CHAPTER II

LITERATURE REVIEWS

Tumor Angiogenesis

Angiogenesis, the formation of new blood capillaries from pre-existing vessels, is an important mechanism for supplying nutrients to cells that are distant from existing blood vessels (Folkman and Shing, 1992). Angiogenesis is critically important during embryonic development (Breier, 2000) and certain physiological circumstances in the adult, including wound healing (Iruela-Arispe and Dvorak, 1997). In addition to its important role in normal physiological processes, angiogenesis contributes to the pathology of a number of diseases (Patz, 1980; McLaren *et al.*, 1996; Fava *et al.*, 1994), including tumor progression (Carmeliet and Jain, 2000). This is because angiogenesis provides nutrients that maintain the viability of diseased tissue.

Tumor angiogenesis is the proliferation of a network of blood vessels that penetrates into cancerous growths. Tumor angiogenesis actually starts with cancerous tumor cells releasing molecules that send signals to surrounding normal host tissue. This signaling activates certain genes in the host tissue that, in turn, make proteins to encourage growth of new blood vessels. Once researchers knew that cancer cells can release molecules to activate the process of angiogenesis, the challenge became to find and study these angiogenesis-stimulating molecules in animal and human tumors. It is now recognized that the endothelial cell, by paracrine mechanisms, produces growth factors that stimulate the proliferation of the tumor cell population. Thus, there is a bi-directional reciprocal signal of endothelial and tumor cell growth. From such studies more than a dozen of different proteins, as well as several smaller molecules, have been identified as *"angiogenic factors"* meaning that they are released by tumors as signals for angiogenesis.

Tumor growth and metastasis are angiogenic dependent. Tumor-associated angiogenesis allows the tumor to maintain its growth advantage and also facilitates metastatic spreading by establishing connections to the existing vasculature. A

correlation has been observed between the density of microvessels in primary breast carcinoma and nodal metastases with respect to survival (Weidner *et al.*, 1991; Horak *et al.,* 1992). Similarly, a correlation has been reported between vascularity and invasive behavior in a number of other tumors (Wakui *et al.*, 1992; Macciarini *et al.*, 1992). These findings indicate that the number of vessels in tumor sections may be a prognostic factor for cancer patients (Biglar *et al.*,1993).

Molecular mechanisms of tumor angiogenesis

Endothelial cells are the main targets and actors of the process of tumor angiogenesis (Folkman and Shing, 1992; Hanahan and Folkman, 1996; Cockerill *et al.*, 1995; Ortega *et al.*, 1999). Normal endothelial are quiescent cells, characterized by a very low proliferation index and a long life span (Ortega *et al.*, 1999; Ortega *et al.*, 1997).

Most primary solid tumors go through a dormant state in which the maximum size attainable is ≅ 1-2 mm in diameter. This size is maintained because tumor cells use pre-existing host blood vessels but do not allow formation of new vessels. Instead, dormant tumors undergo vascular regression by disruption of endothelial cell / periendothelial cell interactions and endothelial cell apoptosis. When tumor cells become angiogenic, the tumor expands progressively and disseminates metastatic cells. Much of interest in angiogenesis comes from the notion that for tumors to grow beyond a critical size, they must recruit endothelial cells from the surrounding stroma to form their own endogenous microcirculation and that this process is driven by the metabolic requirements of the rapidly growing tumor itself. Thus, during tumor progression, two phases can be recognized: a prevascular phase and a vascular phase. The transition from the prevascular to the vascular phase is referred to as the *"angiogenic switch"*. This angiogenic switch has been observed in different types of cancers. The *"angiogenic switch"* depends on a net balance of positive and negative angiogenic factors in the tumor (Hanahan and Folkman, 1996). It is now widely accepted that the *"angiogenic switch"* is "off" when the effect of angiogenic molecules is balanced by that of anti-angiogenic molecules, and is "on" when the net balance is tipped in favour of angiogenesis (Hanaban and Weinberg, 2000; Bouck *et al.*, 1996). In tumors, the switch to an angiogenic phenotype is known to be critical

for disease progression. Unless a tumor can stimulate the formation of new blood vessels, it remains restricted to a microscopic size. Inflammation and hypoxia are widely accepted as key elements in the induction of angiogenesis.

From such studies more than a dozen of different proteins, as well as several smaller molecules, have been identified as *"angiogenic factors"* meaning that they are released by tumors as signals for angiogenesis. Thus, the angiogenic phenotype may result from the production of growth factors, such as fibroblast growth factor-2 (FGF-2) and vascular endothelial growth factor (VEGF) by tumor cells and/or the downregulation of negative modulators, like thrombospondin-1 (TSP-1), in tissues with a quiescent vasculature. Angiogenic phenotype serves the development of malignant tumor at multiple stages. Tumor cells may overexpress one or more of the positive regulators of angiogenesis, may mobilize an angiogenic protein from the extracellular matrix (ECM), may recruit host cells such as macrophages (which produce their own angiogenic proteins), or may engage in a combination of these processes. Tumor angiogenesis is mediated by tumor-secreted angiogenic growth factors that interact with their surface receptors expressed on endothelial cells (ECs). The most commonly found angiogenic growth factors such as VEGF and bFGF, when encounter ECs, they bind to the tyrosine kinase receptors on ECs membrane. Binding leads to dimerization of the receptors and activation of autophosphorylation of tyrosines on the receptor surface and thereby initiates the several signaling proteins (including PI3- kinase, Src, Grb2/m-SOS-1 (a nucleotide exchange factor for Ras) and signal transducers and activators of transcriptions (STATs) each of which contains src-homology-2 (SH-2) domains (Sebti and Hamilton, 2000). Binding of the SH-2 regions of these proteins to the phosphotyrosines on the receptor tyrosine kinases (RTKs) activates several pathways that are crucial for triggering the cell cycle machinery. The most well studied pathway passes through the GTP-binding protein Ras and activates the mitogen activated protein kinase (MAPK) cascade and subsequently transcription factors in the nucleus (Sebti and Hamilton, 2000). Up-regulation of an angiogenic factor is not sufficient in itself for a tumor cell to become angiogenic, however, certain negative regulators or inhibitors of vessel growth may need to be down-regulated (Dameron *et al.*, 1994). If there is a preponderance of angiogenic factors in the local milieu, the neovasculature may persist as capillaries, or differentiate into mature venules or arterioles. If instead, the local milieu changes such

that there is a preponderance of angiostatic factors, the neovessels can regress. The angiostatic factors that mediate regression can do so either by inducing apoptosis or cell cycle arrest of ECs. Thus, the switch to the angiogenic phenotype is regulated by a change in the local equilibrium between positive and negative regulators of the growth of microvessels (Bussolino *et al.*, 1997; Dameron *et al.*, 1994).

Angiogenesis is a complex process involving extensive interplay between cells, soluble factors and ECM components. The construction of a vascular network requires different sequential steps, which can be summarized as follows (Lieken *et al.*, 2001):

1. The release of proteases from "activated" endothelial cells
2. Degradation of the basement membrane surrounding the existing vessel
3. Migration of the endothelial cells into the interstitial space
4. Endothelial cell proliferation
5. Lumen formation
6. Generation of new basement membrane with the recruitment of pericytes
7. Fusion of the newly formed vessels
8. Initiation of blood flow

Endothelial Cell Activation

The activation of endothelial cells by VEGF or bFGF sets in motion a series of steps toward the creation of new blood vessels. First, the activated endothelial cells produce *matrix metalloproteinases (MMPs),* a special class of degradative enzymes. These enzymes are then released from the endothelial cells into the surrounding tissue. The MMPs break down the ECM—support material that fills the spaces between cells and is made of proteins and polysaccharides. Breakdown of this matrix permits the migration of endothelial cells. As they migrate into the surrounding tissues, activated endothelial cells begin to divide. Soon they organize into hollow tubes that evolve gradually into a mature network of blood vessels.

Despite the abundances of angiogenic factors present in different tissues, endothelial cell turnover in a healthy adult organism is remarkably low with a turnover in the order of thousands of days. The maintenance of endothelial quiescence

is thought to be due to the presence of endogenous negative regulators. Moreover, positive and negative regulators often co-exist in tissues with extensive angiogenesis. These observations have led to the hypothesis that activation of the endothelium depends on a balance between these opposing regulators. If positive angiogenic factors dominate, the endothelium will be activated, whereas quiescence Thus, the angiogenic process can be divided in an activation phase (initiation and progression of the angiogenic process) and a phase of resolution (termination and stabilization of the vessels). It is not yet clear whether the resolution phase is due to upregulation of endogenous inhibitors or exhaustion of positive regulators.

Among the factors that affect endothelial cell activation status, either positively or negatively, are cytokines. On the basis of the observation that a given tissue can profoundly influence the way in which its cellular components respond to a given cytokine, it has been suggested that cytokines should be seen as "specialized symbols" in a language of intercellular communication, whose meaning is controlled by context. Context is determined by (at least) three parameters: (1) the presence and concentration of other cytokines in the pericellular environment of the responding cell; (2) interactions between cells, cytokines, and the extracellular matrix; and (3) the geometric configuration of the cells (and thus their cytoskeleton). With respect to angiogenesis, the notions of both the angiogenic switch as well as context are proving to be central to our understanding of the molecular mechanisms that govern this process.

The cytokines that have been the most extensively studied in the context of angiogenesis are VEGF, aFGF, and bFGF. The finding that *in vitro* VEGF and FGF positively regulate many endothelial cell functions, including proliferation, migration, extracellular proteolytic activity, and tube formation, has led to the notion that these factors are direct-acting positive regulators. However, although a role for VEGF in developmental and tumor angiogenesis has been clearly established, much controversy still exists as to whether the FGFs are relevant to the endogenous control of neovascularization *in vivo*. Furthermore, although a large number of factors have been demonstrated to be active in the experimental setting, it does not necessarily follow that they are relevant to the endogenous regulation of new blood vessel formation in the intact organism. In the case of molecules that are active during the phase of

activation, only one, namely VEGF, meets most of the criteria required for the definition of a vasculogenic or angiogenic factor.

The ultimate target for both positive and negative regulators is the endothelial cell. This has led to the notion that angiogenesis regulators may act either directly on endothelial cells or indirectly by inducing the production of direct-acting regulators by inflammatory and other nonendothelial cells. Thus, in contrast to VEGF and FGF (which are direct endothelial cell mitogens), TGF-ß and TNF-α inhibit endothelial cell growth in vitro and have therefore been considered as direct-acting negative regulators. However, both TGF-ß and TNF-α are angiogenic *in vivo*, and it has been demonstrated that these cytokines induce angiogenesis indirectly by stimulating the production of direct-acting positive regulators from stromal and chemoattracted inflammatory cells. In this context, then, TGF-ß and TNF-α are considered to be indirect positive regulators (Pepper *et al.*, 1996). In view of its capacity to directly inhibit endothelial cell proliferation and migration, reduce extracellular proteolysis, and promote matrix deposition in vitro, TGF-ß has also been proposed to be a potential mediator of the phase of resolution. In vitro, TGF-ß also promotes the organization of single endothelial cells embedded in three-dimensional collagen gels into tubelike structures, a phenomenon that is likely to be representative of the phase of resolution.

Other cytokines that have been reported to regulate angiogenesis *in vivo* include HGF, EGF/TGF-β, PDGF-BB, interleukins (IL-1, IL-6, and IL-12), interferons, GM-CSF, PlGF, proliferin, and proliferin-related protein. Chemokines that regulate angiogenesis *in vivo* have to date only been identified in the -C-X-C- family and include IL-8, platelet factor IV, and gro-ß. Angiogenesis can also be regulated by a variety of noncytokine or nonchemokine factors, including enzymes (angiogenin and PD-ECGF/TP), inhibitors of matrix-degrading proteolytic enzymes (TIMPs) and of PAs (PAIs), extracellular matrix components/coagulation factors or fragments (thrombospondin, angiostatin, hyaluronan, and its oligosaccharides), soluble cytokine receptors, prostaglandins, adipocyte lipids, and copper ions (Table 2.1).

Table 2.1. Positive and negative regulators of angiogenesis.

	Positive regulators	**Negative regulators**
Growth factors	Angiogenin Angiotropin Epidermal growth factor Fibroblast growth factor (acidic and basic) Granulocyte colony-stimulating factor Hepatocyte growth factor/scatter factor Platelet-derived growth factor-BB Tumor necrosis factor-alpha Vascular endothelial growth factor	
Proteases and protease inhibitors	Cathepsin Gelatinase A, B Stromelysin Urokinase-type plasminogen activator (uPA)	Tissue inhibitor of metalloprotease (TIMP-1, TIMP-2) Plasminogen activator-inhibitor-1 (PAI-1)
Trace elements	Copper	Zinc
Oncogenes	*c-myc* *ras* *c-src* *v-raf* *c-jun*	p53, Rb
Signal transduction enzymes	Thymidine phosphorylase Farnesyl transferase Geranylgeranyl transferase	
Cytokines	Alpha v Beta 3 integrin	Angiopoietin-2

	Angiopoietin-1 Angiostatin II (AT 1 receptor) Endothelin (ETB receptor) Erythropoietin Hypoxia Nitric oxide synthase Platelet-activating factor Prostaglandin E Thrombopoietin	Angiotensin Angiotensin II (AT -2 receptor) Caveolin-1, caveolin-2 Endostatin Interferon-alpha Isoflavones Platelet factor-4 Prolactin (16 Kd fragment) Thrombospondin Troponin-1

Role of basement membrane proteolysis

Endothelial cells line the interior of blood vessels and are ensheathed by a basement membrane, a specialized form of the extracellular matrix comprised of collagen, glycoproteins such as fibronectin and laminin, and heparan sulfate proteoglycans. A crucial early step in the neovascularization process is the dissolution of the basement membrane at the site of endothelial outgrowth from the parent vessel, which facilitates cell migration and stromal invasion. The phenomenon of cell invasiveness is not only unique to angiogenesis, but is also an essential component of both tumor metastasis and embryonic morphogenesis.

Basement membrane degradation, extracellular matrix invasion, and capillary lumen formation are essential components of the angiogenic process, all of which are dependent on a cohort of proteases and protease inhibitors produced by endothelial and nonendothelial cells. Extracellular proteolysis has also been implicated in the regulation of cytokine activity, and one of the consequences of matrix degradation is the generation of a variety of degradation products, many of which themselves have biological activity (Pepper *et al.*, 1996; Senger *et al.*, 1996; Flaumenhaft *et al.*, 1992). A number of enzymatic systems have been implicated in extracellular proteolytic events, many of the relevant enzymes belong to one of two families: the serine

proteases, in particular the PA (plasminogen activator)/plasmin system, and the MMPs (Basbaum and Werb, 1996). Although many mechanisms, including transcriptional and translational controls and secretion and activation of proenzymes, are involved in the regulation of extracellular proteolysis, one mechanism that appears to be particularly relevant to cell migration and morphogenesis is spatial localization. Spatial localization, which appears to have evolved to concentrate proteolysis near the cell surface as well as to restrict its activity to the immediate pericellular environment, can be achieved by two mechanisms: first, by binding to cell-surface receptors and matrix-binding sites, and second, by the coproduction of protease inhibitors (Pepper *et al.*, 1996). By preserving matrix integrity and thereby ensuring normal tissue architecture, protease inhibitors play an important permissive role during angiogenesis. However, since the net balance of proteolysis required for invasion is always likely to be positive, it has been suggested that antiproteolysis could be effective in inhibiting angiogenesis. In this respect, the requirement for MMP (Albini *et al.*, 1994; Galardy *et al.*, 1994, Johnson *et al.*, 1994) and PA (Soff *et al.*, 1995; Min *et al.*, 1996) activity during experimentally induced angiogenesis *in vivo* has been clearly demonstrated. Taken together, these studies provide evidence for a causal role for the MMP and PA/plasmin systems during angiogenesis *in vivo* and point to the potential therapeutic benefits that can be gained from inhibiting angiogenesis by interfering with extracellular proteolytic cascades.

Endothelial Cell Migration and Proliferation

Following proteolytic degradation of the ECM, endothelial cells start to migrate through the degraded matrix. They are followed by proliferating endothelial cells, which are stimulated by a variety of growth factors, some of which are released from the degraded ECM. Other ECM products, such as peptide fragments of fibrin or hyaluronic acid also stimulate the angiogenic process. Therefore, a local collapse of the ECM results in an increased extracellular concentration of soluble mediators of endothelial cell migration and proliferation.

Angiogenesis is under the control of diffusable, locally acting factors (peptides) which promote angiogenesis (angiogenesis promoters) or inhibit angiogenesis (angiogenesis inhibitors). In normal humans, there exists a fine balance between these

inducers and inhibitors; hence angiogenesis is under control. These promoters and inhibitors are sub-classified depending on their source and mode of action. Angiogenesis promoters are all from natural source, i.e. they are produced by the human body. They are classified further as follows: -

1. Factors stimulating growth of endothelial cells (EC) or Primary Factors: These genetically determined factors are expressed in response to hypoxia. The factors include VEGF and placenta growth factor (PGF).
2. Factors produced by EC's in response to stimulation by VEGF and PGF or Secondary Factors: These include non-specific growth factors like basic fibroblast growth factor (bFGF), acid fibroblast growth factor (aFGF), transforming growth factor-a (TGF-α), TGF-β and proteolytic enzymes like matrix metalloproteinases (MMP's) and serine proteases that break the extracellular matrix (ECM) for a new capillary branch to develop.

VEGF and FGF-2 have been found to be prominent angiogenic factors. The processes of cell invasion, migration and proliferation do not only depend on angiogenic enzymes, growth factors and their receptors, but are also mediated by cell adhesion molecules (Ziche *et al.*, 1994). To initiate the angiogenic process, endothelial cells have to dissociate from neighbouring cells before they can invade the underlying tissue. During invasion and migration, the interaction of the endothelial cells with the ECM is mediated by integrins. Also, the final phases of the angiogenic process, including the construction of capillary loops and the determination of the polarity of the endothelial cells, which is required for lumen formation, involve cell-cell contact and cell-ECM interaction.

Cell adhesion molecules can be classified into four families depending on their biochemical and structural characteristics. These families include the selectins, the immunoglobulin supergene family, the cadherins and the integrins. Members of each family are implicated in neovascularization (Ziche *et al.*, 1994).

Integrins are a group of cell adhesion receptors, consisting of non-covalently associated alpha- and beta- subunits, which can heterodimerize in more than 20

combinations. Endothelial cells thus express several distinct integrins, allowing attachment to a wide variety of ECM proteins. Integrin αvβ3 was found to be particularly important during angiogenesis. αvβ3 is a receptor for a number of proteins with an exposed Arg-Gly-Asp (RGD) sequence, including fibronectin, vitronectin, laminin, von Willebrand factor (vWF), fibrinogen and denatured collagen. In addition, αvβ3 has been shown to bind MMP-2, in an RGD-independent way, thereby localizing MMP-2-mediated matrix degradation to the endothelial cell surface (Ziche *et al.*, 1994). αvβ3 is nearly undetectable on quiescent endothelium, but is highly upregulated during cytokine or tumor-induced angiogenesis. In activated endothelium αvβ3 suppresses the activity of both p53 and the p53-inducible cell-cycle inhibitor $p21^{WAF1/CIP1}$, while increasing the Bcl2:Bax ratio, resulting in an anti-apoptotic effect (Stromblad *et al.*, 1996). Consequently, αvβ3 was found to promote melanoma growth by regulating tumor cell survival. Another receptor, which has recently been implicated in angiogenesis is integrin αvβ5. Antibodies directed against αvβ3 were found to specifically block FGF-2 or TNF-α -induced angiogenesis, whereas antagonists of αvβ5 blocked VEGF-induced angiogenesis. This implies that specific cytokines may stimulate angiogenesis by distinct signaling pathways that may be mediated by specific integrins.

Capillary formation and vessel maturation

After proteolytic degradation of the basement membrane and endothelial cell migration, the newly-forming capillaries synthesize a new basement membrane. During this process extracellular proteolysis must be locally inhibited to permit the deposition and assembly of ECM components. Once a capillary sprout is formed, degradation of the newly formed ECM again occurs at the tip of the sprout, which then allows further invasion. Thus, capillary formation results form alternate cycles of activation and inhibition of extracellular proteolysis. The endothelial cells also form branches, which connect with other branches to from capillary loops. Further stabilization of the new capillaries requires the recruitment of pericytes and smooth muscle cells, which is regulated by platelets-derived growth factor (PDGF). Finally, when sufficient neovascularization has occurred, angiogenic factors are downregulated or the local concentration of inhibitors increases. As a result, the

endothelial cells become quiescent and the vessels remain or regress if no longer needed. Thus, angiogenesis requires many interactions that must be tightly regulated in a spatially and temporally manner.

The morphologic events of capillary growth include endothelial cell–induced degradation of the basement membrane of the parent venule, directional locomotion in concert with other endothelial cells, endothelial mitosis, lumen formation, development of sprouts and loops, generation of new basement membrane, and recruitment of pericytes (Montesano *et al.,* 1983). This sequence is similar to the morphologic steps of angiogenesis in a wound healing or in embryo development. However, many tumors impose modifications on a new capillary bed that differs from the angiogenesis induced by nonneoplastic cells. Tumor growth and invasion of the tissue are mediated by proteinases, such as uPA and MMPs, produced by the tumor. Thus, positive angiogenic factors are produced by tumor cells and endothelial cells, released by attracted inflammatory cells such as mast cells and macrophages and/or mobilized from the ECM. So, different self-amplifying loops exist to maintain the angiogenic phenotype in the tumor. There are two major differences between pathological and normal angiogenesis. First, in diseased tissue, the regulatory mechanisms which "turn off" neovascularization in healthy tissue do not function normally; there is a shift in the balance of positive and negative angiogenesis regulators towards the positive molecules. The second major difference between pathological and normal angiogenesis is that the vessels formed in diseased tissue are highly disorganized, and their walls have numerous openings. This is because tumor vessels are not able to mature through the recruitment of smooth muscle cells and pericytes, leading to the formation of leaky vessels in the tumor. This may be due to an imbalance of angiogenic regulators, such as VEGF and angiopoietins. Consequently, tumor blood flow is chaotic and variable and leads to hypoxic and acidic regions in tumors. These conditions lower therapeutic effectiveness, modulate the production of angiogenic stimulators and inhibitors, and select for cancer cells that are more malignant and metastatic.

Regulation of Tumor Angiogenesis

Angiogenesis is regulated by both *activator* and *inhibitor* molecules. In normal condition the angiogenic inhibitors predominate and cause endothelial cell quiescence. Should a need for new blood vessels arise, angiogenesis activators increase in number and inhibitors decrease. This prompts the growth and division of vascular endothelial cells and, ultimately, the formation of new blood vessels. The switch to the angiogenic phenotype involves a change in the local equilibrium between positive and negative regulators of the growth of microvessels.

In both normal and pathological angiogenesis, hypoxia is the main force initiating the angiogenic process. Hypoxia induces the expression of VEGF and its receptor *via* hypoxia-inducible factor-1α (HIF-1α) (Carmeliet *et al.*, 1998) and is also an attractant for macrophages. In a tumor, the angiogenic phenotype can be triggered by hypoxia resulting from the increasing distance of the growing tumor cells to the capillaries or from the inefficiency of the newly formed vessels. Also, several oncogenes such as *v-ras*, *K-ras, v-raf*, *src, fos* and *v-yes* induce the upregulation of angiogenic factors like VEGF, insulin-like growth factor-1 (IGF-1) and TGF-α (Jiang *et al.*, 1997; Kerbel *et al.*,1998). Moreover, oncogene products may act directly as angiogenic factors. This is the case for the protein product of FGF-4/hst-1. In addition, oncogenes can indirectly promote angiogenesis by increasing the production of cytokines and proteolytic enzymes. In contrast, the tumor suppressor gene *p53* was found to cause degradation of HIF-1α, inhibition of VEGF production (Mukhopadhyay *et al.*, 1995), and stimulation of the inhibitor TSP-1 (Dameron *et al.*, 1994). Regulatory genes may be mutated or non functional, allowing for tumor cells to develop an uncontrolled response to hypoxia resulting in the synthesis and secretion of high levels of pro-angiogenic factors.

The stromal and inflammatory cells present in the tumor microenvironment are also involved in the regulation of the angiogenic process. Stromal mesenchymal cells may synthesize and secrete either pro- or anti-angiogenic factors susceptible to contribute to the angiogenic balance within the neoplastic tissue. Inflammatory cells, and particularly macrophage (Harmey *et al.*, 1998), are also important sources of pro-angiogenic factors.

Vascular Endothelial Growth Factor (VEGF)

Vascular endothelial growth factor (VEGF) is a 40 to 45 kDa homodimeric protein with a signal sequence secreted by a wide variety of cells and the majority of tumor cells. VEGF, also known as vascular permeability factor (VPF), is a heparin-binding angiogenic growth factor, and is highly expressed in various types of tumors. It may increase ECs permeability by enhancing the activity of vesicular-vacuolar organelles, clustered vesicles in ECs lining small vessels that facilitate transport of metabolites between luminal and abluminal plasma membranes (Kohn *et al.*, 1992). Alternatively, VEGF may enhance permeability through mitogen-activated protein (MAP) kinase signal transduction cascade by loosening adhering junctions between ECs in a monolayer via rearrangement of cadherin/catenin complexes (Esser *et al.*, 1998; Kevil *et al.*, 1998). In addition, recent studies have shown that VEGF enhances ECs permeability by activating PKB/Akt, endothelial nitricoxide synthase (eNOS), and MAP kinase dependent pathways using human umbilical vein endothelial cell (Lal *et al.*, 2001). Increased vascular permeability may allow the extravasation of plasma proteins and formation of ECM favorable to endothelial and stromal cell migration.

The VEGF family currently includes five members in addition to the prototype VEGF, namely, PlGF, VEGF-B, VEGF-C, VEGF-D, and Orf virus VEGFs (Ferrara, 1999; Persico, *et al.*,1999; Ogawa, *et al.*, 1998; Meyer, *et al.*, 1999). The VEGFs mediate angiogenic signals to the vascular endothelium via high-affinity RTKs. To date, three receptors for the VEGFs have been identified. All three are relatively specific for endothelial cells and demonstrate structural and functional similarities to the PDGF receptor family. These receptors are currently designated VEGFR-1, VEGFR-2, and VEGFR-3 and were originally named *flt* (*fms*-like tyrosine kinase), KDR (kinase insert domain-containing receptor)/*flk-1* (fetal liver kinase-1), and *FLT4*, respectively (Shibuya *et al.*, 1999; Neufeld *et al.*, 1999; Shibuya *et al.*, 1990; Terman *et al.*, 1991; Aprelikova *et al.*, 1992; Galland *et al.*, 1992). All have seven immunoglobulin homology domains in their extracellular part and an intracellular tyrosine kinase signaling domain split by a kinase insert (Figure 2.1). In adults,

VEGFR-1 and VEGFR-2 are expressed mainly in the blood vascular endothelium, whereas VEGFR-3 is restricted largely to the lymphatic endothelium.

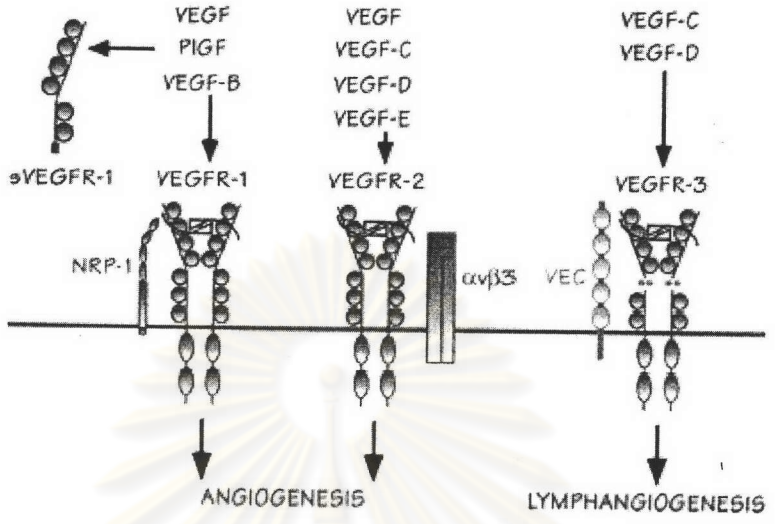

Figure 2.1. The VEGFs and their receptors (Veikkola *et al.*, 2000)

Cellular mechanisms of VEGF action

VEGF, after binding to its high affinity receptors (Flt-1/VEGFR-1, Flk-1/KDR/ VEGFR-2), promotes the formation of the second messenger via hydrolysis of inositol, thus induces the autophosphorylation of the receptors in the presence of heparin-like molecules, and open phosphatidylinositol metabolic signal transduction pathways, activates MAP kinases in EC and thereby VEGF exerts its mitogenic effect by promoting EC proliferation (Ferrara, 2001). VEGF induces a balanced system of proteolysis that can remodel ECM components necessary for angiogenesis. VEGF stimulates EC production of urokinase-like plasminogen activator (uPA), tissue type plasminogen activator (tPA) and plasminogen activator inhibitor-1 (PAI-1)(Mandriota *et al.*, 1995; Pepper *et al.*, 1991), proteolytic enzymes, tissue factors, and interstitial collagenase (Unemori *et al.*, 1992). Plasminogen activators activate plasminogen to plasmin, which can break down ECM components. In addition to remodeling the basement membrane, uPA bound to uPAR also mediates intracellular signal transduction in ECs. Tang *et al.* (1998) have demonstrated that uPAR occupancy on

ECs results in the phosphorylation of focal adhesion proteins and the activation of MAP kinase (Tang *et al.*, 1998) through which uPA influences EC migration and proliferation (Figure 2.2). Moreover, VEGF has been shown to exhibit its angiogenic effect by inducing expression of the $\alpha1\beta1$, $\alpha2\beta1$ and $\alpha v\beta3$-integrins, which promote cell migration, proliferation and matrix reorganization (Figure 2.2), and $\alpha1\beta1$, $\alpha2\beta1$ and $\alpha v\beta3$ antagonists may prove effective on inhibiting VEGF-driven angiogenesis associated with cancers and other pathologies through apoptosis (Senger *et al.*, 1996, 1997).

VEGF, in addition to a very specific mitogen for vascular EC, is a potent pro-survival factor for ECs in newly formed immature vessels. Several endothelial survival factors (VEGF, angiopoietin-1 and $\alpha v\beta3$) suppress p53, p21, p16 and p27, and proapoptotic protein Bax, whereas they variably activate the survival PI3k/Akt, p42/44 MAP kinases, bcl-2, A1 and survivin pathways (Figure 2.2). It was reported that p42/p44 MAP kinases promoted VEGF expression by activating its transcription via recruitment of the AP-1/Sp1 (activator protein-1) complex on the proximal region (-88/-66) of the VEGF promoter and by direct phosphorylation of hypoxia inducible factor-1 alpha (HIF-1α). Pharmacological inhibition of PI3K or transfection with a dominant-negative Akt mutant abolished the antiapoptotic effect of VEGF on ECs. In addition to the PI3K/Akt pathway, ras-dependent signaling pathways might also play an important role at least for VEGF signaling. Thus, H-rasV12G down-regulation leads to profound tumor regression, which is initially characterized by massive apoptosis of tumor- and host-derived ECs (Chin *et al.*, 1999). Therefore, apoptosis induction is resistant to enforced VEGF expression, suggesting that VEGF requires an intact Ras-dependent signaling pathway to mediate its apoptosis inhibitory effect (Chin *et al.*, 1999). And also, VEGF via the KDR/Flt-1 receptor induces enhanced expression of the serine-threonine protein kinase Akt (Gerber *et al.*, 1998), a downstream target of PI3-kinase, which potently blocks apoptosis by interfering with various apoptosis signaling pathways (Khwaja, 1999; Fujio and Walsh, 1999), promotes EC migration (Morales-Ruiz *et al.*, 2000), and enhances the expression of the hypoxia-inducible factor (HIF), which is known to stimulate VEGF expression, suggesting a potent proangiogenic effect. These findings have identified the VEGFR2 and the PI3K/Akt signal transduction pathway as crucial elements in promoting EC

survival induced by VEGF. The downstream effector pathways mediating the antiapoptotic VEGF effect include Akt-dependent activation of the endothelial nitric oxide synthase (NOS)(Dimmeler *et al.*, 1999; Fulton *et al.*, 1999), resulting in an enhanced endothelial NO synthesis, which, in turn promotes EC survival (Figure 2). Gupta *et al.* (2003) demonstrated that the VEGF-induced activation of the MAPK/extracellular signal-regulated kinase (ERK) pathway and inhibition of the stress-activated protein kinase/c-Jun amino-terminal kinase pathway is also implicated in the antiapoptotic effect mediated by VEGF (Figure 2). Interestingly, the activation of the PI3K/Akt pathway mediates not only the antiapoptotic effect but also the migratory effect of VEGF on ECs via Akt-dependent phosphorylation and activation of eNOS (Figure 2).

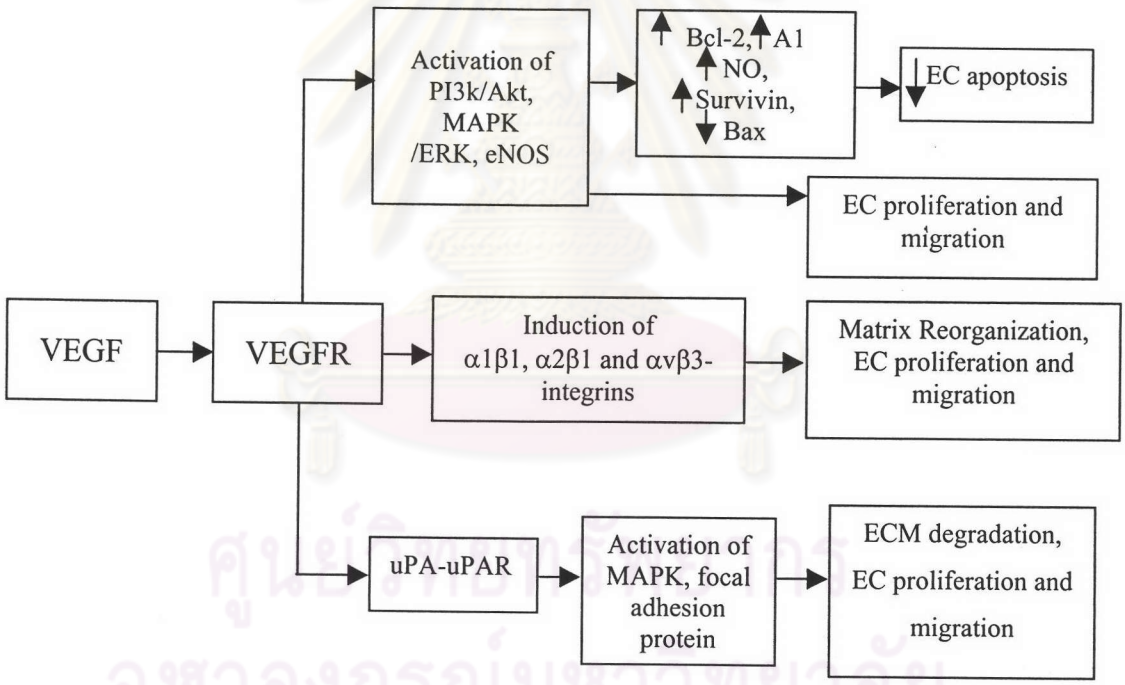

Figure 2.2. Mechanisms of action of VEGF in angiogenesis (Gupta *et al.*, 2003)

Regulation of VEGF expression

The expression of VEGF mRNA is highest in hypoxic tumor cells adjacent to necrotic areas. Hypoxia-induced transcription of VEGF mRNA is apparently mediated, at least in part, by the binding of HIF-1 to an HIF-1 binding site located in the VEGF promoter, and by the activation of a stress inducible PI3K/Akt pathway. In fact, progressive growth of tumor creates ongoing hypoxia, which up-regulates several pro-angiogenic compounds including VEGF, bFGF, IL-8, TNF-α, TGF-b *etc*. These compounds, via several mechanisms such as increase of vessel hyperpermeability, release of plasma proteins, induction of proteases, fibrin formation, EC proliferation, migration etc, promote angiogenesis and fibrinolysis resulting in continued tumor growth and dysfunctional vasculature, which further positively feedback to create continuing hypoxia inside tumors (Figure 2.3).

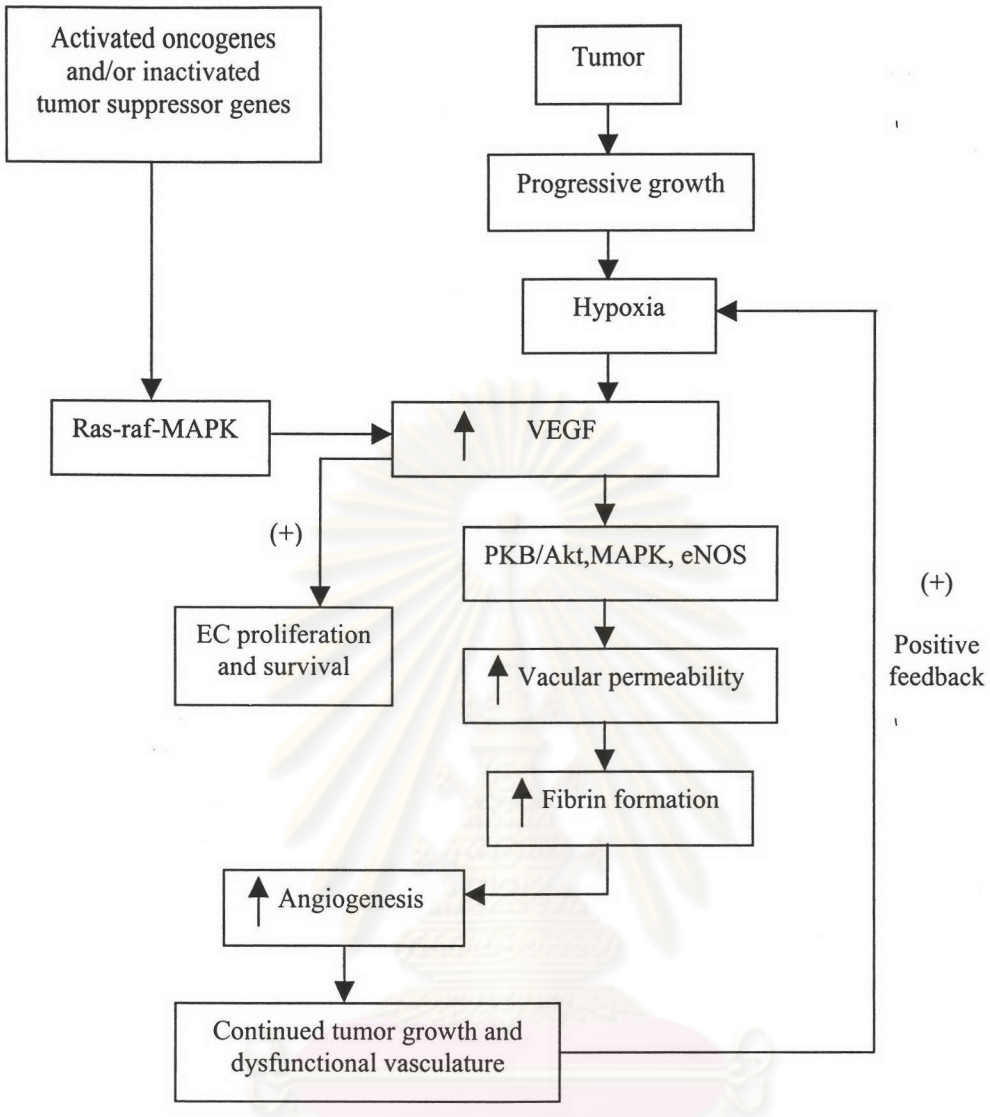

Figure 2.3. The triggering mechanism in tumor angiogenesis: inactivated tumor suppressor genes/activated oncogenes versus hypoxia (Gupta *et al.*, 2003)

Role of VEGF in Tumor Angiogenesis

VEGF has received attention as a target for therapeutic angiogenesis (Ferrara and Davis-Smyth, 1997). There is a correlation between tumors with higher densities of blood vessels and metastasis and poorer clinical outcome. Expression of VEGF is associated with tumor growth, angiogenesis, and metastasis. The expression of VEGF correlates both temporally and spatially with the onset of neovascularization (Ferrara and Davis-Smyth, 1997). Elevations in VEGF levels have been detected in the serum

of some cancer patients (Kondo *et al.*, 1994), and a correlation has been observed between VEGF expression and microvascular density in primary breast cancer sections (Toi *et al.*, 1994). A postoperative survey indicated that the relapse-free survival rate of patients was correlated with VEGF-poor tumors suggesting that VEGF expression is associated with stimulation of angiogenesis and with early relapse in primary breast cancer. Moreover, there is compelling evidence that circulating VEGF levels are of prognostic significance in a variety of tumor types (Adams *et al.*, 2000; Bian *et al.*, 2000; Loncaster *et al.*, 2000; Mineta *et al.*, 2000; Stockhammer *et al.*, 2000; Yoshikawa *et al.*, 2000; Broll *et al.*, 2001; Hirai *et al.*, 2001). Furthermore, an essential role for VEGF in tumor angiogenesis has been demonstrated in animal models by the findings that neutralizing VEGF antibodies and dominant-negative VEGF receptors inhibit both angiogenesis and the progression of the disease (Kim *et al.*, 1993; Millauer *et al.*, 1994). These results are consistent with the hypothesis that angiogenesis is necessary for tumor growth, and that VEGF is a potent stimulator of the angiogenic response.

Cyclooxygenase-2 (COX-2)

Cyclooxygenase (COX) is the rate-limiting enzyme involved in the conversion of arachidonic acid to prostaglandin H_2, the precursor of various compounds including prostaglandins, prostacyclin, and thromboxanes. Two COX genes, COX-1 and COX-2, have been identified that share > 60% identity at the amino acid level (Hla and Neilson, 1992). COX-1 is constitutively expressed in many tissues and responsible for various physiological functions including cytoprotection of the stomach, vasodilatation in the kidney, and production of a proaggregatory prostanoid, thromboxane A_2, by platelets. On the other hand, COX-2 is an inducible immediate-early gene originally found to be induced by various stimuli, such as mitogens and cytokines, and growth factors (Jones *et al.*, 1993; Hamasaki *et al.*, 1993; DuBois *et al.*, 1994)(Figure 2.4). Conventional NSAIDs inhibit both COX-1 and COX-2; hence they also disrupt COX-1 dependent homeostatic functions. Therefore, molecular-based targeting strategies were employed to develop specific COX-2 inhibitors to circumvent the gastric and renal toxicities caused by mixed COX inhibitors.

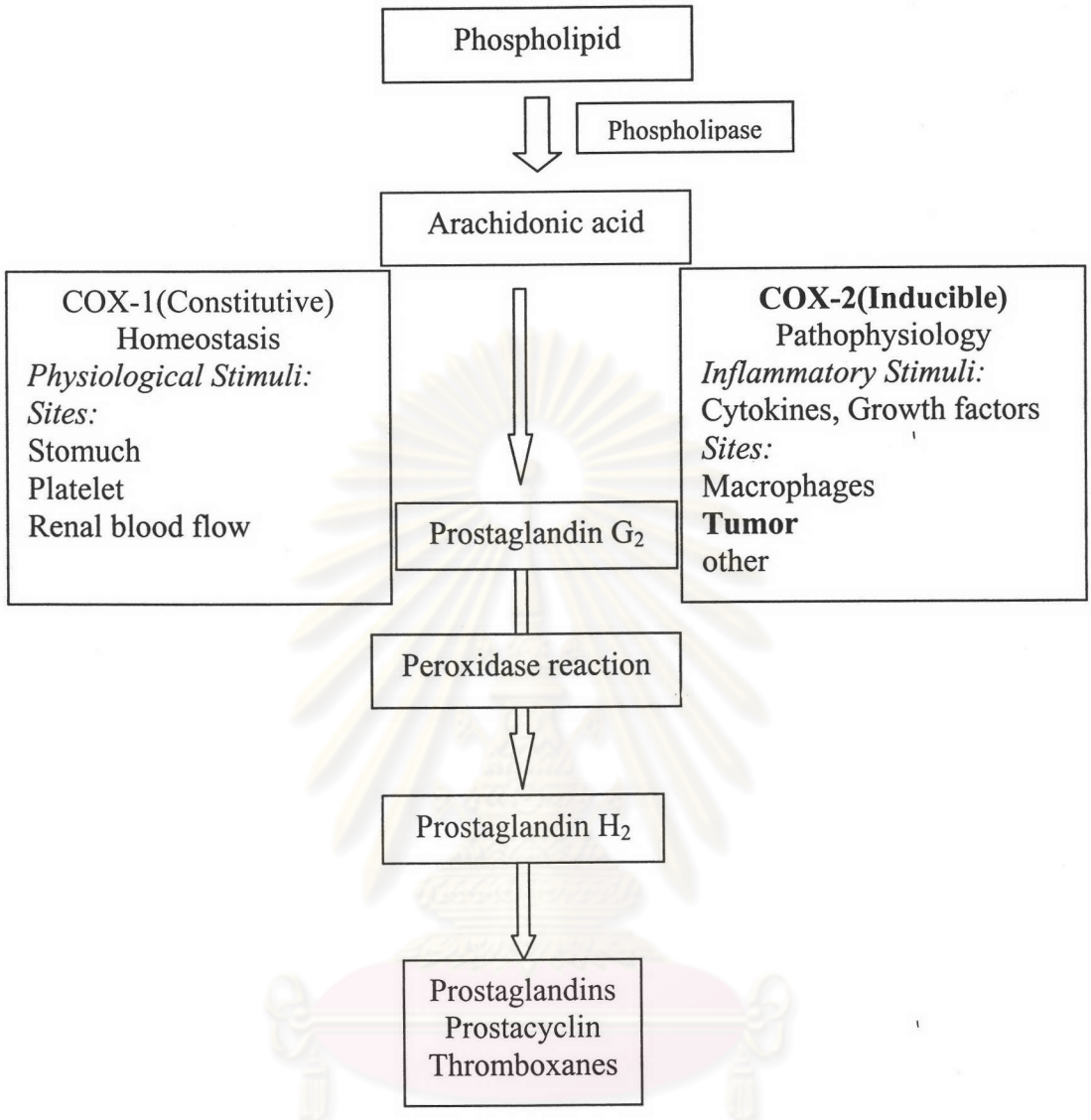

Figure 2.4. Bifunctional role of the COX enzyme (prostaglandin H synthase) in the biosynthesis of prostaglandins and thromboxanes, and physiological and pathophysiological effects of the COX isoenzymes (Davies *et al.*, 2002)

Regulation of COX-2 Expression

The genes for COX-1 and COX-2 are located on human chromosomes 9 and 1, respectively (Kraemer *et al.*, 1992). Whereas COX-1 represents a housekeeping gene, which lacks a TATA box (Kraemer *et al.*, 1992), the promotor of the immediate-early gene COX-2 contains a TATA box and binding sites for several transcription factors including nuclear factor-κB (NF-κB), the nuclear factor for

interleukin-6 expression (NF-IL-6) and the cyclic AMP response element binding protein (Appleby *et al.*, 1994). Thus, the expression of COX-2 is regulated by a broad spectrum of mediators involved in inflammation. Whereas lipopolysaccharide, proinflammatory cytokines (interleukin-1β, tumor necrosis factor), and growth factors may induce COX-2, glucocorticoids, interleukin-4, interleukin-13, and the anti-inflammatory cytokine interleukin-10 have been reported to inhibit the expression of this enzyme (Lee *et al.*, 1992;Onoe *et al.*, 1996;Niiro *et al.*, 1997).

COX-2 is also regulated at the post-transcriptional level. Recently, a 3'-untranslated region of its mRNA has been shown to certain multiple copies of adelylate- uridylate-rich elements that may confer post-transcriptional control of COX-2 expression by acting as an mRNA instability determinant or as a translation inhibitory element (Dixon *et al.*, 2000). Loss of this post-transcriptional regulation of COX-2 through mutation of proteins that specifically interact with the COX-2 adelylate- and uridylate-rich elements may lead to COX-2 overexpression and has been proposed as a crucial factor involved in carcinogenesis.

Increased expression of COX-2 in malignancy is likely to occur via multiple routes. COX-2 induction by lipopolysaccharide (LPS) has been shown to occur through both the mitogen-activated protein kinase (MAPK) and protein kinase C-ζ (PKC-ζ) pathways (Mestre *et al.*, 2001). It has also been shown that ceramide-stimulated activation of MAPK can activate c-Jun N-terminal kinase (JNK), which in turn can lead to increased COX-2 gene expression in human mammary epithelial cells (Subbaramaiah *et al.*, 1998). This occurs via a cAMP response element (CRE) in the COX-2 promoter. Further evidence for the role of MAPK and c-Jun pathways in tumour necrosis factor-α (TNF-α)-stimulated COX-2 expression in human epithelial cells was provided recently by Chen *et al.* (2001). These pathways are illustrated in Figure 2.5. Transient transfection experiments have demonstrated that nuclear factor κB (NFκB), nuclear factor IL-6 (NF-IL6) and CRE promoter sites mediate gene transcription independently in response to LPS treatment (Mestre *et al.*, 2001). LPS can activate different pathways to induce COX-2 gene transcription: through NFκB via extracellular signal related kinase (ERK-2), p38 and JNK pathways, through NF-IL6 via a p38 pathway, and through CRE via ERK-2 and JNK pathways. Moreover,

PKC-ζ signalling seems to mediate transcription after LPS treatment through all three promoter sites. Therefore, individual signaling pathways, such as ERK-2, p38, JNK or PKC-ζ, appear to be sufficient to mediate COX-2 gene transcription by virtue of their ability to recruit transcription factors to at least two promoter sites. This may indicate redundancy in the signalling pathways and promoter elements regulating COX-2 transcription, at least in endotoxin-treated cells of macrophage/monocyte lineage. Associations have also been made between mutated *ras* gene and COX-2 expression in human breast cancer cell lines (Gilhooly and Rose, 1999), and c-*myb* expression is upregulated in colon tumours and breast cancers (Guerin *et al.*, 1990;Ramsay *et al.*, 1992); c-*myb* overexpression causes a modest induction of COX-2 promoter activity (Ramsay *et al.*, 2000).

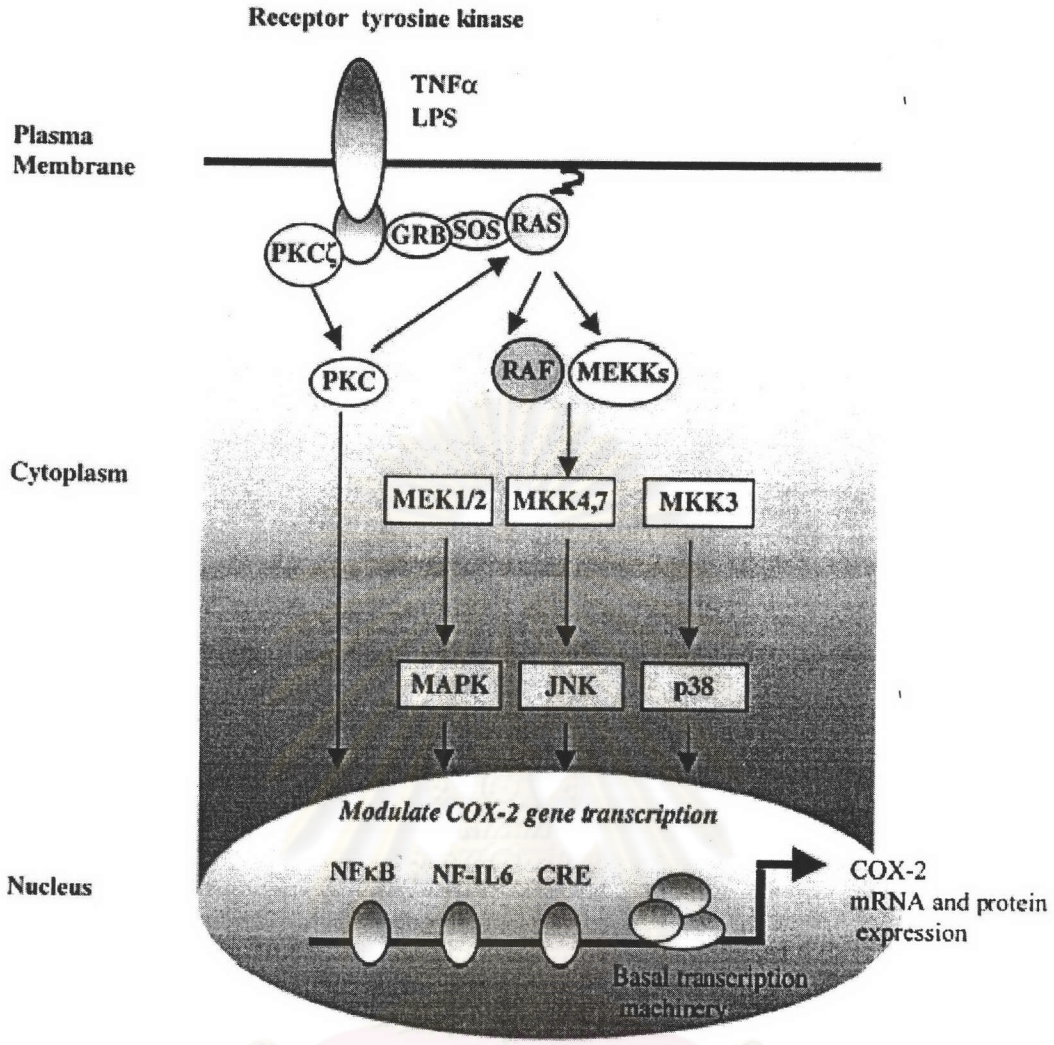

Figure 2.5. Signal transduction pathways influencing COX-2 expression. TNF-α, tumor necrosis factor-α; LPS, lipopolysaccharide; PKC-ζ, protein kinase C-ζ; MAPK, mitogen-activated protein kinase; CRE, cAMP response element (Davies *et al.*, 2002)

Role of COX-2 in Carcinogenesis and Tumor Angiogenesis

Recent studies have highlighted the potential role of COX-2 in carcinogenesis. The induction of COX-2 was reported in carcinomas of the colon, and in subsequent years, increased levels of COX-2 were found in carcinomas of stomach, breast, esophagus, and lung (Eberhart *et al.*, 1994; Ristimaki *et al.*, 1997; Wilson *et al.*,1998; Hida *et al.*, 1998; Wolff *et al.*, 1998). In contrast, the levels of COX-1 are mostly similar between normal and tumor tissues. Importantly, overexpression of COX-2 in human carcinomas appears to be of functional significance because double knockout

mice for adenomatous polyposis coli and COX-2 genes showed a reduction in the size and frequency of intestinal polyps (Oshima *et al.*, 1996). There is also cumulative evidence that selective COX-2 inhibitors prevent carcinogenesis in experimental animals (Boolbol *et al.*, 1996). These findings suggest that COX-2 may be associated with carcinogenesis and/or the progression of certain types of human malignancies.

COX-2 is overexpressed along the continuum of oncogenesis and is likely to be a key player in a number of biologic pathways leading to cancer. COX-2 was consistently and more intensely observed in metastatic lesions compared with the corresponding primary tumor. In general, COX-2 is expressed in 40% to 80% of neoplastic cells in human cancers and the extent and intensity of expression is greater in cancerous than in noncancer cells. Moreover, well- and moderately-differentiated cancers have significantly higher COX-2 expression than poorly differentiated cancers. COX-2 is also detected in non-cancerous cells immediately adjacent to tumor cells and in the angiogenic vasculature within tumors and in pre-existing blood vessels adjacent to tumors (Koki *et al.*, 1999). In contrast, COX-2 is not detected in the vasculature of normal tissues (Buckman *et al.*, 1998).

With regard to the action of COX-2, Tsujii *et al.* (1998) found that COX-2-derived prostaglandins may modulate the production of angiogenic factors by colon cancer cells, thereby inducing newly formed blood vessels that sustain tumor cell viability and growth. Moreover, overexpression of COX-2 in epithelial cells has been shown to result in resistance to apotosis, which in turn leads to dysregulation of growth and normal cell death.

One of the mechanisms by which COX-2 supports tumor growth is inducing the angiogenesis necessary to supply oxygen and nutrients to tumors > 2 mm in diameter (Hanahan and Folkman, 1996). Current evidence indicates that COX-2 promotes tumor-specific angiogenesis (Leahy *et al.*, 2002; Masferrer *et al.*, 2000; Liu *et al.*, 2000), inhibit apoptosis (Souza *et al.*, 2000; Tsujii and DuBois, 1995), and induces proangiogenesis such as VEGF (Shweiki *et al.*, 1992; Liu *et al.*, 1995), inducible nitrogen oxide synthase (iNOS) promotor (Mellilo *et al.*, 1995), IL-6 (Yan *et al.*, 1995), IL-8 (Karakurum *et al.*, 1994), and Tie-2 (Tian *et al.*, 1997).

Sawaoka and his co-workers (1999) demonstrated that NS-398 (COX-2 specific antagonist) suppresses growth of gastric cancer cells *in vitro*. In addition, COX-2 inhibitors induce apoptosis of cancer cells *in vivo* but not *in vitro*. They therefore hypothesized that angiogenesis is involved in the difference between the *in vivo* and *in vitro* effects of COX-2 inhibitor. Later, they examined the effects of COX-2 inhibitor on angiogenesis of COX-2 overexpressing tumor. The results demonstrated that COX-2 inhibitors significantly suppress angiogenesis of COX-2 overexpressing cancer xenografts in athymic mice. Immunohistochemical examinations revealed a close association of the angiogenic index with apoptotic index in the tumor. Consequently, these COX-2 inhibitors appear to induce apoptosis by suppressing angiogenesis.

In a rat model of angiogenesis, the COX-2 inhibitor, celecoxib, blocked corneal blood vessel formation while SC-560, a specific COX-1 inhibitor, had no effect (Masferrer *et al.,* 1999, 2000). Celecoxib dose-dependently inhibited the angiogenic response with an EC_{50} of 0.3 mg/kg/day, and with maximal inhibitory activity of 80% at a dose of 30 mg/kg/day. Plasma levels were determined 4 h after the last dose and found to be approximately 0.3 and 2.0 μg/ml for the 3 and 30 mg/kg/day, respectively. To determine whether the angiogenic activity of celecoxib was due to the inhibition of PG synthesis, they tested an inactive isomer (1,3- *versus* 1,5-diarylpyrazole) of celecoxib. This compound was completely devoid of antiangiogenic activity at a maximal dose of 30 mg/kg/day. This raised the possibility that elevated COX, especially COX-2, plays a role in the growth of certain colon cancer cells due to its ability to act as a tumor promoter via stimulation of angiogenesis.

In spite of their efficacy as anti-angiogenic agent, the precise mechanism(s) for the effect of COX-2 inhibitors remains unclear. Recent finding have demonstrated that COX-2 inhibitor may be due, at least in part, to their ability to inhibit angiogenic factor production. In one such study, Williams *et al.* (2000) demonstrated that mice lacking the COX-2 gene had deficient production of VEGF by fibroblasts and treatment of wild-type fibroblasts with selective COX-2 inhibitor suppressed VEGF production. Overexpression of COX-2 in colon cancer cells induced expression of

VEGF and other angiogenic factors and this effect was inhibited by the COX-2-specific antagonist NS-398 (Tsujii *et al.,* 1998). Consistent with these findings, prostaglandins enhanced VEGF production in many different cells (Ben-Av *et al.,* 1995). COX-2 is also involved in the regulation of VEGF-induced vascular permeability and endothelial cell proliferation (Murohara *et al.,* 1998; Jones *et al.,* 1999). Taken together, these results indicated that COX-2 is crucial for tumor angiogenesis.

Curcumin

Curcumin (diferuloylmethane) is a major yellow pigment that has been isolated from the ground rhizome of the *Curcuma* species, Zingiberaceae. Seven major species of *Curcuma* including *Curcuma longa* Linn., *C. xanthorrhiza* Roxb., C. *wenyujin, C. sichuanensis*; *C. kwangsiensis*; *C. aeruginosa* Roxb.; and *C. elata* Roxb. have been cultivated in China and their composition of curcuminoids were analyzed (Chen and Fang, 1997). Three major curcuminoids namely curcumin, demethoxycurcumin and bisdemethoxycurcumin (Figure 2.6) occur naturally in these *Curcuma* species. It seems that *C. longa* L. (turmeric) has the highest concentration of curcumin as compared to the other species. Turmeric is widely used as a spice and coloring agent in several foods, such as curry, mustard, bean cake, cassava paste and potato chips, as well as in cosmetics and drugs. Curcumin has been demonstrated to have potent antioxidant (Kunchandy and Rao, 1990; Subramanian *et al.*, 1994; Sreejayan, 1994) and anti-inflammatory activity (Huang *et al.*, 1988, 1991, 1997; Shih and Lin, 1993), and to inhibit the carcinogen-DNA adduct (Conney *et al.*, 1991) and tumorigenesis in several animal models (Huang *et al.*, 1992, 1994, 1995; Rao *et al.*, 1995).

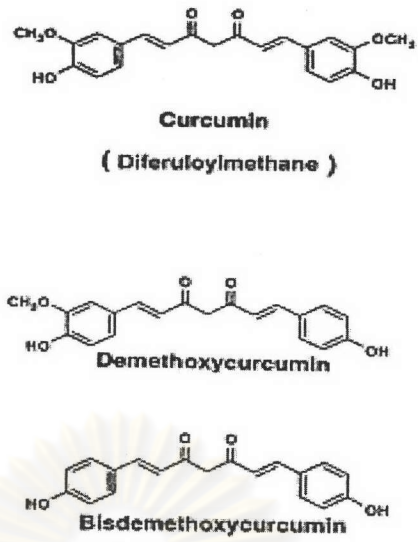

Figure 2.6. Chemical structure of curcumin, demethoxycurcumin and bisdemethoxycurcumin (Chen and Fang, 1997)

Biological Activities of Curcumin

Curcumin has been demonstrated to have potent antioxidant (Kunchandy and Rao, 1990;Subramanian *et al.*, 1994;Sreejayan, 1994). Animals fed curcumin showed decreased levels of lipid peroxides and subsequent reduction in the processes of inflammation (Sreejayan and Rao, 1994). These studies showed that curcumin prevents the production of tissue-damaging free radicals. Curcumin has more potent superoxide anion scavenging activity than demethoxycurcumin and bisdemethoxycurcumin (Reddy and Lokesh, 1992). The same study also included comparison of curcumin to fat-soluble vitamin E. The results showed curcumin to be eight times more powerful than vitamin E in preventing lipid peroxidation.

Another major biological property of tumeric and curcuminoids is its anti-inflammatory activity, which is comparable in strength to steriodal drugs and nonsteriodal anti-inflammatory (NSAIDs) drugs such as indomethacin and phenylbutazone (Ghatak and Basu, 1972; Srimal and Dhawan, 1985). Curcuminoids inhibit enzymes which participate in the synthesis of inflammatory substances in the body derived from arachidonic acid. Arachidonic acid is a compound metabolized in the body to yield important hormone-like substances which play major roles in the

process of inflammation. Arachidonic acid can be converted by the action of the enzyme cyclooxygenase to prostaglandins (PG) and thromboxanes (TX), and by action of the enzyme lipoxygenase to hydroxyeicosatetraenoic acids (HETE) and leukotrienes (LT). Some of the prostaglandins like PGE_2 and PGI_2 dilate the blood vessels, while certain leucotrienes LTB_4, LTC_4 and LTD_4 increase vessel permeability resulting in tissue swelling, which characterizes inflammation. Increased levels of some prostagalandins like PGE produce reddness, swelling and pain of the inflammed part of the body.

Turmeric extracts and curcumin have been also found to be cancer preventing compounds in different tumor models. Recently, curcumin has been considered a potentially important chemopreventive agent against cancer (Kelloff *et al.,* 1997). Animal studies have demonstrated that curcumin inhibits carcinogenesis in various tissues, including skin (Huang, *et al.,* 1997), colorectal (Rao *et al.,* 1995 ; Huang *et al.,* 1994), oral (Tanaka *et al.,* 1994), forestomach (Huang *et al.,* 1994 ; Singh *et al.,* 1998) and mammary cancers (Singletary *et al.,* 1998 ; Pereira *et al.,* 1996). The genetic changes in carcinogenesis in these organs involve different genes, but curcumin is effective in preventing carcinogenesis in several organs.

It has been reported that the anti-tumor effect of curcumin may be related to the modulation of arachidonic acid metabolism. Huang *et al.* (1988), studying the effect of curcumin , chlorogenic acid, cafefeic acid and ferulic acid on tumor protion in mouse skin by 12-O-tetradecanoyol-13-acetate (TPA), observed that all these compounds inhibit the epidermal ornithine decarboxilase (ODC) and epidermal DNA synthesis, being curcumin the most efficient. In 1991 (Huang *et al.*, 1991) it was suggested that curcumin was a potent inhibitor of TPA- and arachidonic acid-induced inflammation and of lipoxygenase and cyclooxygenase activities in mouse epidermis. The IC_{50} for curcumin-dependent inhibition of these enzyme activities was 5-10 μM. In this the results indicated that curcumin inhibited the epidermal metabolism of arachidonic acid via the lipoxygenase and cyclooxygenase pathways.

The inhibition of phorbol 12-myristate 13-acetate (PMA)- or chenodeoxycholate (CD)-induced COX-2 by curcumin in several human gastrointestinal cell lines (SK-GT-4, SCC450, IEC-18 and HCA-7) was studied (Zhang *et al.*, 1999). Treatment with curcumin (1, 5, 10, and 20 μM) suppressed CD (400μg)- and PMA (50 ng/ml)-mediated induction of COX-2 m-RNA as well as protein level and synthesis of prostaglandin E_2 in these cell lines. The results revealed increased rates of COX-2 transcription after treatment with CD or PMA , and these effects were inhibited by curcumin at concentration of 5 μM or above . Treatment with CD or PMA increased the binding of AP-1 to DNA. This effect was also inhibited by curcumin (Huang *et al.*, 1991; Zhang *et al.*, 1999). Furthermore, the activity of COX-2 was found to be directly inhibited by curcumin *in vitro* (Zhang *et al.*, 1999). These findings may provide new insights into the inhibition of COX-2 by curcumin.

Curcumin, a carotenoid pigment isolated from the root of tumeric that is known to inhibit the progression of chemically induced colon and skin cancers, may act by inhibiting angiogenesis. Curcumin's mechanism of action is not fully understood. Several possible mechanisms of the observed anti-angiogenic effects of curcumin have been examined. Arbiser and his co-worker (1998) tested the molecule for its ability to inhibit the proliferation of primary endothelial cells with or without the presence of bFGF, a potent angiogenic factor. They assayed curcumin's ability to inhibit proliferation of an immortalized endothelial cell line, and to inhibit phorbol ester-stimulated VEGF mRNA production. The capacity of curcumin to inhibit bFGF-induced corneal neovascularization in the mouse cornea was also investigated. It was found that curcumin inhibit bFGF-induced proliferation of endothelial cells *in vitro* and angiogenesis *in vivo*. In addition, curcumin demonstrated significant inhibit of bFGF-mediated corneal neovascularization in the mouse. They concluded that curcumin directly inhibits angiogenesis *in vivo* and *in vitro*. Thus, the ability of curcumin in inhibiting carcinogenesis in several organs may be mediated in part through angiogenesis inhibition. Although the exact mechanisms of action of curcumin remain to be elucidated, the inhibitory effects of curcumin on tumor angiogenesis may be mediated through the main pathway of cyclooxygenase pathway.

Biotransformations of Curcumin

Previous studies have investigated the pharmacokinetic properties of curcumin in mice (Pan *et al.*, 1999). After intraperitoneal adminstration of curcumin (0.1 g/kg) to mice, about 2.25 μg/ml of the curcumin appeared in the plasma during the first 15 min. One hour after administration, the levels of curcumin in the intestine, spleen, liver and kidneys were 177, 26, 27, and 7.5 μg/g, respectively. In 2001, Ireson and his co-workers compared curcumin metabolism in human and rat hepatocytes in suspension with that in rats *in vivo*. Analysis by high-performance liquid chromatography with detection at 420 and 280 nm permitted characterization of metabolites with both intact diferoylmethane structure and increased saturation of the heptatrienone chain. The major metabolites in suspensions of human or rat hepatocytes were identified as hexahydrocurcumin and hexahydrocurcuminol. In rats, *in vivo,* curcumin administered i.v. (40 mg/kg) disappeared from the plasma within 1 h of dosing. After p.o. administration (500 mg/kg), parent drug was present in plasma at levels near the detection limit. The major products of curcumin biotransformation identified in rat plasma were curcumin glucuronide and curcumin sulfate whereas hexahydrocurcumin, hexahydrocurcuminol, and hexahydrocurcumin glucuronide (Figure 2.7.) were present in small amounts. To test the hypothesis that curcumin metabolites resemble their progenitor in that they can inhibit COX-2 expression, curcumin and four of its metabolites at a concentration of 20 μM were compared in terms of their ability to inhibit phorbol ester-induced PGE_2 production in human colonic epithelial cells. Curcumin reduced PGE_2 levels to preinduction levels, whereas tetrahydrocurcumin, previously shown to be a murine metabolite of curcumin, hexahydrocurcumin, and curcumin sulfate, had only weak PGE_2 inhibitory activity, and hexahydrocurcuminol was inactive. The results suggest that (*a*) the major products of curcumin biotransformation by hepatocytes occur only at low abundance in rat plasma after curcumin administration; and (*b*) metabolism of curcumin by reduction or conjugation generates species with reduced ability to inhibit COX-2 expression.

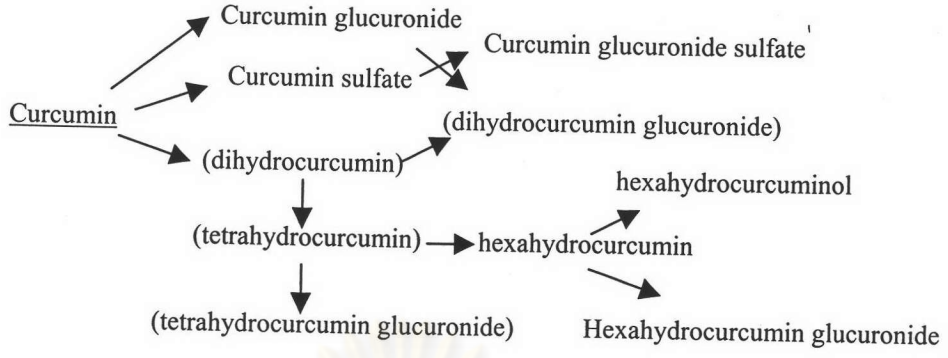

Figure 2.7. Pathways of metabolism of curcumin (Ireson *et al.*, 2001)

In 2001, Sharma *et al.* observed differences in levels of curcumin and its conjugates in plasma and tissue after two administration modes in rats. Administration of dietary curcumin was compared with that of an intragastric (i.g.) bolus. For dietary administration, curcumin and corn oil were mixed with the diet furnish 2% for each. Rats received this level of curcumin in the diet for 14 days. For administration by gavage, curcumin was administered at 500 mg/kg once only or daily for 7 consecutive days. The result of this study demonstrated that dietary curcumin elicited concentrations of the drug in the colon mucosa of between 0.3 and 1.8 μmol/g, whereas plasma levels were around the limit of detection. Curcumin suspended in a solvent mixture and given by i.g. bolus furnished levels of drug in the plasma were 3-6 fold higher than those seen after dietary admistration. Conversely, colon mucosal levels of curcumin after i.g. bolus were only fraction of those observed after dietary administration. Overall, this part of the study suggests that the pharmacokinetic behavior of curcumin after administration of an i.g. bolus of curcumin in suspension is clearly unrepresentative of that of curcumin mixed into the diet. This finding is important because, although in intervention studies curcumin is generally given as a constituent of the diet, studies of its pharmacokinetics and metabolism have been performed mostly with the drug formulated in suspension, using DMSO, aqueous carboxymethyl cellulose solution, or arachis oil as solvent, and administered as an i.g. bolus or via the i.p. route (Ravindranath and Chandrasekhara, 1980; Shoba *et al.*, 1998; Pan, M.H *et al.*, 1999). The results suggest that should oral curcumin be advocated in the chemoprevention of malignancies remote from the liver

or gastrointestinal tract, improvement of its oral bioavailability might be necessary, perhaps by formulating it as a solution.

Curcumin exerts its effects on growth, COX-2 expression, and transcription factor activity in cells *in vitro* at concentrations of 5 μM or above (Plummer *et al.,* 1999; Zhang *et al.,* 1999). The colon mucosa level of curcumin after 14 days feeding observed in this study, 1.8 μmol/g, is more than 300-fold higher than the minimal concentration shown to be active *in vitro*. Although the dose used in this study, 2% in the diet, has been used frequently in rodent intervention studies (Singh *et al.,* 1998; Huang *et al.,* 1994), it is at least 10 times higher than the highest estimated daily human intake of curcumin as dietary constituent (Sambaiah *et al.,* 1982) and does not reflect normal dietary use.

In recent intervention studies, dietary level of 0.1 and 0.2% curcumin have been demonstrated to protect rodents from colon cancer (Rao *et al.,* 1995; Plummer *et al.,* 1999; Kawamori *et al.,* 1999). The dose of curcumin required for efficacy in humans equivalent to the 0.2% dietary concentration or 300 mg/kg/day, which was active in mice, when calculated on the basis of equivalent body surface area (900 mg/m^2 in the mouse), would be 1.6 g per person per day, assuming a body surface area of 1.8 m^2 accompanying a body weight of 70 kg (Freireich *et al.,* 1966). This putative efficacious clinical dose of curcumin is well within the dose range, 0.5, 1.2, 2.1, and 8 g pd for up to 6 weeks, which according to the literature has been administered to humans apparently without adverse effect (Soni and Kuttan, 1992; Cheng *et al.,* 1998).