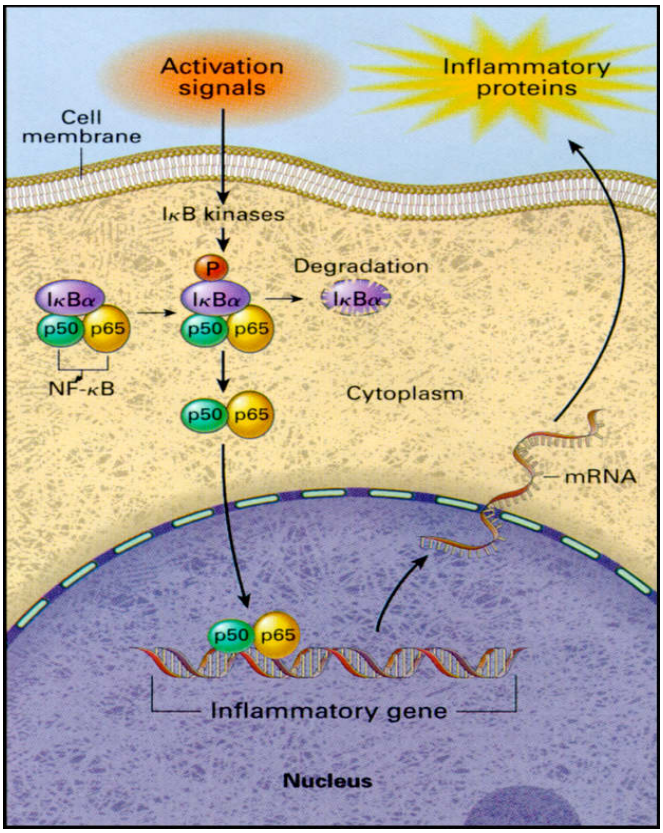

Figure 4. NF-κB signal transduction pathway. The phosphorylated inhibitory proteins IκB are degradated. NF-κB complexes are separated in the cytoplasm from IκB, and become activated. Hetero- or homodimers of NF-κB subunit are translocated into the nucleus to activate target genes.

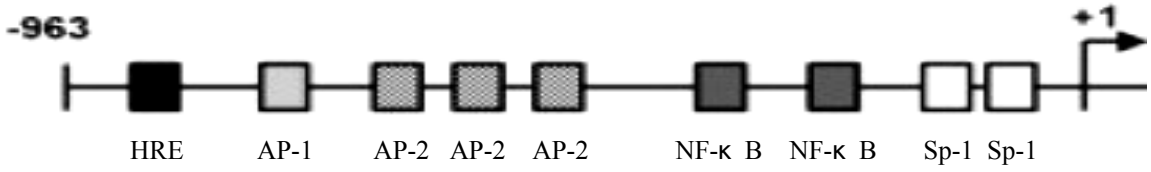

Figure 5. The different activating factors perform in various combinations to activate and express VEGF gene.

3. Cytokines, growth factors, hormones, and oncogenes

Several major growth factors, including epidermal growth factor, transforming growth factor-a (TGF-a), TGF-b, fibroblast growth factor (FGF), and PDGF up-regulate VEGF mRNA expression under various conditions (Pertovaara, Kaipainen et al. 1994; Frank, Hubner et al. 1995; Warren, Yuan et al. 1996) . Also, inflammatory cytokines such as IL-1-a and IL-6 induce VEGF expression in several cell types, including synovial fibroblasts (Ben-Av, Crofford et al. 1995; Cohen, Nahari et al. 1996), supporting the fact that VEGF plays an important role to mediate angiogenesis and vascular permeability in inflammation.

Hormones are also important regulators of VEGF gene expression. Several reports showed that Thyroid-stimulating hormone (TSH) and sex steroids are important stimulus for regulating VEGF expression in hormone-sensitive tissues (Shweiki, Itin et al. 1993; Soh, Sobhi et al. 1996; Ferrara, Chen et al. 1998; Shifren, Mesiano et al. 1998). Estradiol is a direct transcriptional activator of VEGF via binding to the specific estrogen response elements in the VEGF gene (Mueller, Vigne et al. 2000).

4. Differentiation and transformation

Specific transforming events also result in induction of VEGF gene expression.

Role of VEGF in Angiogenesis

Angiogenesis is a complex and multilevel process. It formats the new capillary from pre-existing blood vessels characterized by expansion of the endothelium by proliferation, migration, and remodeling. Angiogenesis is regulated strictly by stimulatory (angiogenic) and inhibitory (angiostatic) factor to control the correct development of blood vessels (Josko and Mazurek 2004).

1. Embryonic and postnatal development

Angiogenesis is a fundamental requirement for organ development and differentiation during embryogenesis (Hamilton et al 1962, Gilbert 1988). The critical role of VEGF and angiogenesis is emphasized by embryonic lethality between day11 and day12 after loss of a single VEGF allele. Administration of soluble of VEGFR-1 chimeric protein initiated at day 1 or day 8 postnatally inhibits VEGF expression, and results in almost complete growth arrest and more apoptotic endothelial cells in the liver.

2. Endochondral bone formation and skeletal growth.

VEGF is expressed by chondrocytes of hypertrophic cartilage layer (Figure 6)(Harper and Kalgsbrun 1999). The VEGF gradient is needed for directional growth and cartilage invasion by metaphyseal blood vessels (Gerber, Vu et al. 1999; Carlevaro, Cermelli et al. 2000)

Administration of a soluble VEGFR-1 chimeric protein or anti-VEGF monoclonal antibody, almost completely suppressed blood vessel invasion (Ryan, Eppler et al. 1999) (Gerber, Vu et al. 1999). VEGF produced by osteoblasts in response to BMPs couple angiogenesis to bone formation (Deckers, van Bezooijen et al. 2002). VEGF blockade inhibits bone repair (Street, Bao et al. 2002). VEGF also has direct chemotactic and other effects on osteoblasts and osteoclasts (Engsig, Chen et al. 2000; Mayr-Wohlfart, Waltenberger et al. 2002). VEGF-dependent blood vessel recruitment is essential for cartilage resorption in the process of bone formation (Carano and Filvaroff 2003).

3. Angiogenesis is also a fundamental requirement for wound healing and reproductive functions in the adult (Folkman and Klagsbrun 1987; Folkman, Szabo et al. 1991)

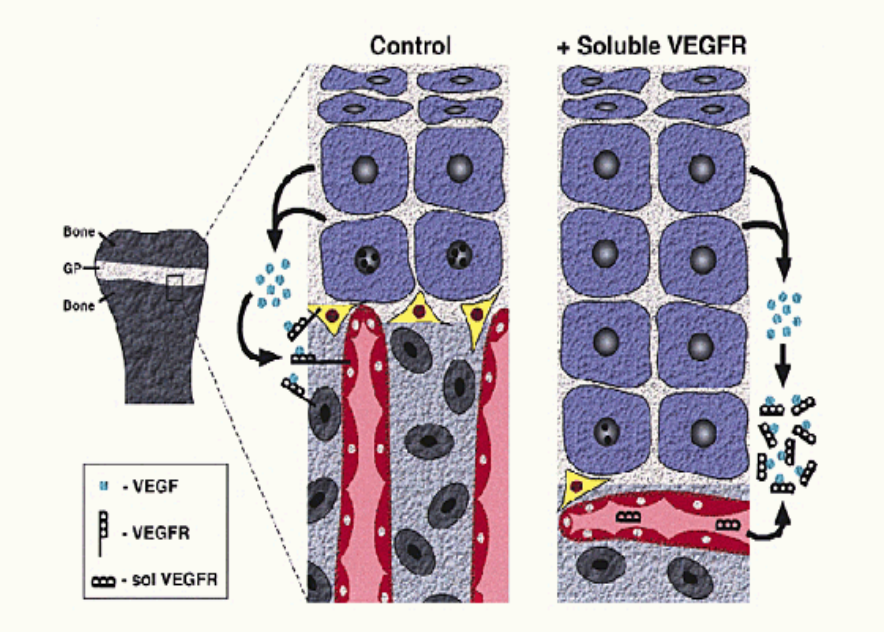

Figure 6. VEGF plays an important role in bone growth. Endochondral ossification occurs as the chondrocytes (blue) mature in the growth plate. These hypertrophic chondrocytes secrete VEGF, and then undergo apoptosis. VEGF binds to VEGF receptor, which are expressed by chondroclasts (yellow), osteoblasts (gray), and the endothelial cells in vessel for vascular invasion. Chondroclasts aid in the resorption of hypertrophic cartilage, paving the way for osteoblasts to migrate in and deposit bone matrix. These events are mediated by VEGFs, which bind to VEGF receptors. In these events the growth plate is replaced by advancing bone. Derived from Nature Medicine. Volume 5. Nu mber 6. June 1999

Role of VEGF in Pathogenesis

Many tumors express and secrete VEGF (Ferrara 2004) to stimulate vascular growth and get nutrition from vessel (Figure 2.8). Anti-VEGF monoclonal antibodies exert a potent inhibitory effect on the growth of several tumor cell lines in nude mice (Kim et al 1993). In addition, tumor-associated stroma is also an important source of VEGF production (Gerber et al 2000, Fukumura et al 1998, Tsuzuki et al 2000, Kishimot et al 2000). VEGF blockade using an anti-VEGF monoclonal antibody (bevacizumab) decrease tumor perfusion, vascular volume, microvascular density and circulating endothelial a progenitors in colorectal cancer patients (Willett et al 2004).

VEGF up-regulation has been implicated in various inflammatory disorders (Dvorak 2002), such as wound healing and psoriasis (Detmar et al 1995). VEGF is also involved in the pathogenesis of RA, an inflammatory disease in which angiogenesis plays a significant role, and levels of VEGF were increased in the synovial fluid of RA patients (Koch et al 1994, Fava et al 1994).

Role of VEGF in Osteoarthritis

The structure of the joint

In the joint, the end of the bone is very thin layer normal cartilage. The normal cartilage has two main components. One is the extracellular matrix (ECM). ECM is rich in collagens (mainly type II, IX and XI) and proteoglycans (mainly aggrecan). The second component is chondrocyte cells. These cells embed in the matrix. The matrix components are responsible for tensile strength and resistance to mechanical of the articular cartilage. In the Joint Capsule inner layer there is Synovium, which is composed of synovial

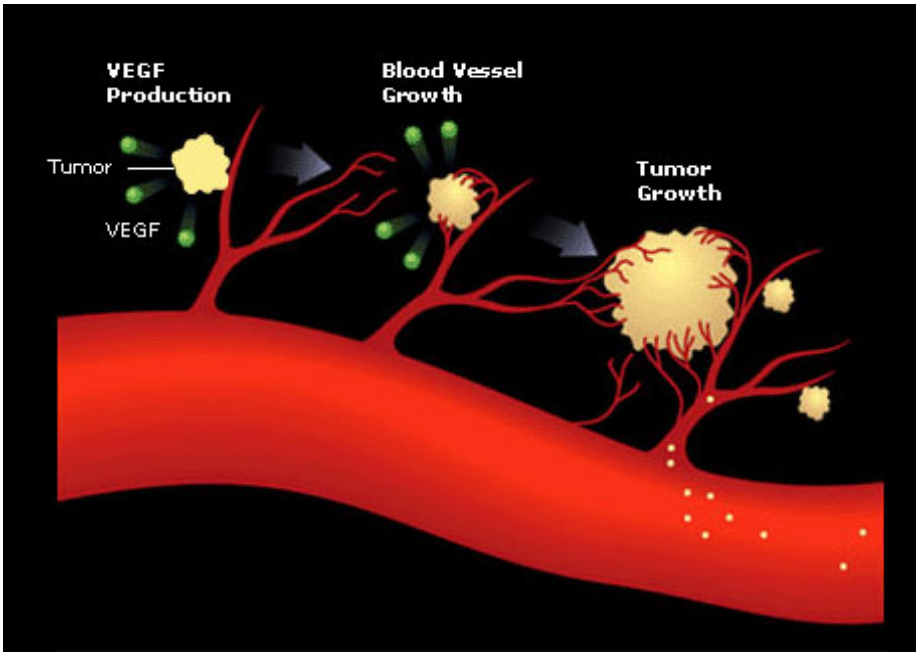

Figure 7. Tumors express and secrete VEGF to stimulate vascular growth and get nutrition from vessel.

fibroblasts. Synovium secretes many of the proteins found in the synovial fluid. Synovial fluid is a lubricant for joint's movement and protects joints from friction (Figure 8).

Characteristics of Osteoarthritis

Osteoarthritis can be defined as a gradual loss of articular cartilage, combined with thickening of the subchondral bone, bone outgrowths at joint margins and mild, chronic nonspecific synovial inflammation (Figure 9).

Abnormal cartilage and bone erosion is a common phenomenon in Osteoarthritis. During the progression of Osteoarthritis, the bone is degenerated, and massive loss of the articular cartilage is observed. Another interesting feature of Osteoarthritis is the osteophyte formation. Synovial and capsular lesions are other characteristics. During the progression of Osteoarthritis, Synovium and capsule are swelling, and with inflammation, and the synovial layer invades in the synovial fluid.

Incidence of Osteoarthritis

The specific cause of the Osteoarthritis is unclear. Over many years with activity and use of a joint, the cartilage may become torn, even wear away entirely. All people will develop OA to some degree when the aging process advances. The most common sites for OA include to base of the knees, the hip, the hands and shoulder. Angiogenesis and inflammation are important processes in the pathophysiology of OA. Osteoarthritic cartilage exhibit reduced resistance to vascular invasion. More blood vessels are found superficially in the articular cartilage as the severity of OA diseases. A striking association between the presence of bone erosion and increased intra-articular blood flow at the site of bone damage has been shown in RA by using Power Doppler

ultrasonography (Figure 10). The vascular signal is represented by the red color, and vascularized erosion in tissue is indicated by the arrow (Taylor, 2002).

VEGF is expressed in chondrocytes of cartilage in joints affected with OA. Osteophyte formation and extension of the subchondral growth plate have been observed in OA, and these processes are very similar to endochondral ossification, which is highly dependent on VEGF (Figure 11). VEGF expression plays an important role in the formation of new vessels in OA cartilage.

Serum amyloid A-activating factor-1 (SAF-1)

Serum amyloid A (SAA) protein is linked to various pathophysiological conditions, including rheumatoid arthritis and atherosclerosis. Serum amyloid A-activating factor-1 (SAF-1) plays a major role in aberrant transcriptional induction of SAA in response to inflammation (Ray and Ray, 1996). Many inflammatory agents including LPS, PMA, and cytokines like IL-1 and IL-6, which trigger SAA overexpression, induce both the DNA binding and transactivation potential of SAF-1 (Ray and Ray, 1997, 1998; Ray et al., 1999, 2000, 2001, 2002). Cytokine-responsive induction of SAF-1 activity is mediated by a mitogen-activated protein (MAP) kinase signaling pathways (Ray et al., 2002). Phosphorylation of endogenous SAF-1 in response to IL-1 and IL-6 was markedly inhibited by the addition of MAP kinase inhibitors. SAF-1 contains one MAP kinase phosphorylation site and MAP kinase directly phosphorylated SAF-1.

To study the biological function of SAF-1 in vivo, and investigate whether SAF-1 is associated with high level of VEGF expression, a transgenic mouse model that over-expresses an inflammation-responsive transcription factor SAF-1 (figure 12) was created

(Ray et al, 2004). These transgenic mice are highly sensitive to develop OA, and develop a severe form of arthritis when challenged with the Lyme disease agent. SAF-1 transgenic mice have markedly high rate of synovial inflammation, macrophage cell infiltration, and angiogenesis. High level of SAF-1 and VEGF expression are observed in joint. Massive cartilage degradation was also noticed when SAF-1 is locally over-expressed (Figure 13). To test whether this phenomenon exists in the diseased tissue sample, similar experiments were performed using human OA patient cartilage (Figure 14). These slides show that high level of expression of SAF-1 is clearly associated with VEGF expression in OA in human patients.

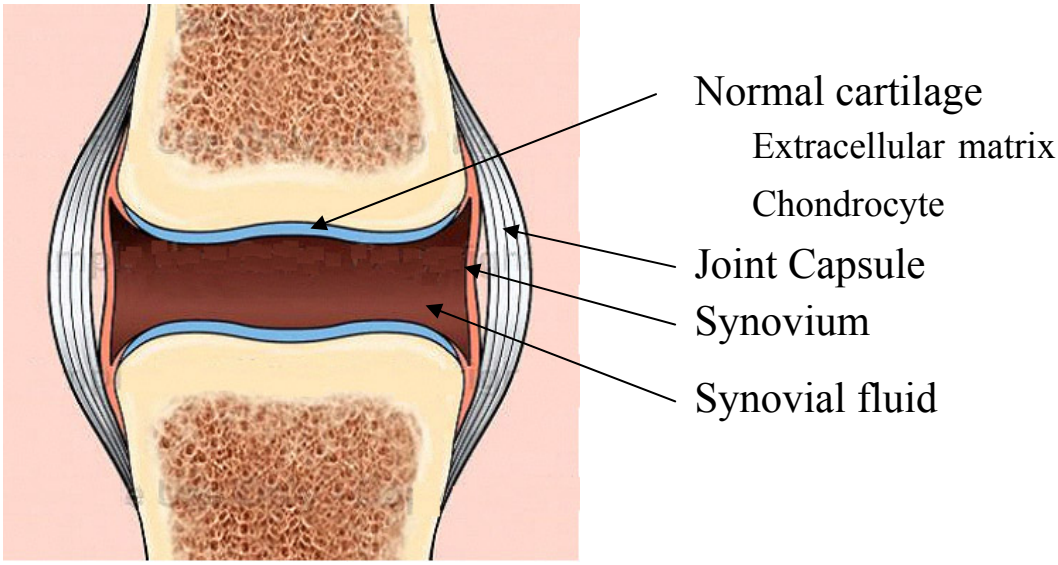

Figure 8. Normal Synovial Joint.

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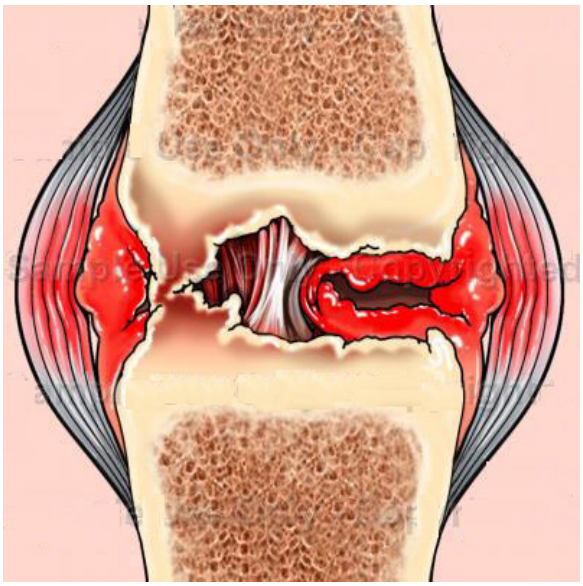

Figure 9. Angiogenesis in OA.
Angiogenesis and inflammation are important processes in the pathophysiology of OA.
Osteoarthritic cartilage exhibit reduced resistance to vascular invasion.

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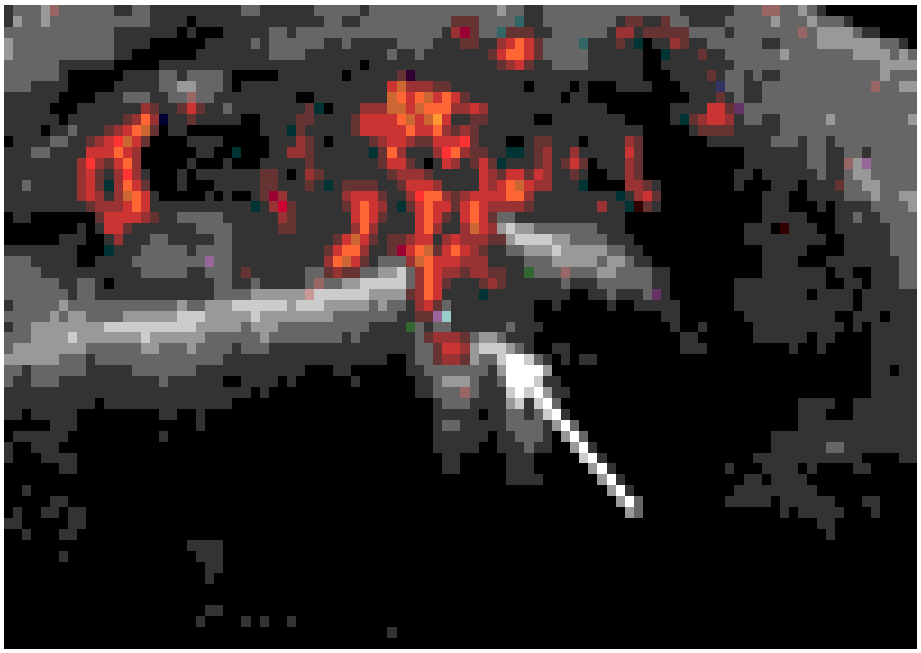
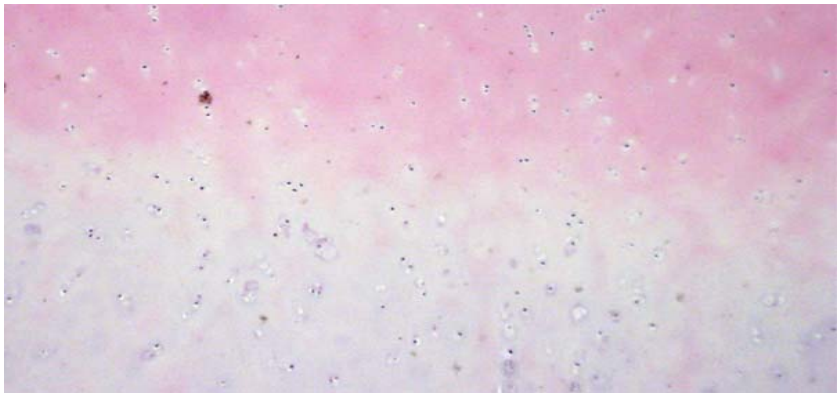


Figure 10. Power Doppler ultrasonography. The red color represents vascular signal. The arrow indicates vascularized erosion in tissue. Derived from Taylor P.C. Arthritis Research, 2002.

H&E staining



Anti-VEGF staining

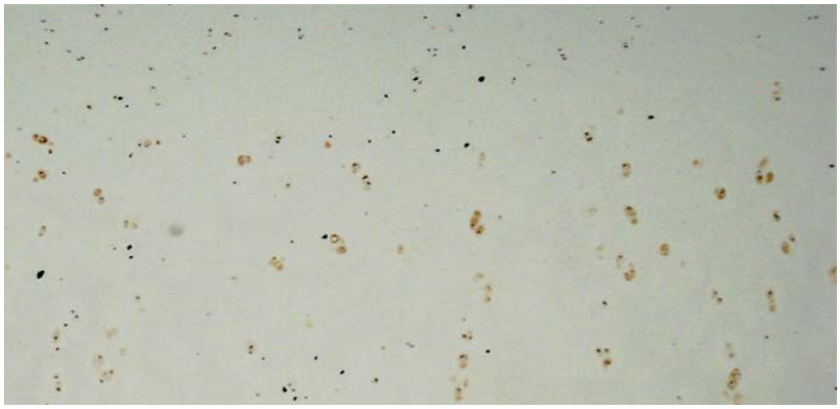


Figure 11. VEGF expresses in cartilage from a human OA patient. The upper panel is Hematoxylin and Eosin staining. The area stained pink represents the top of the cartilage or the superficial later, while the area stained blue represents deep layer of the cartilage which is proximal to calcified cartilage and bone. More densely packed cells are usually noted at the deep layer, which is evident from the blue stains of nuclei. The lower panel is Anti-VEGF staining. The brown color spots show VEGF expression in OA.

Figure 12. CMV immediate-early promoter was used to directly express SAF-1 transgene in multiple organs.

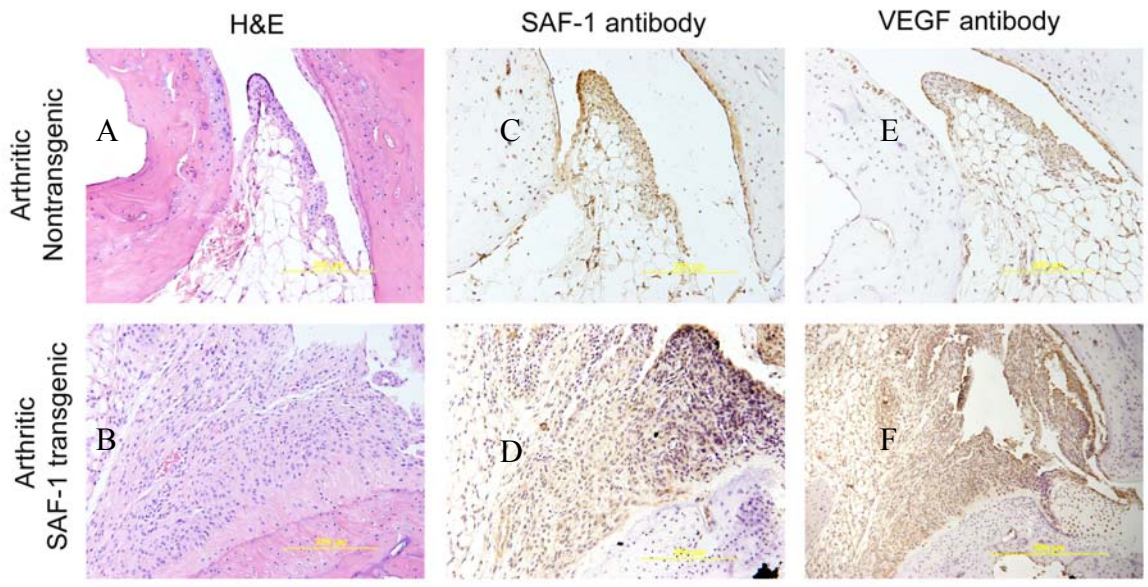


Figure 13. High level of expression of SAF-1 was associated with VEGF expression. Panels A. C. E are slides from the joint of arthritic non-transgenic mice. Panels B.D.F are slides from the joint of arthritic SAF-1 transgenic mice. Panels A and B show H&E staining. Panels C and D show SAF-1 level detected by immunohistochemical analysis. Panels E and F show VEGF level detected by immunohistochemical analysis. These experiments were done by using serial section of the joint tissue.

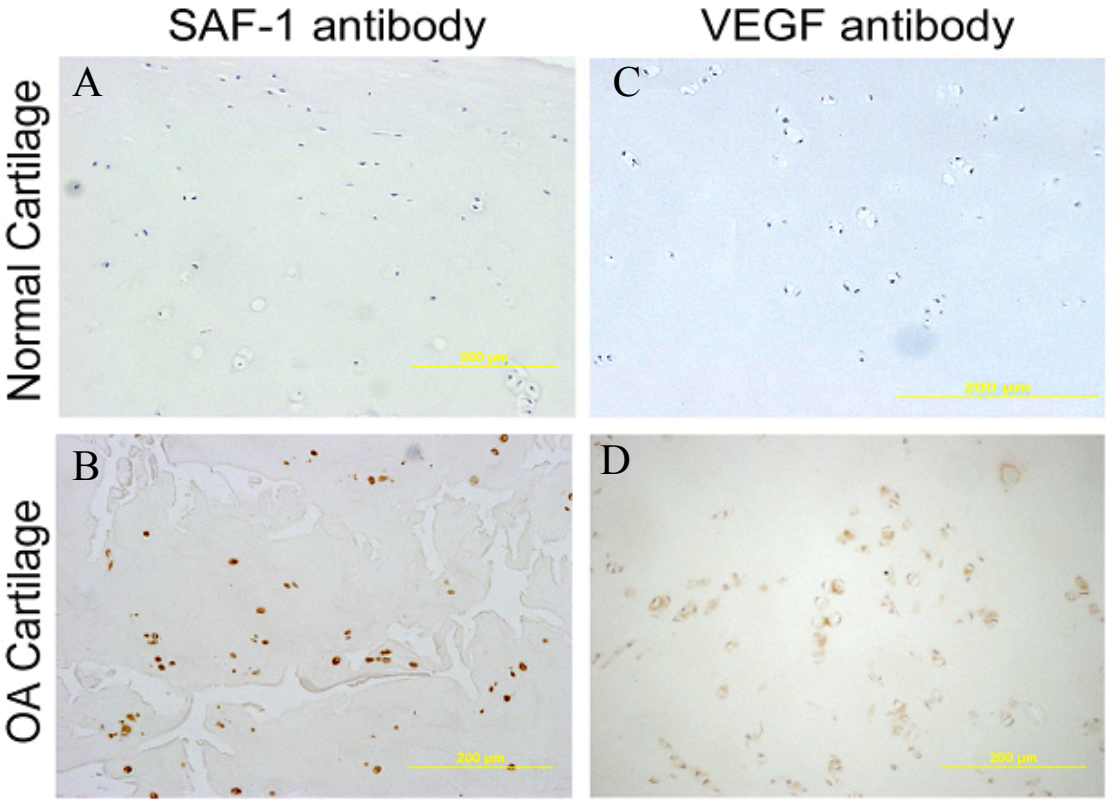


Figure 14. High level of expression of SAF-1 associated with VEGF expression in OA in human patients. When compared between panels A and B, there is high level of SAF-1 expression in OA cartilage than normal cartilage. Similarly when panels C and D are compared, there is high level of VEGF expression in OA cartilage than normal cartilage.

Hypothesis:

Base on these previous data, SAF-1 transgenic mice have high level of SAF-1 expression and high level of VEGF expression. Further, high level expression of SAF-1 is clearly associated with high level VEGF expression. Thus, we hypothesize that SAF-1 regulates VEGF expression.

Chapter 3

METHODS AND MATERIAL

Generating construct containing VEGF promoter (HUMAN):

An about 1.2 kb DNA fragment corresponding to human VEGF promoter region -1179 to +21 was amplified by PCR. Forward primer is 5' TGGTCGACGCTCTGGGCAGCTGGCCTAC 3'. Reverse primer is 5' GAGGATCCCGGCTGCCCCAAGCCTC 3'. The PCR mixture was made using 7.6 µl of 3.3 X XL Buffer II, 2.0 µl of 10 mM dNTP, 1.2 µl of 25mM $Mg(OAC)_2$, 0.5 µl of rth DNA polymerase, 10.3ul of dH2O. 1.25 µl of the each of both the forward and reverse primers 20 pmol/µl were added. 1.0 µl of 129µg/ml human genemic DNA was added as the template.

The PCR products were digested using enzyme SalI and BamHI, and ligated in to pTZ19U vector.

Cloning into pTZ 19U plasmid.

pTZ19U vector, which contains promoter for T7 RNA polymerase, was chosen for cloning purpose. In the pTZ19U vector, the T7 promoter has 2-4 nucleotides within the multiple cloning sites (MCS) in the *lac Z* gene. Thus screening for β-galactosidase activity identifies recombinant clones. The VEGF promoter region was cloned in the pTZ19U plasmid for the preparation of probe used in Electrophoretic Mobility Shift Assay (EMSA) and DNAse I Footprint assay.

Constructing CAT reporter plasmid

pBLCAT3 plasmid was used for the construction of VEGF-CAT (Chloramphenicol Acetyl Transferase) reporter plasmid. A promoter of the gene for the

initiation of transcription is required for the pBLCAT3. This plasmid is suitable for monitoring the promoter enhancer function of a DNA element when inserted at the 5' end of the CAT reporter gene. For the expression of the reporter gene in pBLCAT3, one must provide a basal promoter element, i.e. a TATA box for the assembly of the transcription initiation complex. There is a multiple cloning site in this plasmid located upstream of the CAT reporter gene.

In order to determine the promoter activity of VEGF regulated by SAF-1, the VEGF promoter was placed in the upstream of CAT reporter gene, which encoded a protein, called CAT. CAT has the ability to neutralize the antibiotic chloramphenicol by transfering acetyl groups to chloramphenicol and producing Chloramphenicol-Acetates, and thus changes its shape into a harmless form. When the VEGF promoter is activated, the expression of reporter CAT gene will be enhance.

SAF-1 plasmid

The pcMV-SAF-1 expression plasmid was made by inserting a full-length SAF-1 cDNA under the control of cytomegalovirus promoter in a pcDNA3 vector (Invitrogen, Carlsbad, CA).

pRSETA vector was used to clone the fragment of the SAF-1 cDNA sequence, which codes the full length SAF-1 protein. In transient transfection, this plasmid produces a functional SAF-1 protein in the transfected cells. Since pRSET A vector has some advanced feature, such as, small size of the vector (2.9 kb), high copy number/cell (200-250 copies), and high level expression of recombinant proteins (20mg purified protein/liter of culture) are made.

Small scale DNA preparation for screening the clones

An individual colony was grown in 2.0 ml of 2X YT medium containing 0.5 µg/µl ampicillin in 37°C shaker for overnight. 1.5 ml of the culture was transferred to a microfuge tube and the cells were collected by centrifugation at 14,000 rpm for 2 minutes. The supernatant was removed and the cells were resuspended in 100ul of solution (50mM glucose, 25mM Tris-HCl, pH 8.0, and 10 mM EDTA), then added 200 µl of 0.2 N NaOH containing 1% SDS lysing buffer, and gently mixed by inversion until the supernatant become clear looking. 150ul of 3.0 M potassium acetate, pH 4.8, was added, and mixed by briefly vortex, then centrifuge for 5 minutes. The supernatant was transferred in a clean tube, 4.0ul of 5ug/ul DNAse free RNAse A was added and incubated at 37°C for 1.5 hours to digest RNA. About 200ul of phenol/chloroform was added in the mixture, vortexed vigorously for 3 minutes, and centrifuged at 14,000rpm for 5 minutes. The upper layer aqueous phase was transfered into a new microfuge tube, 1.0 ml of 100% ethanol was added, mixed well by inversion, then kept at -20°C for overnight.

Next day, the DNA pellet was collected by centrifugation at 14,000 rpm for 10 minutes, and the supernatant was discarded. The DNA pellet was wash 3 times with 0.5 ml of 80% ethanol and the DNA pellet was dried. The pellet was dissolved with 16.8 µl of sterile distilled water. 3.2 µl of 5.0 M NaCl and 20 µl of 13% PEG (polyethylene glcol) was added, mixed well, and kept at 4°C for a couple of hour or overnight. The mixture was centrifuged and the supernatant was discarded. The DNA pellet was washed 3 times and then dried. 20ul of dH_2O was added to dissolve the DNA, and stored at -20°C for future use.

Isolation of DNA fragment

The plasmid DNA fragment was digested using the desired restriction enzymes. 0.1 µl of the loading dye (10X TBE with bromophenol blue) was added to stop the reaction and heated at 75°C for 3 minutes. Electrophoresis was done in a low melting temperature agarose gel with 1X TBE running buffer. The gel was stained with ethidium bromide, and the desired DNA fragment band was cut and put in an Eppendorf tube. The piece of gel was heated at 75°C until completely melted. 0.1 volume of 3.0M sodium acetate, pH 5.5, and saturated phenol were added. After incubated at 4°C overnight, the mixture was centrifuged and the upper aqueous layer was transferred into a new Eppendorf tube. The aqueous layer was extracted some times using n-butanol to reduce the volume. 2.5 volumes of 100% ethanol was added to precipitate the DNA, and incubated at -80°C for a couple of hours or at -20°C for overnight. The DNA was centrifuged at 14000rpm for 10 minutes, and the supernatant was discarded. The DNA pellet was washed three times using 0.5 ml of 80% ethanol, and then it was dried. The DNA was dissolved in 10ul of sterile distilled water, and purified using a coarse Sephadex G-50 mini-column. The DNA was stored at -20°C for further use.

Large scale DNA preparation from the positive clones

Once the positive clones are confirmed, cultures were transferred to a flask containing 30 ml of 2XYT medium with ampicillin (80ug/ml), and put in a 37°C shaker incubator for overnight. The culture was transferred to the Sorvall tube and centrifuged at 5000rpm for 10 minutes. The supernatant was removed and the cells were resuspended with 2.0 ml of 50mM glucose, 10mM EDTA and 25 mM Tris-HCl, pH 8.0. After incubating 5 minutes at room temperature, 4.0 ml of 0.2 N NaOH solution containing 1% SDS was added for lysing the cells, gently mixed by inversion and kept on ice for 5

minutes. 3.0 ml of ice cold 3M potassium acetate solution (pH 4.8) was added, gently mixed by inversion and incubated on ice for 15 minutes. The mixture was centrifuged at 10,000rpm for 10 minutes and the supernatant was transferred to a clean tube. DNA was precipitated by adding 2.5 volumes of ice cold absolute ethanol and incubating at -80°C for 1 hour or at -20°C for overnight. The mixture was centrifuged at 2,500 rpm for 15 minutes and the supernatant was discarded. The pellet was washed with 80% ethanol twice and dried. The pellet was dissolved in 500 µl sterile distilled water, and transferred to an eppendorf tube. 5 µl of 10mg/ml DNAse free RNase A was added and incubated at 37°C for 1 hour. Equal volume of phenol/ chloroform was added, and the aqueous phase was removed in a fresh eppendorf tube. 2.5 volumes of 100% ethanol was added and incubated at -80°C for several hours or at -20°C for overnight. The solution was centrifuged at 14,000rpm for 10 minutes and the supernatant was discarded. The pellet was washed with 80% ethanol twice and dried. 336 µl of sterile distilled water was added for dissolving the pellet. 64ul of 5M NaCl and 400ul of 13% PEG were added and incubated at 4°C for overnight. The mixture was centrifuged at 14,000rpm for 15 minutes and the supernatant was removed. The pellet was washed with 80% ethanol three times, and dried. Finally the DNA was dissolved in 100~500ul sterile distilled water depending on the different plasmids and stock at -20°C for further use.

Transformation

First, competent *E.coli* cells were prepared. E. coli cells were grown until the $O.D_{560}$ reaching to about 0.5. The cells were centrifuged at 2,500 rpm for 10 minutes and the supernatant was discarded. 0.5 ml of 0.1 M $MgCl_2$ was added and the suspension was kept at 4°C for 10 minutes. The cells were centrifuge again at 2,500 rpm for 5 minutes

and the supernatant was discarded. 0.2 ml of 0.1 M $CaCl_2$ was added for resuspend the cells, the suspension was stored on ice for overnight. Next day, plasmid DNA was added to the competent cells and kept on ice for 30 minutes to 1 hour. The cells were heat in a water bath at 42°C for 2 minutes. 0.3 ml of 2XYT medium was added and incubated at 37°C for 1 hour. 60µl of 20mg/ml Ampicillin was added and mixed with 20µl of 0.1 M IPTG, 20µl of 30mg/ml X-GAL, spread on a 2XYT plate, and incubated at 37°C for overnight.

Cell Culture

Human chondrocyte cells HTB-94, obtained from the American Type Culture Collection, were derived from a primary grade II chondrosarcoma of the right humerus of a patient. Many of the features of normal chondrocyte retain in these cells. HTB-94 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing a high concentration of glucose (4.5g/L) supplemented with 7% fetal calf serum (FCS).

Transfection

Chondrocyte cells (HTB 94) were grown in 24 well plates by seeding at a density of $5x10^3$ cells per well (50% confluence). Twenty-four hours later, the cells were transfected by adding different concentration of SAF-1 plasmid DNA (amounts indicated in the figure legends) by the calcium phosphate precipitation method (Graham and Van der Eb, 1973).

The DNA calcium phosphate precipitate was allowed to settle on cells overnight. The next day, the cells were washed once with phosphate-buffered saline (PBS) for washing out the DNA calcium precipitate, and the cells were subjected to a hypotonic shock with 15% glycerol in HEPES-buffered saline (HBS) for 1 minute. Then the cells

were washed three times with HBS. Medium (DMEM+4.5g/L glucose+7%FCS) was added to the cells and inducted for 24 hours.

Cell Harvesting

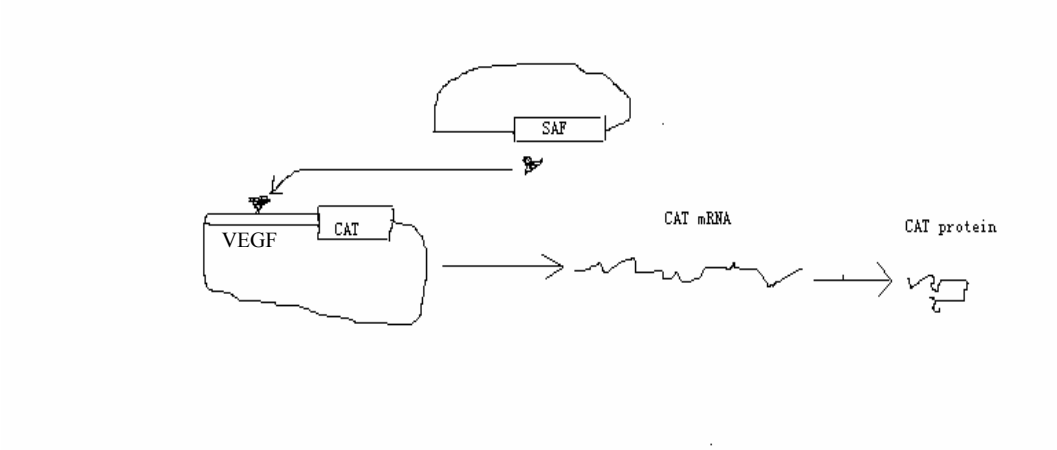
Cells were harvested into Microcentrifuge Tubes, and centrifuged at 2,400 rpm 10 minutes, the supernatant was discarded. After washing by 1xPBS, 100ul of 0.25M Tris pH=8 was added and vortexed the mixture for break the cells. The cells were lysed by 3 freeze-thaw cycles at -80°C and 37°C, and heated at 60°C for 10 minutes and centrifuged at 14,000rpm for 5 minutes. Protein concentrations were measured by using OD at 228.5 nm and 234.5 nm following the formula:

Protein concentration (mg/ml) = (A228.5 nm –A234.5 nm) x 317x Dilution factor (ug/ml)

Then these proteins were used for CAT assay.

CAT Assay

To perform the CAT enzyme assay, the transient transfection was performed. First, we transfected cells in culture with a plasmid DNA that carried the VEGF promoter and a reporter gene (CAT) whose expression is dependent on the promoter function. Then, we co-transfected the cells with a second plasmid DNA that carried the test transcription factor SAF-1. If the test transcription factor SAF-1 is indeed responsible for inducing the VEGF promoter, co-transfection will enhance the reporter gene CAT expression.



When SAF-1 is expressed, it can bind to VEGF promoter. If this happened, then CAT mRNA may be formed. CAT mRNA is then converted to CAT protein which can be performed using CAT Assay.

Cell extracts containing CAT were isolated from the cells under various conditions. Then the cell extracts were mixed with the ^{14}C labeled chloramphenicol. In addition, the substrate acetyl CoA was also added to the mixture and the mixture was incubated at 37°C for overnight. When the CAT reactions were completed, the products were spotted on the thin-layer chromatographic (TLC) plate. The TLC plate was placed into the TLC chamber with a solvent, a mixture of chloroform and methanol (chloroform: methanol = 95:5), and allowed the spots migrate up with solvent until it reached the top of the TLC plate. The TLC plate was exposed to X-ray film.

The amount of acetylation is directly proportional to the amount of CAT enzyme present. Therefore, measuring the amount of acetylated chloramphenicol in different cell extractions will determine how much CAT protein was produced as a result of activated promoters. CAT can add two acetyl groups to each chloramphenicol. All products and

unused substrate will migrate on the TLC surface according to their ability to interact with surface of the TLC (Figure 15).

Since only the radioactive molecules are chloramphenicol and the only molecules visible on the X-ray film are chloramphenicol and all its acetylated forms. The amount of acetylated chloramphenicol can be determined by measuring the radioactivity in each spot on the TLC plate. The C AT assay offers an indirect but quantitative way to measure the amount of transcription driven by the given promoter.

Preparation of radioactive probe

The VEGF pTZ19U plasmid DNA was digested with restriction enzymes HindIII and DraI to create the 5' recessed ends and purified from the agarose gel. The DNA fragment was from -131 to -26 in Human VEGF promoter sequence: 5' CGAGCCGCGTGTGGAGGGGCTGAGGCTCGCCTGTCCCCGCCCCCCGGGGCGG GCCGGGGGCGGGGTCCCGGCGGGGCGGAGCCATGCGCCCCCCC 3' 10ug/ul VEGF DNA (H+D) fragment was incubated with 10uci [$a^{32}P$] dATP and 8 units Klenow DNA polymerase in presence of 10X Klenow Buffer, and incubated at RT for 60 minutes. 5mM dNTP's were added to the reaction mixture, and incubated at room temperature for 10 minutes. The reaction was heated at 75C for 1 minute to stop the reaction. The labeled DNA probe was purified by passing through a Sephadex G-50 (coarse) mini-column to remove free ^{32}P-ATP.

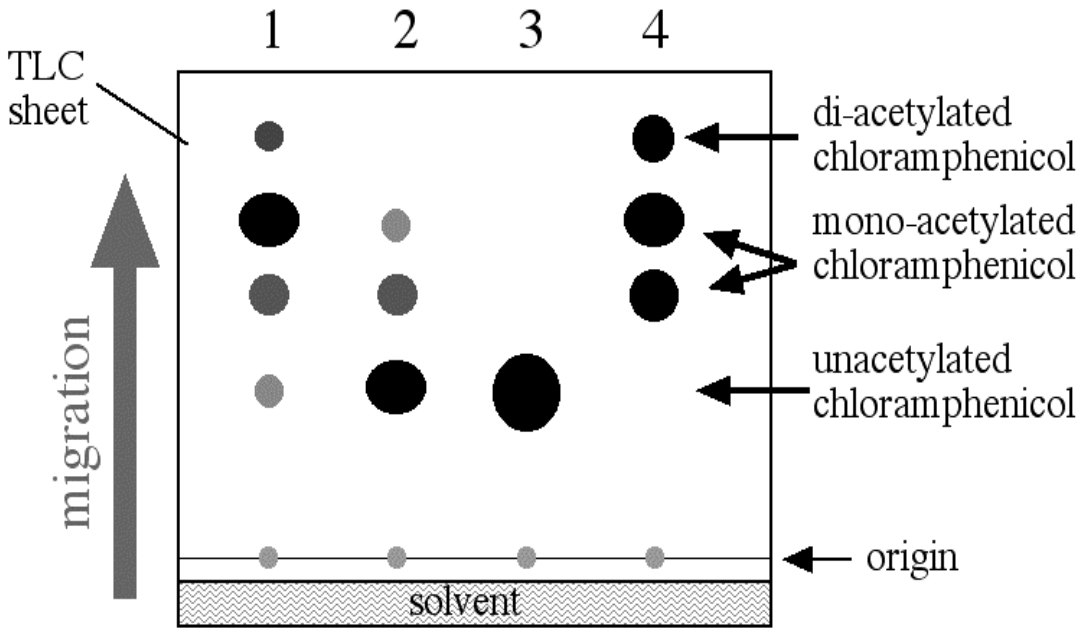


Figure 15. The separation of CAT-derived products. In the TLC, Lane 1 and lane 2 are samples. Lane 3 is negative control. Lane 4 is positive control. Chloramphenicol migates slower than chloramphenicol-acetates. The amount of acetylated chloramphenicol is greater in lane 1 than that in lane2. Therefore, promoter number 1 has more activity than promoter number 2.

Preparation of nuclear extracts

The cells were harvested into Microcentrifuge Tubes, and centrifuged at 2,000 rpm for 5 minutes and washed 3 times with sterile phosphate-buffered saline (PBS). The cells were resuspended in 500 µl of buffer A (10mM HEPES, pH 7.9, 1.5 mM$MgCl_2$, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 µg/ml each of leupeptin, antipain, pepstatin, 0.1 µg/ml chymostatin, 0.3TIU/ml of aprotinin, 0.5 mg/ml benzamidine). The cell suspension was incubated on ice for 15 minutes, vortexed vigorously for 15 seconds, and centrifuge at 3,000 rpm for 5 minutes to collect the nuclei. The supernatant was transferred to a fresh tube and stored as post-nuclear extract. The nuclei pellet was resuspended in 300ul of buffer C (20 mM HEPES, pH 7.9, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% (v/v) glycerol, 0.5 mM PMSF, 0.5 µg/ml each of leupeptin, antipain, pepstatin, 0.1 µg/ml chymostatin, 0.3 TIU/ml of aprotinin, 0.5 mg/ml benzamidine), and vortex vigorously. The mixture was incubate on ice for 30 minutes with occasional overtaxing, then centrifuged at 8,000 rpm for 5 minutes. The supernatant was transferred into a fresh tube and stored as nuclear extract.

Electrophoretic Mobility Shift Assay (EMSA)

EMSA is used in studying gene regulation and determining protein: DNA interactions. EMSA is performed by incubating a protein(s) (such as nuclear or cell extract) with [α^{32}P] end-labeled DNA fragment containing the putative protein binding site. The specificity of the DNA-binding protein for the putative binding site is established by competition experiments using unrelated DNA fragments or oligonucleotides containing a binding site for the protein of interest. In the assay, the

complexes of protein and DNA should migrate through a native gel slower than that of the free DNA fragments or double-stranded oligonucleotides.

EMSA was performed with equal amounts of the nuclear protein prepared from HTB-94 cells. Protein concentrations were estimated by Bradford's method (Bradford, 1976). The OD 228.5 nm and 234.5 nm were measured. The protein concentration was calculated using the following formula:

Protein amount = (A228.5nm-A234.5nm) x 317 x 10^{-3} Dilution factor.

The DNA fragments corresponding to -131 to -26 of VEGF promoter region was labeled using [α^{32}P] dATP and used as the oligonucleotide probe in EMSA reactions. The nuclear extracts were mixed with mixture made using 10X Binding Buffer (70mM Tris HCl, pH 7.5, 30mM NaOAC, pH 7.0, and 10 mM EDTA), 50%(v/v) Glycerol, 0.1MgCl2, 01%(v/v) Nonidet P-40, and 1ug/ul Poly (dI-dC). The reaction mixtures were incubated at room temperature for 15 minutes. 10ug/ul [α^{32}P] labeled double-stranded VEGF DNA (HindIII+DraI) fragment were added, and were incubated at room temperature for 30 minutes. The reaction mixtures were running on a 5% non-denaturing polyacrylamide gel with recirculation of 1X Running Buffer (7mM Tris HCl, pH 7.5, 3mM Sodium acetate, pH 7.0 and 1mM EDTA).

Mutagenesis analysis.

Synthesis of a deletion construct of VEGF promoter oligonucleotide

VEGF forward primer:

5’CCGGGCCCGAGCCGCGTGTGGAGGGGCTGCTTTTTTTTTTAAAAGTCGGCT G 3’

Reverse primer:

GAGGATCCCGCCTGCCCCAAGCCTCCGCGATCCTCCCCGCTACCAGCCGACTT

TTA

Deleteded element: The sequence from -109 to -36 was deleted.

-109

5'AGGCTCGCCTGTCCCCGCCCCCCGGGGCGGGCCGGGGGCGGGGTCCCGGC

GGGGCGGAGCCATGCGCCCCCCC 3'

-36

Annealing:

2 µl oligonucleotide of VEGF Forward Oligo and 2 µl of VEGF Reverse Oligo were mixed at the presence of 4 µl of 5X sequence buffer (200 mM Tris HCl, ph 7.5, 100 mM $MgCl_2$, 250 mM NaCl). The mixture was diluted by adding 12 µl of sterile distilled water, mixed well, spined down at 14000 rpm for 30 seconds, and heated at 75°C and slowly cool to 35°C.

Extension:

20 µl of annealed DNA was diluted by adding 21.5 µl of sterile distilled water, then added 1 µl 10X Klenow Buffer (500mM tris HCl, pH 7.2, 100 mM $MgSO_4$, 1mM DTT), 5 µl of 5 mM dNTPs, 0.5 µl of 0.1 M DTT, and 2 µl of 5U/µl Klenow. The mixture was mixed well, spined down at 14000 rpm for 30 seconds, and incubated at room temperature for one and a half hours. 50 µl of 7.5 M NH_4OAC, and 400 µl of 100% Ethanol were added, mixed well and kept at -20°C overnight. The DNA was centrifuged at 14000rpmfor 30 minutes, and discarded the supernatant. The pellet was washed 3

times in 80% ethanol, dried and resuspended in 20 µl of sterile distilled water. DNA was passed through a coarse Sephadex G-50 mini-column. The DNA was stored at -20°C for future use.

Chapter 4

Results

1. VEGF promoter activity is regulated by SAF-1

Construction of VEGF-CAT: 1.2 VEGF-CAT reporter plasmid was constructed by ligating a 1.2 Kb promoter DNA fragment from -1179 to +21 of human VEGF gene (Tischer et al., 1991) into pBLCAT3 vector. The inserted VEGF promoter DNA was confirmed by DNA sequencing. The physical map of the reporter construct is shown in Figure 16.

Transfection was carried out in the chondrocyte cells (HTB94). The cells were cultured in the presence of DNA for 16 hours. After removing the DNA, cells were incubated in DMEM-HG, +7% FCS medium for 24hours. The cells were then harvested and lysate were prepared for CAT assay.

In order to study VEGF induction, chondrocyte cells were transfected with 1.2 VEGFCAT3 plasmid or pBLCAT3 empty vectors. The result of CAT assay indicated that VEGF promoter promotes reporter CAT gene expression in the chondrocyte cells (Figure 17).

To test whether SAF-1 is able to promote this VEGF promoter- driven transcription, co-transfection assay was performed. In this assay, chondrocyte cells were transfected with 1.2 VEGFCAT3 plasmid along with a mixture of reporter plasmid (pCDSAF-1) which was added at increasing concentration (0.5, 1.0 and 1.5 μg). As a control, empty vector pCDNA3 was added also at the same concentration. Following 16

hours incubation with DNA, the cells were washed and grown for 24 hours in DMEM-HG +7% FCS. CAT assay was performed as described below in Figure 18.

The result of CAT assay showed that when SAF-1 is ectopically expressed at increasing doses, the reporter gene expression was proportionately (concomitantly) increased. As a control, empty vector (pCDNA3) had no effect on CAT activity. This data suggested that SAF-1 is directly responsible for VEGF promoter induction in a dose dependent manner.

From Footprint Assay, we know that SAF-1 binding sequence is located nucleotide position -100 to -30. To test whether SAF-1 binding site in VEGF promoter is essential for gene expression, SAF-1 binding sites were deleted. Chondrocyte cells were transfected with either entire 1.2 VEGFCAT3 plasmid or mutated (-100 to -30) VEGFCAT3 plasmid in which SAF-1 binding sites were deleted. Since IL1β+TGFβ activate SAF-1 in chondrocyte (HTB94) cells, the cells were incubated, after overnight transfection with IL1β+TGFβ for additional 24 hours. In the transfection assays, these two cytokines were added at the following concentrations: (100U+2ng)/ml, (200U+4ng)/ml and (400u+4ug)/ml, respectively. The cells were harvested and CAT activity was measured. Deletion of SAF-1 binding sites completely abolished VEGF promoter activity (Figure 19). This data suggests that SAF-1 binding site is necessary for the induction of VEGF promoter activity by inflammatory cytokines.

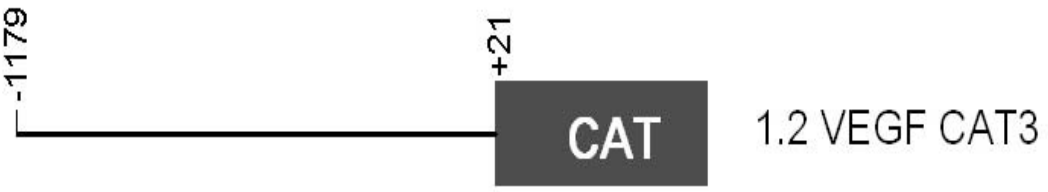


Figure 16. The physical map of the VEGF promoter-CAT reporter construct. Human VEGF DNA fragment containing DNA sequences from nucleotide position -1179 to +21 was ligated in front of the CAT gene in the pBLCAT3 plasmid. The region representing the VEGF DNA plus the CAT coding region is shown.

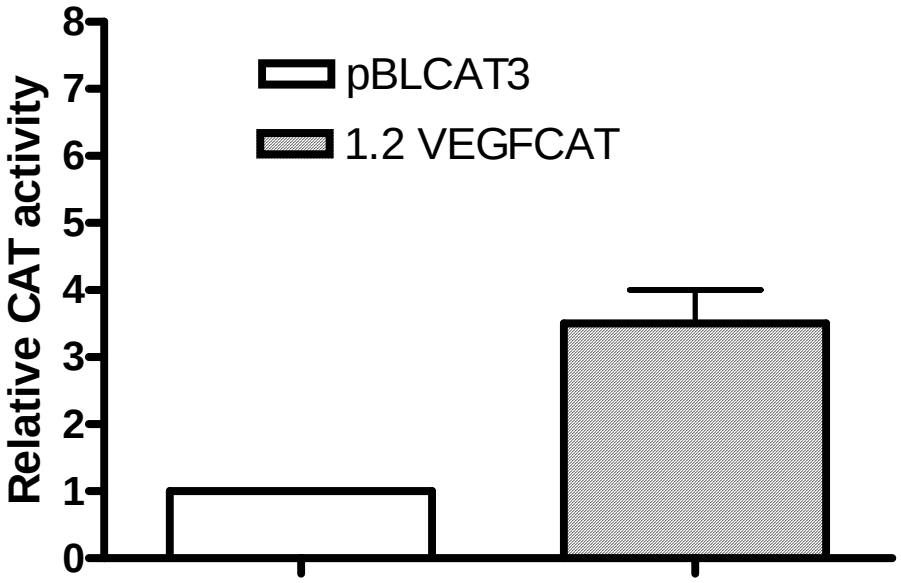


Figure 17. VEGF promoter activity in chondrocyte cells. Chondrocyte cells were transfected with either the empty vector pBLCAT3 or VEGF promoter-containing pBLCAT3 which is designated as 1.2 VEGF CAT3. Cells were harvested and CAT activity was measured as described in chapter 3. Increase in CAT activity in the 1.2 VEGFCAT3-transfected cells were relative to that of the pBLCAT3 was calculated and plotted. This experiment was repeated 3 times with duplicate samples in each experiment. Result in this Figure shows an average of the three experiments.

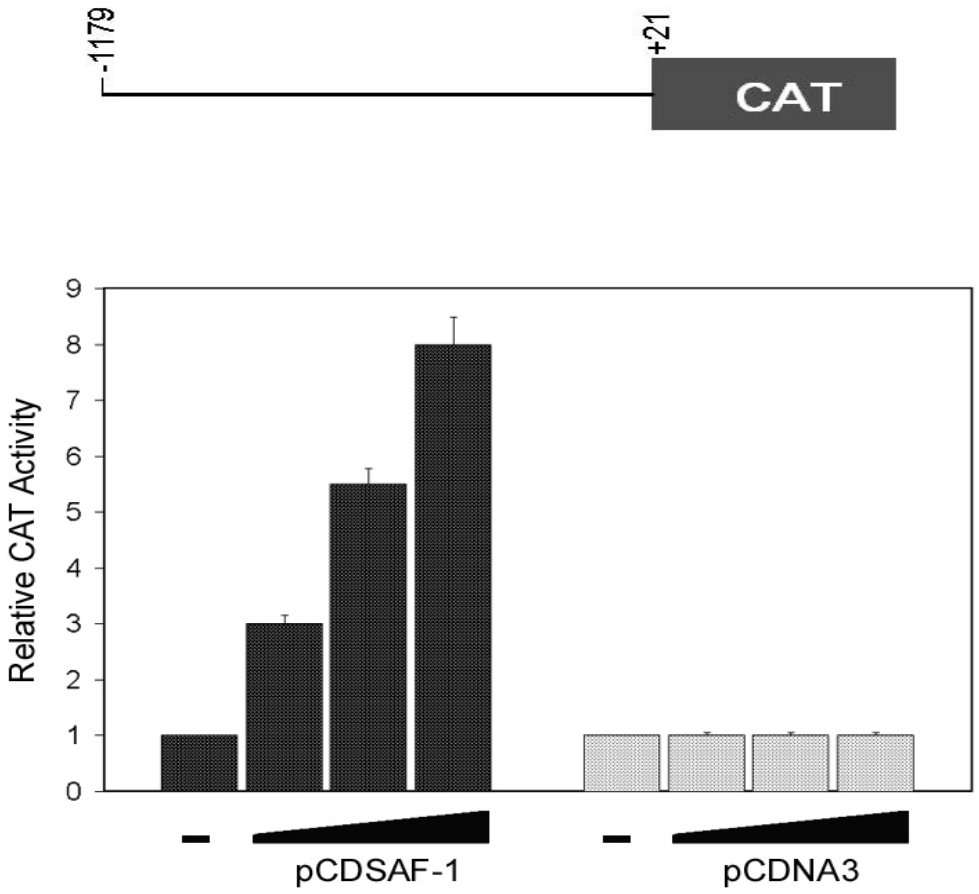


Figure 18. SAF-1 expression increases VEGF promoter activity. Chondrocyte cells were transfected with the 1.2 VEGF CAT3 plasmid. In addition, some of the cells were co-transfected with increasing concentration of pCDSAF-1 or pCDNA3 (0.5 ug, 1.0 ug and 1.5 ug), as indicated. The cells were then grown for 24 hours and CAT activity was determined. Increase in CAT activity in the co-transfected cells relative to that of only reporter-transfected cells was calculated and plotted.

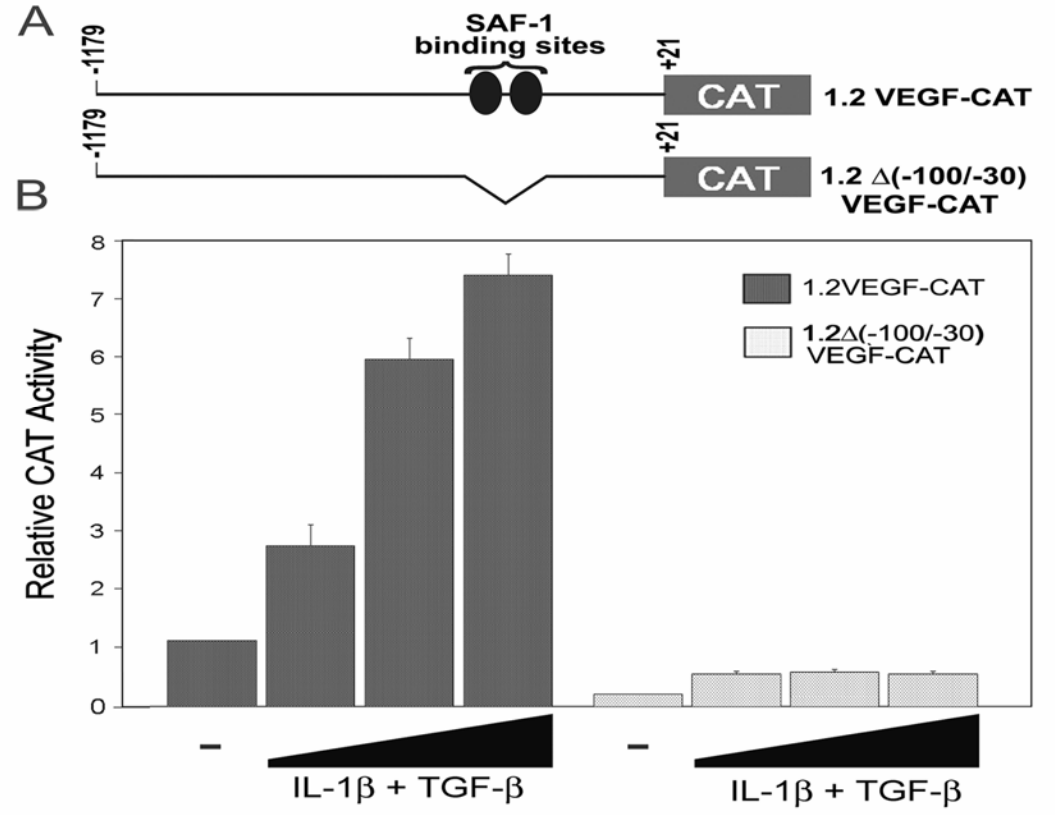


Figure 19. Effect of deletion of SAF-1 binding sites of VEGF promoter. Chondrocyte cells were transfected with either 1.2 VEGFCAT3 or 1.2 mutated (-100 to -30) VEGFCAT3 where DNA sequences from nucleotide position -100 to -30, containing the SAF-1 binding sites, was deleted. Chondrocyte cell-specific endogenous SAF-1 activity was induced by incubating the cells with increasing concentration of IL-1β plus TGF-β, (100U+2ng)/ml, (200U+4ng)/ml and (400u+4ug)/ml, respectively. Cells were harvested following 24h incubation with the cytokines and CAT activity was determined. Results are an average of three independent experiments.

2. Characterization of SAF-1 binding site of VEGF promoter in chondrocyte

EMSA for DNA-binding activity to VEGF promoter was performed. Joint cartilage tissues were obtained from normal human cadavers and OA patients and nuclear extracts were prepared by grinding the tissues under liquid nitrogen in a mortar pestle, and resuspending the grinded tissue in buffer A (10mMHEPES, pH 7.9, 1.5 mM MgCl2, 10mMKCl, 0.5 mMDTT, 0.5 mM PMSF, 0.5 µg/ml each of leupeptin, pepstain and antipain, 0.1 µg/ml chymotrypsin, 0.3 TIU/ml aprotinin and 0.5 mg/ml of benzamidine). The mixture was mixed well and centrifuged at 3000 rpm for 3 minutes. The supernatant was processed to prepare nuclear extract and protein was estimated as described in Chapter 3. EMSA was performed with equal amount of proteins. Radiolabeled VEGF DNA from -108 to -25 which contains a SAF-binding element of the VEGF promoter was prepared by using [α-^{32}P] dCTP to label the double-stranded DNA probe with Klenow fragment of DNA polymerase. Some DNA-binding assay mixture contained 100-fold molar excess (100 pmol) of competitor oligoncleotides for AP-1, AP-2, NF-κB, Sp1 and SAF. AP-1, AP-2, NF-κB and Sp1 consensus oligonucleotides were obtained from promega Corporation, Madison, Wisconsin. SAF consensus oligonucleotide contained the sequence 5'-CCCTTCCTCTCCACCCACAGCCCCCATGG-3'. For antibody interaction studies, anti-SAF-1 antibody or IgG control was added to the reaction mixtures during a pre-incubation period of 30 minutes on ice. The DNA-protein complexes were separated in a non-denaturing 6% polyacrylamide gel, and exposed by autoradiography.

To detect if the chondrocytes in OA cartilage contain VEGF promoter binding proteins, EMSA was performed using nuclear extracts of human cartilage chondrocytes and radiolabeled DNA containing SAF-1 binding site of VEGF promoter. Four DNA-protein complexes were detected in the nuclear extracts of OA patients (Figure 20). This result suggests that high level of DNA-binding proteins is present in OA cartilages.

To further characterize these DNA-protein complexes, a detailed analysis was performed using nuclear extracts of human cartilage chondrocyte of OA patient with several competitive oligonucleotides, such as AP-1, AP-2, NF-κB, Sp1, and SAF. Also anti-SAF-1 antibody and normal IgG were used (Figure 21). In lane 6, when SAF-1 competitor oligo was added, complexes I, II and IV were significantly decreased. In lane 8, when SAF-1 antibody was added, Complexes I, II and IV were also decreased. These data indicate that three SAF-1 binding complexes I, II and IV are present in OA cartilage. Complex III is not affected by anti-SAF-1 antibody and thus it is generated by the action an un-related protein. Identify of this protein is still unknown. Multiple complexes of SAF-1 could be due to the formation of heterodimers of SAF-1 and other member of SAF family, such as SAF-2 and SAF-3.

To test whether inflammatory cytokines stimulate SAF-1 binding, EMSA was performed using nuclear extracts from chondrocytes treated with cytokine (Figure 22). The result shows that the intensity of SAF-1 binding to VEGF promoter was increased with addition of TNF-α and IL1-β.

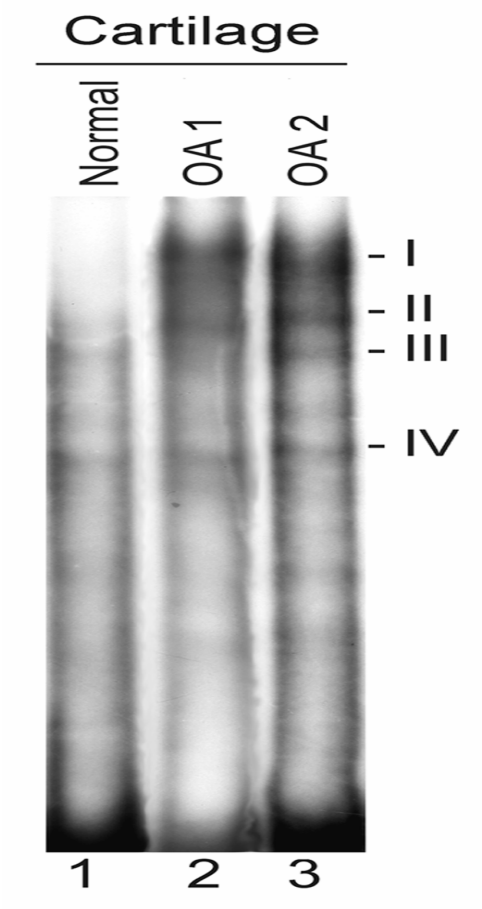


Figure 20. EMSA was performed using nuclear extracts of human cartilage chondrocytes, and radio labeled DNA containing SAF-1 binding sites in VEGF promoter. Four DNA- protein complexes (designated I, II, III, and IV) were detected in the nuclear extracts of OA cartilage in lane 2 and 3. Lane is the normal control. This result suggests that high level of DNA-binding proteins is present in OA cartilage.

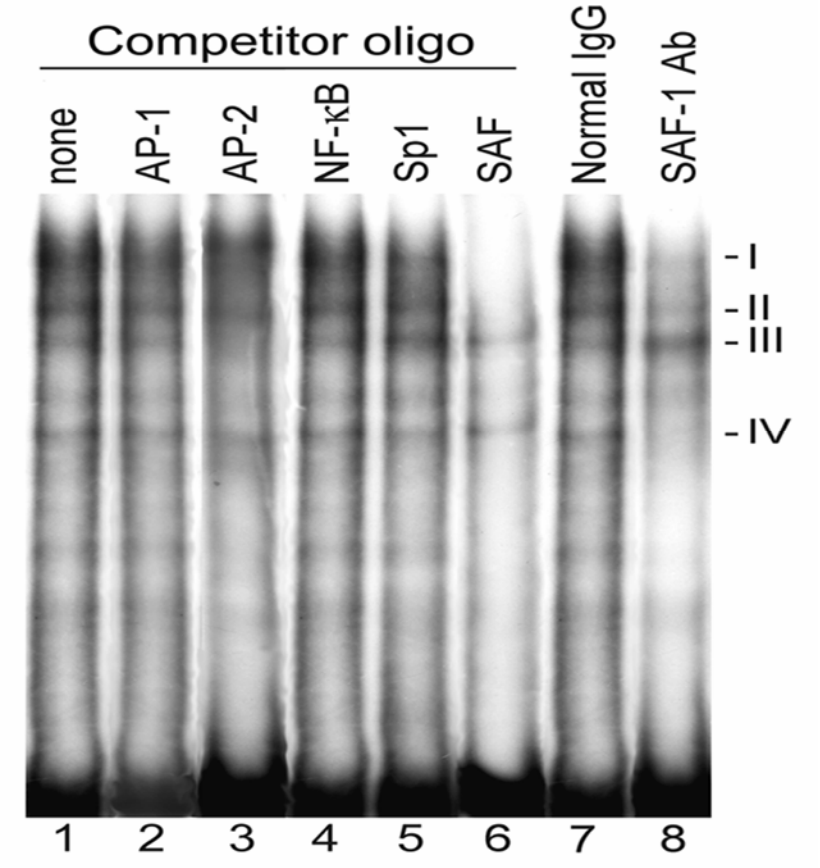


Figure 21. EMSA was performed using nuclear extracts of human cartilage chondrocyte of OA with several different competitor oligonucleotides: AP-1, AP-2, NF-κB, Sp1, SAF, and antibodies: normal IgG and SAF-1 antibody. Four DNA-protein complexes are designated I, II, III, and IV.

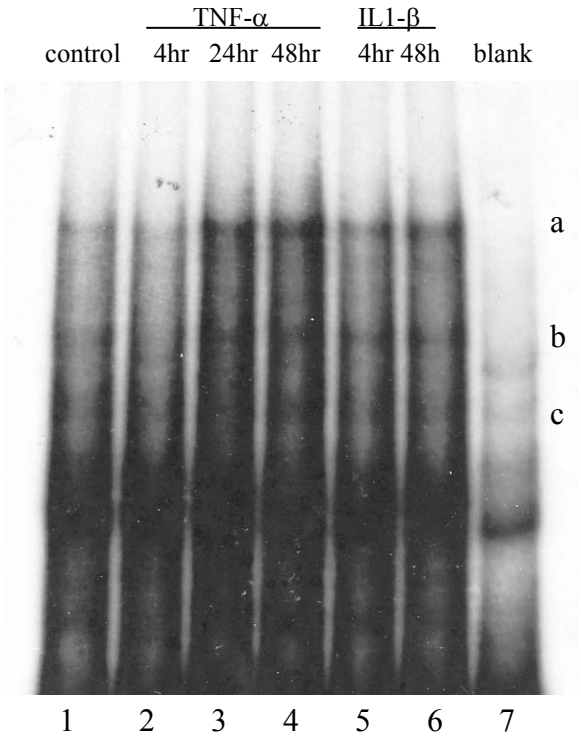


Figure 22. EMSA was performed in chondrocyte with cytokine stimulation. α^{32} P-labeled oligonucleotide containing a VEGF promoter sequence from -26 to -131 was used as a probe with articular Chondrocyte nuclear extracts. In lane 1, nuclear extract of untreated chondrocyte cells were used as control. Lane 2, 3, and 4 contain nuclear extracts of chondrocyte cells treated with TNF-α (4ng) and then incubated 4 hours, 24 hours, and 48 hour at 37°C, respectively. Lane 5, and 6 contain nuclear extracts of chondrocyte cells treated with IL1-β (400U) and incubated for 4 hours, and 48 hours, respectively. Lane 7 contains no nuclear extract as a blank control. Different DNA-protein complexes designated a, b, and c were indicated.

3. **Characterization of SAF-1 binding activity in synovial joint**

Joint fluids are derived from the synovium and inflammatory cells. To study how VEGF expression can be facilitated in this environment, EMSA was performed to with joint fluid from several OA patients. In some of the samples, higher level of DNA-protein complexes were noted (Figure 23).

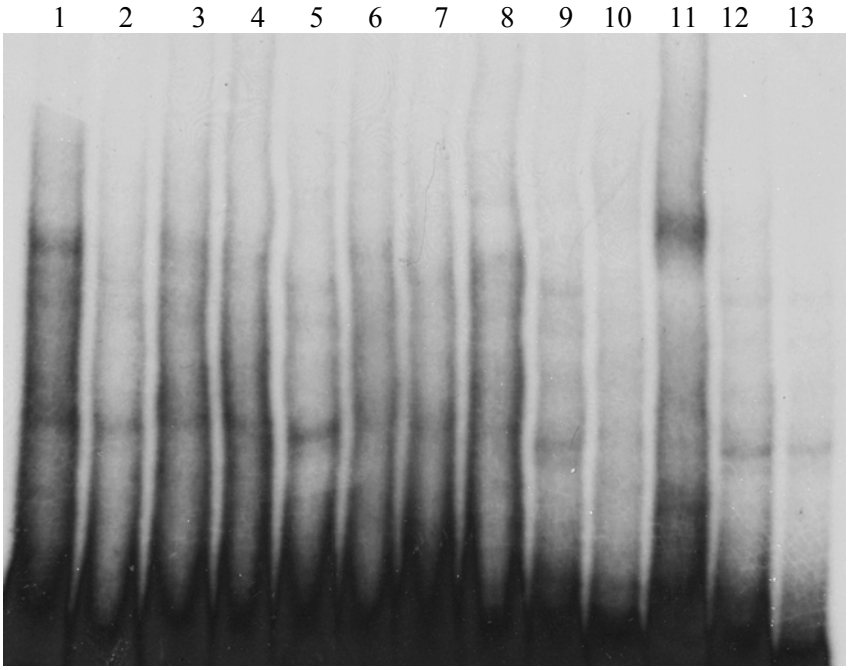


Figure 23. EMSA was performed with joint fluids from several OA patients. In some of the samples, higher lever DNA-protein complexes were noted, such as, sample 8 and 11.

To further characterize these DNA-protein complexes, EMSA was performed with joint fluid of OA patient in presence of competitor oligonucleotides or antibodies (Figure 24). Two different concentration of competitor oligo were included. Three DNA-protein complexes designated A, B and C were indicated. In lane 8, the level of complex A was clearly decreased due to addition of SAF-1 antibody. This result indicates that only complex A corresponds to SAF-1 binding. Interestingly, in lane 3, the level of complex A was increased due to the presence of increasing dose of NF-κB competitor oligo. This result suggests that NF-κB may interrupt SAF-1 binding to VEGF promoter.

These data were further analyzed by quantitative density scanning of the complexes A. In figure 25, it shows the % change in DNA-protein binding activity. The density of complex A was obviously increased by adding NF-κB oligo, and decreased by adding SAF-1 antibody. These data indicate that NF-κB plays a significant role in SAF-1 mediated VEGF expression.

This phenomenon was further investigated to assess the role of NF-κB in VEGF promoter function. When 1.2 VEGF-CAT reporter plasmid was co-transfected with SAF-1 and NF-κB, the CAT activity was clearly decreased compared with that of SAF-1 transfected cells (Figure 26). This result is consistent with the result of EMSA shown in Figure 24. This finding suggests that inducible effect of SAF-1 for VEGF expression is negatively regulated by NF-κB.

Inhibition of SAF-1 indicated that induction of CAT activity due to the ectopic expression of p65 NF-κB suggests that NF-κB likely sequesters SAF-1 and thus makes it unavailable to bind to the VEGF promoter. This is a unique regulatory mechanism.

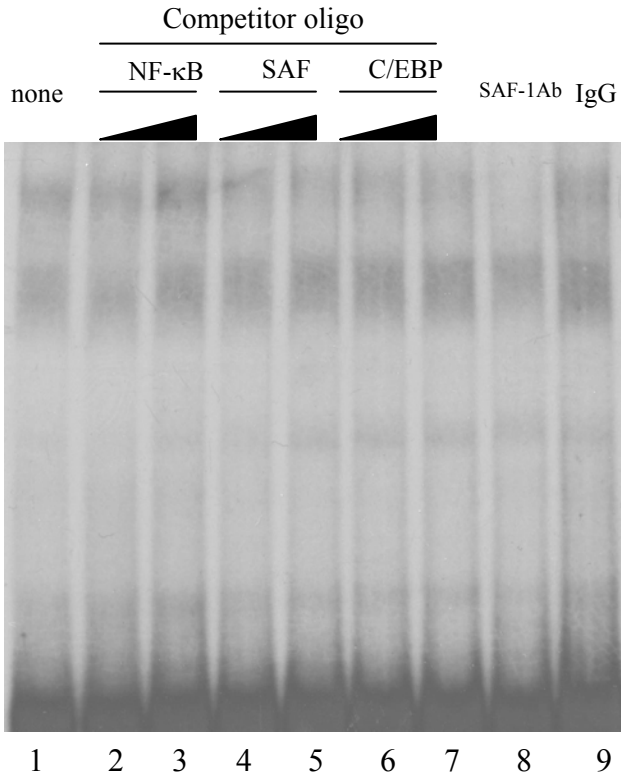


Figure 24. EMSA was performed with joint fluid of OA patient in presence of competitor oligonucleotides or antibodies. Two different concentration of competitor oligo were included(NF-κB: 50ng, 100ng. SAF: 50ng, 100ng. C/EBP: 50ng and 100ng). In lane 3, the level of complex A increased due to increasing dose of NF-κB competitor oligo. In lane 8, the level of complex A was clearly decreased due to the addition of SAF-1 antibody.

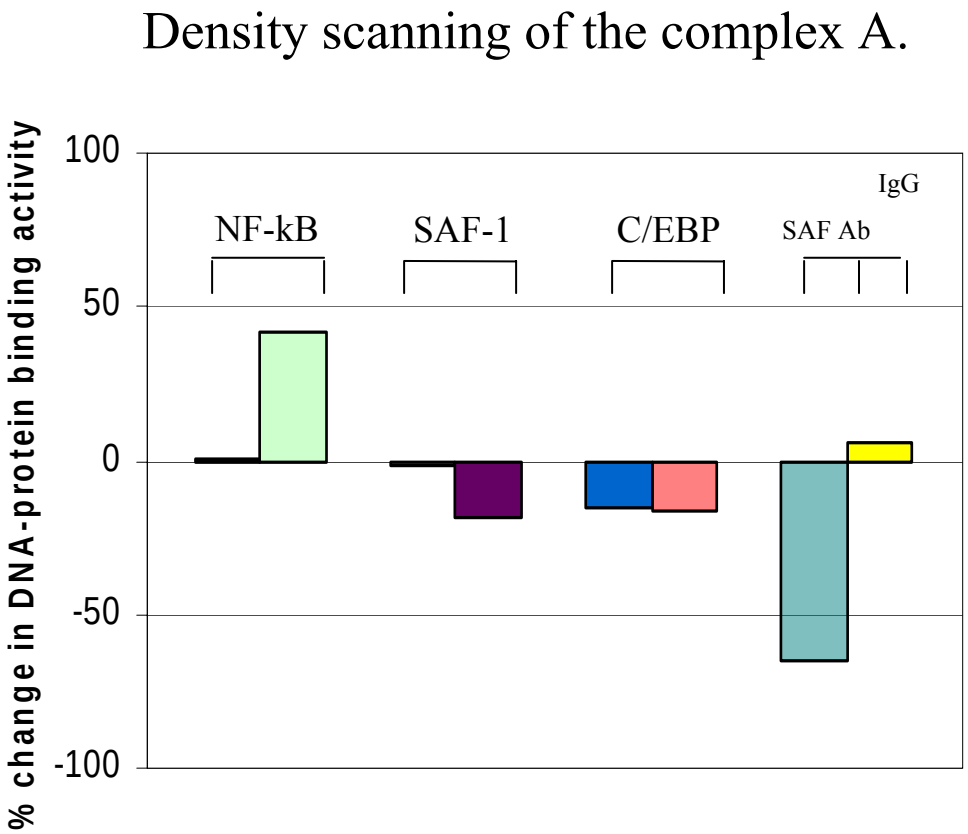


Figure 25. Quantitative analysis of SAF-1 binding to VEGF promoter. The density of complex A from figure 24 were analysis by quantitative density scanning. The data was plotted as % change in the density of DNA-protein complex with addition of oligonucleotide and antibodies.

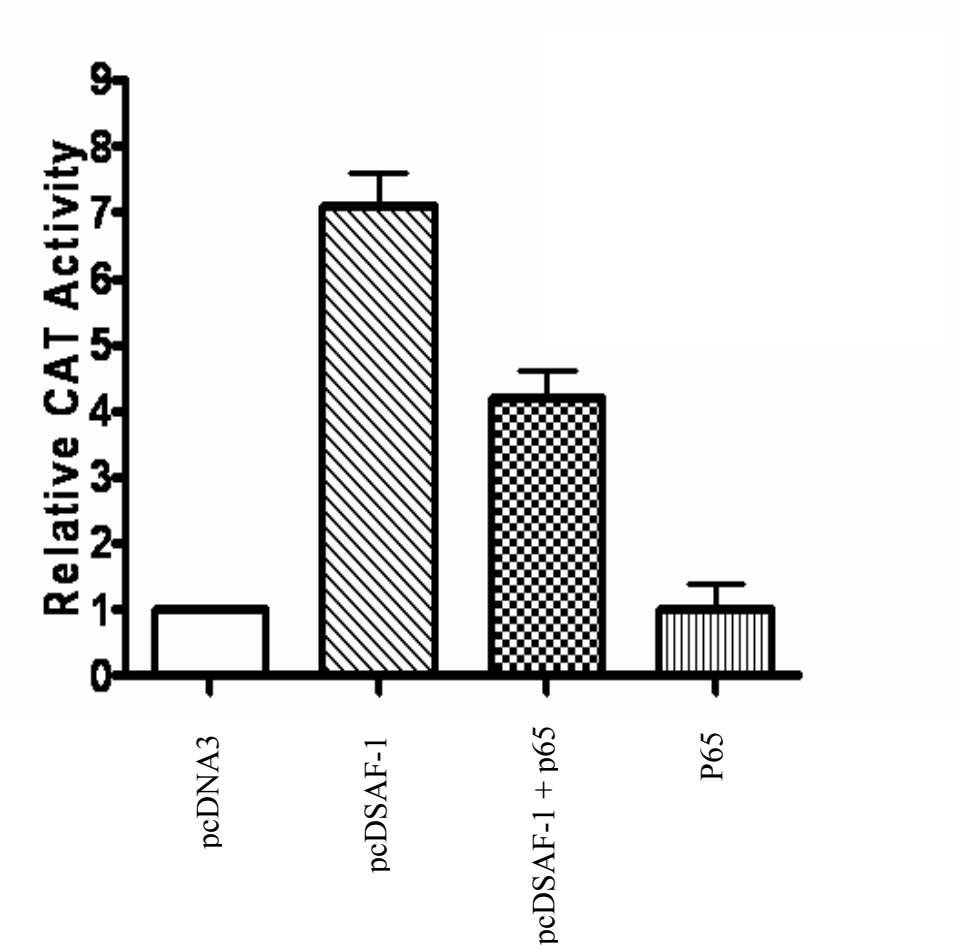


Figure 26. CAT assay was performed to assess the role of NF-κB in VEGF promoter inducing function of SAF-1. 1.2 VEGF-CAT reporter plasmid was co-transfected with SAF-1 alone or with SAF-1 and NF-κB. When 1.2 VEGF-CAT reporter plasmid was co-transfected with SAF-1 and NF-κB, the CAT activity was significantly decreased compared with that of SAF-1 transfected cells.

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Chapter 5

DISCUSSION

This study identified a novel *cis*-acting promoter element, SAF-1 regulating VEGF expression in OA, and the SAF-1 binding sites in the VEGF promoter region were also characterized. This study showed the increased levels of SAF-1 DNA binding protein in the chondrocytes of OA compared to that of normal cartilage, and SAF-1 increases VEGF promoter activity in a dose dependent manner. The intensity of SAF-1 binding to VEGF promoter is also increased in the chondrocytes stimulated by IL-1 and TNF-α. Further it also provided the evidence that NF-κB interrupts SAF-1 binding to VEGF promoter. Consistent with this, the VEGF promoter linked CAT activity induced by SAF-1 was reduced in chondrocytes transfected with NF-κB plasmid. These findings suggest that a novel mechanism regulates VEGF expression in OA, and SAF-1 plays an important role of VEGF expression induced by inflammatory cytokines in the pathogenesis of OA.

Many inflammatory agents including IL-1 and IL-6 alone or in combination are known to increase the DNA binding activity and transcriptional potent of SAF-1(Ray et al. 1999, 2000). Phosphorylation of SAF-1 by the protein kinase is critical for its action. Several protein kinase pathways are involved in the phosphorylation of SAF-1, suggesting that SAF-1 may respond to different inflammatory stimulators (Ray et al 2000; Ray et al 2001; Ray et al 2002). Various inflammatory cells are recruited and produced cytokine at the site of lesion under chronic inflammatory conditions. Angiogenesis and inflammation are important processes in the path physiology of OA. The cartilage of OA exhibits reduced resistance to vascular invasion. VEGF is expressed

in chondrocytes of cartilage in joints affected with OA. Specific mechanism controlling induction of VEGF expression in OA is not clear. The systemic analysis of serially truncated and specifically mutated reporter constructs identified the requirement of SAF-1 binding to VEGF promoter for increasing reporter gene expression induced by inflammatory cytokines, IL-1β, and TGF-β. The SAF-1 DNA binding sites were identified in the VEGF promoter region at -109 to -97, -94 to -65, and -48 to -37. In order to determine if the SAF-1 binding sites are required for inflammatory cytokines induced VEGF expression, chondrocytes were transfected with VEGF promoter construct deleted SAF-1 binding sequence around nucleotide position -100 to -30 in VEGF promoters, and stimulated with IL-1 plus TGF-β. The results showed that mutation of SAF-1 binding sites in the VEGF promoter region completely abolish CAT activity induced by inflammatory cytokines. This result suggests that SAF-1 binding sites are necessary for VEGF expression induced by inflammatory cytokines.

Previous studies showed that SAF-1 and AP-1 synergistically regulated matrix metalloproteinase-9 (MMP9) production (Ray et al. 2005). Increased expression of MMP9 is a key factor in the degradation of cartilage during arthritis. In addition, SAF-1 is also involved in the regulation of MMP1 gene expression and is highly abundant in the articular cartilage chondrocytes of OA patients (Ray et al. 2003). The current study increases our understanding of the mechanism by which SAF-1 involves in the pathogenesis of rheumatoid arthritis (RA) and OA. IL-1 and TNF-α are master cytokines mediated pathology of arthritis (Aggarwal 2003). Anti-TNF-α treatment ameliorates the severity of arthritis (Marinova-Mutafchieva 2002). The current study showed both IL-1 and TNF-α increased the intensity of SAF-1 binding to VEGF promoter, suggesting that

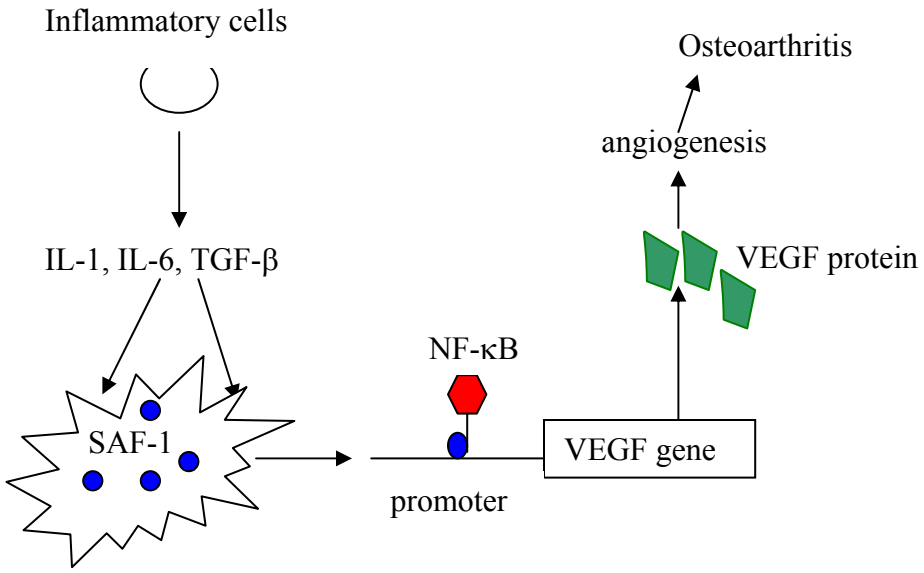
SAF-1 is involved in promoting VEGF expression in chondrocytes induced by IL-1 and TNF-α.

Interestingly, the current study showed that NF-κB from joint fluid interrupts SAF-1 binding to the VEGF promoter. Consistent with this finding, VEGF promoter linked reporter gene expression by SAF-1 decreases in chondrocytes when the cells express NF-κB. Thus NF-κB negatively regulates VEGF expression induced by SAF-1. The mechanism by which this inhibition occurs is unknown. It is possible that NF-κB may directly bind to SAF-1 protein to form complex, inhibiting SAF-1 binding to VEGF promoter. Another possibility is that NF-κB biding site and SAF-1 binding site are so close that NF-κB binding to VEGF promoter blocks SAF-1 physical binding to VEGF promoter due to a steric hindrance.

This study also found that three SAF-1 binding complexes are present in the chondrocyte of OA cartilage. However, only one SAF-1 binding complex is present in the joint fluids of OA patients. Multiple complexes of SAF-1 in chondrocytes of OA cartilage could be due to the formation of heterodimers of SAF-1 or the isoforms of SAF. But in joint fluids of OA patients, only one SAF-1 binding complex is detectable. This could be caused by the degradation of some of the minor SAF isoforms since various proteases exist in the joint fluids.

Since activation of inflammatory cytokines and other inflammatory episodes is recognized features associated with OA, it is conceivable that SAF-1 might play a critical role in the abnormal expression of VEGF and in the pathogenesis of OA. The current study suggested that SAF-1 could be a potential therapeutic target to control the over-

expression of VEGF associate with the pathogenesis of OA. The following model depicts some of the events revealed by the current study.



Inflammatory cells secret cytokines, such as IL-1, IL-6, and TGF-β, which bind to chondrocytes to induce SAF-1 production. SAF-1 transcriptional factors directly bind to VEGF promoter and induce VEGF expression. Subsequently, VEGF promote angiogenesis and Osteoarthritis. NF-κB negatively regulated VEGF expression induced by SAF-1.

APPENDIX

Reagent and Solution

10X PBS, pH 7.4

Sodium chloride	80 g
Potassium chloride	2 g
Phosphate, disodium	14.4 g
Phosphate, monopotassium	2.4 g
Distilled water upto	1000 ml

Autoclave

3M Potassium acetate, pH 4.8

Glacial acetic acid	28 ml
5M Potassium acetate	60 ml
Distilled water	12 ml

Autoclave

1:1 Phenol/Chloroform

Phenol, water saturated	1 volume
Chloroform/isoamylalcohol (24:1)	1 volume

2XYT medium

Triptic peptone	16 g
Yeast extract	10 g
Sodium chloride	5 g
Distilled water upto	1000 g

Autoclave

10X TBE, pH 8.4

Tris	50.4g
Boric acid	27.5g
EDTA disodium	3.72g
Distilled water up to	500ml

Refferences:

Aggarwal B. (2003). Signaling pathways of TNF superfamily: a double-edged sword. Nature Review Immunology. **3**:745-756.

Alon, T., I. Hemo, et al. (1995). "Vascular endothelial growth factor acts as a survival factor for newly formed retinal vessels and has implications for retinopathy of prematurity." Nature Medicine **1**(10): 1024-8.

Ben-Av, P., L. J. Crofford, et al. (1995). "Induction of vascular endothelial growth factor expression in synovial fibroblasts by prostaglandin E and interleukin-1: a potential mechanism for inflammatory angiogenesis." FEBS Letters **372**(1): 83-7.

Carano, R. A. and E. H. Filvaroff (2003). "Angiogenesis and bone repair." Drug Discovery Today **8**(21): 980-9.

Carlevaro, M. F., S. Cermelli, et al. (2000). "Vascular endothelial growth factor (VEGF) in cartilage neovascularization and chondrocyte differentiation: auto-paracrine role during endochondral bone formation." Journal of Cell Science **113**(Pt 1): 59-69.

Clauss, M., M. Gerlach, et al. (1990). "Vascular permeability factor: a tumor-derived polypeptide that induces endothelial cell and monocyte procoagulant activity, and promotes monocyte migration." Journal of Experimental Medicine **172**(6): 1535-45.

Cohen, T., D. Nahari, et al. (1996). "Interleukin 6 induces the expression of vascular endothelial growth factor." Journal of Biological Chemistry **271**(2): 736-41.

Connolly, D. T., D. M. Heuvelman, et al. (1989). "Tumor vascular permeability factor stimulates endothelial cell growth and angiogenesis." Journal of Clinical Investigation **84**(5): 1470-8.

de Vries, C., J. A. Escobedo, et al. (1992). "The fms-like tyrosine kinase, a receptor for vascular endothelial growth factor." Science **255**(5047): 989-91.

Deckers, M. M., R. L. van Bezooijen, et al. (2002). "Bone morphogenetic proteins stimulate angiogenesis through osteoblast-derived vascular endothelial growth factor A." Endocrinology **143**(4): 1545-53.

Dor, Y., R. Porat, et al. (2001). "Vascular endothelial growth factor and vascular adjustments to perturbations in oxygen homeostasis." American Journal of Physiology - Cell Physiology **280**(6): C1367-74.

Dvorak, H. F. (2006). "Discovery of vascular permeability factor (VPF)." Experimental Cell Research **312**(5): 522-6.

Dvorak, H. F., L. F. Brown, et al. (1995). "Vascular permeability factor/vascular endothelial growth factor, microvascular hyperpermeability, and angiogenesis." American Journal of Pathology **146**(5): 1029-39.

Ehrmann, R. L. and M. Knoth (1968). "Choriocarcinoma. Transfilter stimulation of vasoproliferation in the hamster cheek pouch. Studied by light and electron microscopy." Journal of the National Cancer Institute **41**(6): 1329-41.

Engsig, M. T., Q. J. Chen, et al. (2000). "Matrix metalloproteinase 9 and vascular endothelial growth factor are essential for osteoclast recruitment into developing long bones.[erratum appears in J Cell Biol 2001 Jan 22;152(2):following 417]." Journal of Cell Biology **151**(4): 879-89.

Ferrara, N., H. Chen, et al. (1998). "Vascular endothelial growth factor is essential for corpus luteum angiogenesis." Nature Medicine **4**(3): 336-40.

Ferrara, N. and T. Davis-Smyth (1997). "The biology of vascular endothelial growth factor." Endocrine Reviews **18**(1): 4-25.

Ferrara, N. and W. J. Henzel (1989). "Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells." Biochemical & Biophysical Research Communications **161**(2): 851-8.

Ferrara, N., K. A. Houck, et al. (1991). "The vascular endothelial growth factor family of polypeptides." Journal of Cellular Biochemistry **47**(3): 211-8.

Folkman, J. and M. Klagsbrun (1987). "Angiogenic factors." Science **235**(4787): 442-7.

Folkman, J., S. Szabo, et al. (1991). "Duodenal ulcer. Discovery of a new mechanism and development of angiogenic therapy that accelerates healing." Annals of Surgery **214**(4): 414-25; discussion 426-7.

Frank, S., G. Hubner, et al. (1995). "Regulation of vascular endothelial growth factor expression in cultured keratinocytes. Implications for normal and impaired wound healing." Journal of Biological Chemistry **270**(21): 12607-13.

Fujio, Y. and K. Walsh (1999). "Akt mediates cytoprotection of endothelial cells by vascular endothelial growth factor in an anchorage-dependent manner." Journal of Biological Chemistry **274**(23): 16349-54.

Gabrilovich, D. I., H. L. Chen, et al. (1996). "Production of vascular endothelial growth factor by human tumors inhibits the functional maturation of dendritic cells.[erratum appears in Nat Med 1996 Nov;2(11):1267]." Nature Medicine **2**(10): 1096-103.

Gerber, H. P., V. Dixit, et al. (1998). "Vascular endothelial growth factor induces expression of the antiapoptotic proteins Bcl-2 and A1 in vascular endothelial cells." Journal of Biological Chemistry **273**(21): 13313-6.

Gerber, H. P., K. J. Hillan, et al. (1999). "VEGF is required for growth and survival in neonatal mice." Development **126**(6): 1149-59.

Gerber, H. P., A. K. Malik, et al. (2002). "VEGF regulates haematopoietic stem cell survival by an internal autocrine loop mechanism." Nature **417**(6892): 954-8.

Gerber, H. P., A. McMurtrey, et al. (1998). "Vascular endothelial growth factor regulates endothelial cell survival through the phosphatidylinositol 3'-kinase/Akt signal transduction pathway. Requirement for Flk-1/KDR activation." Journal of Biological Chemistry **273**(46): 30336-43.

Gerber, H. P., T. H. Vu, et al. (1999). "VEGF couples hypertrophic cartilage remodeling, ossification and angiogenesis during endochondral bone formation.[see comment]." Nature Medicine **5**(6): 623-8.

Greenblatt, M. and P. Shubi (1968). "Tumor angiogenesis: transfilter diffusion studies in the hamster by the transparent chamber technique." Journal of the National Cancer Institute **41**(1): 111-24.

Grugel, S., G. Finkenzeller, et al. (1995). "Both v-Ha-Ras and v-Raf stimulate expression of the vascular endothelial growth factor in NIH 3T3 cells." Journal of Biological Chemistry **270**(43): 25915-9.

Guerrin, M., H. Moukadiri, et al. (1995). "Vasculotropin/vascular endothelial growth factor is an autocrine growth factor for human retinal pigment epithelial cells cultured in vitro." Journal of Cellular Physiology **164**(2): 385-94.

Harper, J. and M. Kalgsbrun (1999). "Cartilage to bone--angiogenesis leads the way.[comment]." Nature Medicine **5**(6): 617-8.

Hattori, K., S. Dias, et al. (2001). "Vascular endothelial growth factor and angiopoietin-1 stimulate postnatal hematopoiesis by recruitment of vasculogenic and hematopoietic stem cells." Journal of Experimental Medicine **193**(9): 1005-14.

Haywood L. et al. (2003). Inflammation and angiogenesis in osteoarthritis. Arthritis Rheumatism. **48**(8):2713-2177.

Hirota, K., M. Matsui, et al. (1997). "AP-1 transcriptional activity is regulated by a direct association between thioredoxin and Ref-1." Proceedings of the National Academy of Sciences of the United States of America **94**(8): 3633-8.

Houck, K. A., N. Ferrara, et al. (1991). "The vascular endothelial growth factor family: identification of a fourth molecular species and characterization of alternative splicing of RNA." Molecular Endocrinology **5**(12): 1806-14.

Houck, K. A., D. W. Leung, et al. (1992). "Dual regulation of vascular endothelial growth factor bioavailability by genetic and proteolytic mechanisms." Journal of Biological Chemistry **267**(36): 26031-7.

Ide AG, Baker NH, et al. (1939). "Vascularization of the Brown Pearce rabbit epithelioma transplant as seen in the transparent ear chamber." Am J Roentgenol **42**: 891-899.

Jakeman, L. B., M. Armanini, et al. (1993). "Developmental expression of binding sites and messenger ribonucleic acid for vascular endothelial growth factor suggests a role for this protein in vasculogenesis and angiogenesis." Endocrinology **133**(2): 848-59.

Jakeman, L. B., J. Winer, et al. (1992). "Binding sites for vascular endothelial growth factor are localized on endothelial cells in adult rat tissues." Journal of Clinical Investigation **89**(1): 244-53.

Josko, J. and M. Mazurek (2004). "Transcription factors having impact on vascular endothelial growth factor (VEGF) gene expression in angiogenesis." Medical Science Monitor **10**(4): RA89-98.

Keck, P. J., S. D. Hauser, et al. (1989). "Vascular permeability factor, an endothelial cell mitogen related to PDGF." Science **246**(4935): 1309-12.

Keyt, B. A., H. V. Nguyen, et al. (1996). "Identification of vascular endothelial growth factor determinants for binding KDR and FLT-1 receptors. Generation of receptor-selective VEGF variants by site-directed mutagenesis." Journal of Biological Chemistry **271**(10): 5638-46.

Kim, I., S. O. Moon, et al. (2001). "Vascular endothelial growth factor expression of intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), and E-selectin through nuclear factor-kappa B activation in endothelial cells." Journal of Biological Chemistry **276**(10): 7614-20.

Kimura, H., A. Weisz, et al. (2001). "Identification of hypoxia-inducible factor 1 ancillary sequence and its function in vascular endothelial growth factor gene induction by hypoxia and nitric oxide." Journal of Biological Chemistry **276**(3): 2292-8.

Leung, D. W., G. Cachianes, et al. (1989). "Vascular endothelial growth factor is a secreted angiogenic mitogen." Science **246**(4935): 1306-9.

Liu, Y., S. R. Cox, et al. (1995). "Hypoxia regulates vascular endothelial growth factor gene expression in endothelial cells. Identification of a 5' enhancer." Circulation Research **77**(3): 638-43.

Maglione, D., V. Guerriero, et al. (1991). "Isolation of a human placenta cDNA coding for a protein related to the vascular permeability factor." Proceedings of the National Academy of Sciences of the United States of America **88**(20): 9267-71.

Maglione, D., V. Guerriero, et al. (1993). "Two alternative mRNAs coding for the angiogenic factor, placenta growth factor (PlGF), are transcribed from a single gene of chromosome 14." Oncogene **8**(4): 925-31.

Marinova-Mutafchieva L. R. Williams et al. (2002) " Inflammation is preceded by tumor necrosis factor-dependent infiltration of mesnechymal cells in experimental arthritis." Arthritis Rheumatism. **46**:507-513.

Maruotti, N., F. P. Cantatore, et al. (2006). "Angiogenesis in rheumatoid arthritis." Histology & Histopathology **21**(5): 557-66.

Matthews, W., C. T. Jordan, et al. (1991). "A receptor tyrosine kinase cDNA isolated from a population of enriched primitive hematopoietic cells and exhibiting close genetic linkage to c-kit." Proceedings of the National Academy of Sciences of the United States of America **88**(20): 9026-30.

Mayr-Wohlfart, U., J. Waltenberger, et al. (2002). "Vascular endothelial growth factor stimulates chemotactic migration of primary human osteoblasts." Bone **30**(3): 472-7.

Melder, R. J., G. C. Koenig, et al. (1996). "During angiogenesis, vascular endothelial growth factor and basic fibroblast growth factor regulate natural killer cell adhesion to tumor endothelium.[see comment]." Nature Medicine **2**(9): 992-7.

Millauer, B., S. Wizigmann-Voos, et al. (1993). "High affinity VEGF binding and developmental expression suggest Flk-1 as a major regulator of vasculogenesis and angiogenesis." Cell **72**(6): 835-46.

Mueller, M. D., J. L. Vigne, et al. (2000). "Regulation of vascular endothelial growth factor (VEGF) gene transcription by estrogen receptors alpha and beta." Proceedings of the National Academy of Sciences of the United States of America **97**(20): 10972-7.

Muller, J. M., R. A. Rupec, et al. (1997). "Study of gene regulation by NF-kappa B and AP-1 in response to reactive oxygen intermediates." Methods (Duluth) **11**(3): 301-12.

Oberg-Welsh, C., S. Sandler, et al. (1997). "Effects of vascular endothelial growth factor on pancreatic duct cell replication and the insulin production of fetal islet-like cell clusters in vitro." Molecular & Cellular Endocrinology **126**(2): 125-32.

Okada, F., J. W. Rak, et al. (1998). "Impact of oncogenes in tumor angiogenesis: mutant K-ras up-regulation of vascular endothelial growth factor/vascular permeability factor is necessary, but not sufficient for tumorigenicity of human colorectal carcinoma cells." Proceedings of the National Academy of Sciences of the United States of America **95**(7): 3609-14.

Ostendorf, T., U. Kunter, et al. (1999). "VEGF(165) mediates glomerular endothelial repair." Journal of Clinical Investigation **104**(7): 913-23.

Pepper, M. S., N. Ferrara, et al. (1991). "Vascular endothelial growth factor (VEGF) induces plasminogen activators and plasminogen activator inhibitor-1 in microvascular endothelial cells." Biochemical & Biophysical Research Communications **181**(2): 902-6.

Pepper, M. S., N. Ferrara, et al. (1992). "Potent synergism between vascular endothelial growth factor and basic fibroblast growth factor in the induction of angiogenesis in vitro." Biochemical & Biophysical Research Communications **189**(2): 824-31.

Pepper, M. S., S. Wasi, et al. (1994). "In vitro angiogenic and proteolytic properties of bovine lymphatic endothelial cells." Experimental Cell Research **210**(2): 298-305.

Pertovaara, L., A. Kaipainen, et al. (1994). "Vascular endothelial growth factor is induced in response to transforming growth factor-beta in fibroblastic and epithelial cells." Journal of Biological Chemistry **269**(9): 6271-4.

Plouet, J. and H. Moukadiri (1990). "Characterization of the receptor to vasculotropin on bovine adrenal cortex-derived capillary endothelial cells." Journal of Biological Chemistry **265**(36): 22071-4.

Poltorak, Z., T. Cohen, et al. (1997). "VEGF145, a secreted vascular endothelial growth factor isoform that binds to extracellular matrix." Journal of Biological Chemistry **272**(11): 7151-8.

Ray A and B. K. Ray. (1996). A novel cis-acting element is essentail for cytokine-mediated transcriptional induction of the serum amyloid A gene in nonhepatic cells. **16**(4):1584-1594.

Ray B. K. and A. Ray. (1997). Involvement of an SAF-like transcription factor in the activation of serum amyloid A gene in monocyte/macrophage cells by lipopolysaccharide. Biochemistry. 36(15):4662-4668.

Ray A. and B. K. Ray. (1998). Isolation and functional characterization of cDNA of serum amyloid A-activating factor that binds to the serum amyloid A promoter. Mol. Cell. Biol. **18**(12):7323-7335

Ray A. H. Schatten, and BK Ray. (1999). "activation of Sp1 and its functional co-operation with serum amyloid A-activating sequence binding factor in synoviocyte cells trigger synergistic action of interliukin-1 and interliukin-6 in serum amyloid A gene expression." Journal of Biological Chemistry **274**(7): 4300-4308.

Ray A., A. P. Fileds, and B. K. Ray. (2000). "Activation of transcription factor SAF involves its phosphorylation by protein kinase C. " Journal of Biological Chemistry **275**(50): 39727-39733.

Ray B. K., J. Chen, and A. Ray. (2001). Catalytic subunit of protein kinase A is an interacting partner of the inflammation-responsive transcription factor serum amyloid A-activating factor-1. Journal Immunology. **167**(4):2343-23248.

Ray A. Kumar D. and B. K. Ray. (2002). Promoter-binding activity of inflammation-responsive transcription factor SAF is regulated by cyclic AMP signaling pathway. DNA Cell. Biol. **21**(1):31-40.

Ray A. G. Y. Yu, B. K. Ray. (2002). Cytokine-responsive induction of SAF-1 activity is mediated by a mitogen-activated protein kinase signaling pathway. Mol. Cell. Biol. **22**(4):1027-1035.

Ray A. K. Kuroki et al. (2003)" Induction of matrix metalloproteinase 1 gene expression is regulated by inflammation-responsive transcription factor SAF-1 in Osteoarthritis." Arthritis & Rheumatism. 48(1):134-145.

Ray B et al. (2004) Serum amyloid A-activating factor-1(SAF-1) transgenic mice are prone to develop a severe form of inflammation-induced arthritis. Journal Immunology. **173**(7):4684-4691.

Ray A., B. Bal, and B. K. Ray.(2005). " Transcriptional induction of matrix metalloproteinase-9 in the chondrocyte and synoviocyte cells is regulated via a novel mechanism: evidence for functional cooperation between serum amyloid A-activating factor-1 and AP-1." Journal of Immunology. 175:409-4048.

Roberts, W. G. and G. E. Palade (1995). "Increased microvascular permeability and endothelial fenestration induced by vascular endothelial growth factor." Journal of Cell Science **108**(Pt 6): 2369-79.

Ryan, A. M., D. B. Eppler, et al. (1999). "Preclinical safety evaluation of rhuMAbVEGF, an antiangiogenic humanized monoclonal antibody." Toxicologic Pathology **27**(1): 78-86.

Safran, M. and W. G. Kaelin, Jr. (2003). "HIF hydroxylation and the mammalian oxygen-sensing pathway." Journal of Clinical Investigation **111**(6): 779-83.

Seetharam, L., N. Gotoh, et al. (1995). "A unique signal transduction from FLT tyrosine kinase, a receptor for vascular endothelial growth factor VEGF." Oncogene **10**(1): 135-47.

Semenza, G. L. (2002). "Signal transduction to hypoxia-inducible factor 1." biochemical pharmacology **64**: 993-998.

Senger, D. R., S. J. Galli, et al. (1983). "Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid." Science **219**(4587): 983-5.

Shen, H., M. Clauss, et al. (1993). "Characterization of vascular permeability factor/vascular endothelial growth factor receptors on mononuclear phagocytes." Blood **81**(10): 2767-73.

Shibata, A., T. Nagaya, et al. (2002). "Inhibition of NF-kappaB activity decreases the VEGF mRNA expression in MDA-MB-231 breast cancer cells." Breast Cancer Research & Treatment **73**(3): 237-43.

Shibuya, M., S. Yamaguchi, et al. (1990). "Nucleotide sequence and expression of a novel human receptor-type tyrosine kinase gene (flt) closely related to the fms family." Oncogene **5**(4): 519-24.

Shifren, J. L., S. Mesiano, et al. (1998). "Corticotropin regulates vascular endothelial growth factor expression in human fetal adrenal cortical cells." Journal of Clinical Endocrinology & Metabolism **83**(4): 1342-7.

Shweiki, D., A. Itin, et al. (1993). "Patterns of expression of vascular endothelial growth factor (VEGF) and VEGF receptors in mice suggest a role in hormonally regulated angiogenesis." Journal of Clinical Investigation **91**(5): 2235-43.

Soh, E. Y., S. A. Sobhi, et al. (1996). "Thyroid-stimulating hormone promotes the secretion of vascular endothelial growth factor in thyroid cancer cell lines." Surgery **120**(6): 944-7.

Street, J., M. Bao, et al. (2002). "Vascular endothelial growth factor stimulates bone repair by promoting angiogenesis and bone turnover." Proceedings of the National Academy of Sciences of the United States of America **99**(15): 9656-61.

Terman, B. I., M. E. Carrion, et al. (1991). "Identification of a new endothelial cell growth factor receptor tyrosine kinase." Oncogene **6**(9): 1677-83.

Tran, J., J. Rak, et al. (1999). "Marked induction of the IAP family antiapoptotic proteins survivin and XIAP by VEGF in vascular endothelial cells." Biochemical & Biophysical Research Communications **264**(3): 781-8.

Unemori, E. N., N. Ferrara, et al. (1992). "Vascular endothelial growth factor induces interstitial collagenase expression in human endothelial cells." Journal of Cellular Physiology **153**(3): 557-62.

Vaisman, N., D. Gospodarowicz, et al. (1990). "Characterization of the receptors for vascular endothelial growth factor." Journal of Biological Chemistry **265**(32): 19461-6.

Vincenti, V., C. Cassano, et al. (1996). "Assignment of the vascular endothelial growth factor gene to human chromosome 6p21.3." Circulation **93**(8): 1493-5.

Waltenberger, J., L. Claesson-Welsh, et al. (1994). "Different signal transduction properties of KDR and Flt1, two receptors for vascular endothelial growth factor." Journal of Biological Chemistry **269**(43): 26988-95.

Wang, G. L., B. H. Jiang, et al. (1995). "Hypoxia-inducible factor 1 is a basic-helix-loop-helix-PAS heterodimer regulated by cellular O2 tension." Proceedings of the National Academy of Sciences of the United States of America **92**(12): 5510-4.

Wang, G. L. and G. L. Semenza (1995). "Purification and characterization of hypoxia-inducible factor 1." Journal of Biological Chemistry **270**(3): 1230-7.

Warren, R. S., H. Yuan, et al. (1996). "Induction of vascular endothelial growth factor by insulin-like growth factor 1 in colorectal carcinoma." Journal of Biological Chemistry **271**(46): 29483-8.

Zhang, X., J. P. Gaspard, et al. (2001). "Regulation of vascular endothelial growth factor by the Wnt and K-ras pathways in colonic neoplasia." Cancer Research **61**(16): 6050-4.