Chapter 1
Review of literature
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1.1. INTRODUCTION

Cancer is caused by a combination of somatic and genetic changes in a single cell that together result in uncontrolled growth and proliferation. (Hanahan et al, 2000). Continuous division of these cells results in the formation and growth of tumors. A second important process that leads to cancer is that cancerous cells acquire the ability to leave the primary tumor and invade and form colonies at secondary sites (Lodish et al, 2002). This process, known as metastasis, is caused by changes in the cell-substrate and cell-cell adhesion properties of tumor cells (Tepass, 2001). These cells find a way to dislodge themselves from the tumor into the circulatory system and travel within the system until they attach themselves to a secondary site (Lodish et al, 2002).

1.2. ETIOLOGY OF CANCER

A combination of lifestyle, exposure to environmental carcinogens and the overall balance between inherited resistance and sensitivity genes is likely to determine the susceptibility of an individual to cancer. Cancer is believed to result from a series of genetic alterations leading to progressive disorder of the normal controlling growth, differentiation, cell death, or genomic instability. The response of the cell to genetic injury and its ability to maintain genomic stability by means of a variety of DNA repair mechanisms are therefore essential in preventing tumor initiation and progression. Many chemical and physical carcinogens can induce one or more of a variety of mutations in cells when given chronically. A good number of cancer causing chemicals are man-made and used either as industrial agents, pesticides, pharmaceutical chemicals or as food additives. Chemical carcinogens are extremely diverse structures and include both natural and synthetic products. Physical carcinogens such as x-ray, γ-ray and UV ray may cause the formation of pyrimidine dimers, apurinic sites with consequent break in DNA and formation of free radicals, which cause break, leading to somatic mutations. A large number of DNA and RNA viruses have proved to be oncogenic in animals, while only a few viruses have been linked with human cancer.
1.3. IMMUNE SYSTEM AND CANCER

Cancers are caused by the progressive growth of the progeny of a single transformed cell. Therefore, curing cancer requires that all the malignant cells be removed or destroyed without killing the patient. An attractive way to achieve this would be to induce an immune response against the tumor that would discriminate between the cells of the tumor and their normal cellular counterparts. Tumor immunology is a branch of immunology, which study the role of immune system against the tumor development and progression. Tumor recognition is a complex, challenging problem for the immune system, which must distinguish proper cellular growth and organization from neoplastic transformation. This process involves recognition of tumor antigens by effector cells and induction of immunity.

1.3.1. Tumor Immunosurveillance

Having reached the blood stream, metastatic cells are not guaranteed success in founding new colonies. Less than 1 in 10,000 cancer cells that enter the bloodstream actually found a new tumor (Ruoslahti, 2002). Some cells die due to mechanical stress, but the most significant danger to migrating cells is recognition then destruction by leukocytes. Tumor immunosurveillance is a crucial process to limit growth of transformed cells. Several chemokines also play important role in immune homeostasis and migration of immune cells and thus also in tumor immunosurveillance as well as in the formation and propagation of tumors. Several lines of evidence from recent years support the existence of cancer immunosurveillance, especially studies examining effector pathways of the immune system, including T cells, NK cells and the IFN-γ pathway.

1.3.2. Tumor escape mechanisms

Successful growth of tumors is considered as having “escaped” from immunosurveillance, generating the basis for immunoediting or immunosculpting theory (Dunn et al, 2004&2005). Tumors use different mechanisms for escaping from immunosurveillance. On one hand, tumors are thought to behave as immunologically normal tissues thus not being recognized by the immune system. According to this theory, tumor cells do not provide sufficient danger signals to activate the immune system because they express neither microbial immune recognition patterns nor
liberate stress signals to alarm the innate immune cells (Restifo et al, 2002).

Alternatively, tumor cells have been reported to impair the function of the immune system through various mechanisms, including hiding from the immune system in immune sanctuaries, immune ignorance through downregulation of, e.g. major histocompatibility complex (MHC) class I molecules, production of immunomodulatory cytokines such as transforming growth factor-β or IL-10, inhibition of the function and activation of immune cells, protection against lytic activity of immune effectors and inhibition of dendritic cell differentiation, function or survival (Dunn et al, 2004; Zou, 2005). Despite these mechanisms, suppression of tumor establishment or progression by the immune system is clearly observed in cancer patients as well as tumor bearing animals (Yang and Carbone, 2004).

Most of the humoral responses cannot prevent tumor growth. However effector cells, such as T cells, macrophages and natural killer cells have relatively effective tumoricidal abilities. Effector cell activity is induced by antigen-presenting cells and is supported by cytokines (eg: interleukins and interferons).

1.3.3. NK cells

NK cells, which mediate innate immunity are rich in perforin- or granzyme-containing granules, are conspicuously absent from most tumor infiltrates or even precancerous lesions (Whiteside et al, 1998). Although NK cells represent ‘the first line’ of defense against pathogens (Lanier, 2003) and mediate potent antitumor cytotoxicity in vitro, in tumor milieu, they are infrequent, despite the fact that tumor cells frequently downregulate expression of HLA antigens and are enriched in MICA and MICB molecules (Chang et al, 2005). These features make the tumor susceptible to NK cell-mediated cytotoxicity (Lee et al, 2004) and their paucity in tumor infiltrates may be an example of the evasion mechanism preventing NK-cell recruitment to the tumor site.

1.3.4. B-cells

B-cells appear to be necessary for efficient T-cell priming and tumor resistance. In vitro some tumor cells are killed by a process involving coating with antibody, opsonization and subsequent phagocytosis by macrophages; this process may be enhanced by the presence of complement. Alternatively, antibody-coated
tumor cells may be killed in the absence of phagocytosis by antibody dependent cell mediated cytotoxicity (ADCC) when co-cultured with macrophages, NK cells or neutrophils.

1.3.5. T-cells

Tumor-infiltrating lymphocytes, containing various proportions of CD$^3+$CD$^4+$ and CD$^3+$CD$^8+$ T cells, are usually a major component of the tumor microenvironment (Whiteside, 2007). Many of these T cells are specific for tumor-associated antigens, as indicated by clonal analyses (Miescher et al, 1987) and tetramer staining of CD$^8+$ T cells isolated from human tumors (Albers et al, 2002).

1.3.6. Tumor Associated Macrophages

Macrophages present in tumors are known as tumor associated macrophages or TAMs. They are re-programmed to inhibit lymphocyte functions through release of inhibitory cytokines such as IL-10, prostaglandins or reactive oxygen species (ROS) (Mantovani et al, 2003; Martinez et al, 2008).

1.3.7. Complement system

This group of plasma proteins was initially identified on the basis of their ability to ‘complement’ the bacterial activities of antibodies. It is a chief component of both innate and humoral branch of immune system and is of substantial relevance in tumor cell destruction (Carroll, 2004). Complement system is activated by three different pathways: classical, alternative and lectin and leads to a cascade of reactions that ultimately results in the induction of inflammatory responses, phagocyte chemotaxis, opsonization, as well as target cell lysis (Kuby, 2002). Intrinsic interactions among the complement activation products and cell surface receptors provide a basis for the regulation of both B and T cell responses.

1.3.8. Antibody – Dependent Cellular Cytotoxicity (ADCC)

In ADCC, the target tumour cells, which are coated with IgG antibodies, are selectively lysed by killer cells, a special type of lymphomonocytic cell. Thus, the antibody molecule provides the specific recognition signal while the otherwise quiescent and non-specific effector cells are directed to the target cells to provide the cytotoxic event.
1.4. TUMOR METASTASIS

The human body is an undeniably complex system composed of 30 trillion cells. Given the myriad opportunities for cell processes to malfunction, the transformation of some cells into cancers is hardly surprising. Cancer cells not only demonstrate abnormal proliferation but can also develop the ability to migrate from their original site to organs throughout the body. This event, known as metastasis, is the hallmark of malignant cancers. In this process, cancerous cells break through numerous barriers to travel the body’s circulatory highway and invade other organs. These metastasizing cells form new colonies in vital organs around the body, becoming secondary tumors that destroy neighboring cells by robbing them of nutrition. The metastasis accounts for over 90% of lethality in cancer patients (Weigelt et al, 2005). Even if cancer is forced into remission by today’s best treatment methods, the threat of metastasis remains. Unlike local tumors, metastasis cannot be effectively combated with surgery.

The process of metastasis can be divided into three basic steps: departure, penetration into blood or lymphatic vessels and establishment of new colonies. (Figure 1.1). Each step involves numerous complex molecular interactions. In order for malignant cells to leave the primary site, they must overcome the adhesive interactions that keep trillions of normal cells fixed in their proper locations. There are two primary mechanisms of cell adhesion that a metastatic cell must overcome: cell-cell adhesion and cell-ECM adhesion.

Disruption of cell–cell adhesion is an important first step in the stepwise process of metastasis when a metastatic cell detaches from the primary tumor. The exterior portions of E-cadherins form interlocking bonds with E-cadherins on neighboring cells, resembling a “zipper.” Overcoming this cell-cell adhesion is likely a critical early step in metastasis since restoration of E-cadherins to murine cancer cells in studies blocks their ability to form tumors.

The second type of cellular adhesion is between the cell and the extracellular matrix (ECM). Normal cells require binding to a surface to survive and proliferate, a phenomenon known as anchorage dependent growth. One of the defining features of cancer cells is anchorage independent growth. Adhesion between the cell and the
Figure 1.1 Metastatic cascade (Steeg, 2003)
ECM is mediated by integrins, molecules that span the membrane (like E-cadherins) (Hart and Saini, 1992). Integrins are emerging as important mediators of malignant phenotypes during oncogenic transformation.

1.4.1. Cellular Motility

Metastasis fundamentally involves the movement of cells from one site to another. A molecular depiction of cell migration in in vivo models has emerged, which involves dynamic cytoskeletal changes, cell-matrix interactions, localized proteolysis, actin-myosin contractions and focal contact disassembly (Friedl and Wolf, 2003). Several recent studies have implicated components of this cell motility machinery in metastatic progression.

1.4.2. Cellular Invasion

1.4.2.1. Disruption of basement membrane and extra-cellular matrix

Basement membranes that underlie epithelial and endothelial cell layers are a dense meshwork composed of several glycoproteins and proteoglycans (such as type IV collagen, laminin, perlecan). Tumor cells that are able to proteolytically disrupt the basement membrane can progress to overt malignancy and metastasis. The activity of extracellular matrix proteases is normally under tight control through specific localization, autoinhibition and secreted tissue inhibitors (Liotta and Kohn, 2001). Cancerous cells use diverse mechanisms to disrupt this tight regulation and unleash proteolytic activities on the basement membrane and interstitial extracellular matrices. In addition to facilitating tumor invasion, extracellular proteases may generate a diverse array of bioactive cleaved peptides. These products can modulate migration, cancer cell-proliferation and survival and tumor angiogenesis. A positive correlation with tumor aggressiveness has been shown for a variety of degradative enzymes, including heparanases, seryl-, thiol-(cathepsins) and metal-dependent enzymes.

1.4.2.2. Matrix metalloproteinases:

Matrix metalloproteinases (MMPs) are a family of neutral metalloenzymes secreted as latent proenzymes. They require activation through proteolytic cleavage of the amino-terminal domain and their activity depends on the presence of Zn$^{++}$ and Ca$^{++}$ (Coussens and Werb, 1996; Kelly et al, 1998). Five MMP subclasses have been defined, grouped according to the substrate specificity: interstitial collagenases,
gelatinases, stromelysins, membrane-type MMPs (MT-MMPs) and elastases. (Chambers and Martisian, 1997). Increased MMP activity has been detected and shown to correlate with invasion and metastatic potential in a wide variety of cancers, including ovary, lung, prostate, breast and pancreas cancers. (Coussens and Werb, 1996; Liotta et al, 1976; Liotta et al, 1980). Type IV collagen is a critical component of basement membrane architectural scaffolding, on which laminin, heparin sulfate, proteoglycan and minor component of basement membrane are assembled. There are at least two gelatinases: the 72 KD MMP-2 and the 92 KD MMP-9 (Chambers and Martisian, 1997) degrade type IV collagen as a primary substrate and are distinguished by their capacity to degrade gelatin as well. MMP-2 and MMP-9 are distinguishable by immunogenic, molecular regulation and biochemical criteria but not by substrate specificity. Both are secreted as latent pro-enzymes, can be activated by organomercurial compounds with the concomitant autoproteolytic removal of amino-terminal fragment. Other metalloproteases, such as stromelysin, matrilysin and interstitial collagenases are also important in metastasis; they proteolyse other matrix proteins and may degrade type IV collagen in the pepsin-sensitive nonhelical domains in a less specific fashion. (Chambers and Martisian, 1997). In addition MMPs are inhibited by members of the endogenous tissue inhibitor of metalloproteinase (TIMP) family (Stetler-Stevenson et al, 1989). There are now five member of TIMP family. The relationship between the levels of activated MT-MMPs, MMPs and free TIMPs determines the balance between matrix degradation and matrix formation or stabilization. There are selective specific interaction between TIMPs and MMPs as well as more general interactions, such as those between TIMP-1 and TIMP-2 and activated MMPs, where both may be inhibitory. The functions of TIMP-1 and TIMP-2 may not be limited to metalloproteinase blockade. These proteins may also act as cytokines and recognize specific receptors. It was demonstrated that TIMP inhibited angiogenesis in vivo and both capillary endothelial cell proliferation and migration in vitro (Murphy et al, 1993; Schanper et al, 1993).

Progression of invasive carcinomas requires collusion between tumor cells and multiple nontransformed cell types residing in (or being recruited to) the tumor stroma. Indeed, several histopathological markers of stromal cell cooption with
tumors, such as fibrosis and leukocytic infiltration are frequently correlated with an increased likelihood of metastatic relapse. It could be possible that tumor cells that can convert reactive stromal infiltrates from preservers of homeostasis into accomplices in malignancy earn a selective advantage in the primary tumor and at sites of metastasis.

1.4.2.3. Intravasation

In order to metastasize, cancer cells must invade tumor associated vasculature to gain access to distant sites in the body. This is facilitated by the need of developing tumors to establish neo-vasculature in order to grow beyond the diffusion limit of preexisting blood vessels (Hanahan and Folkman, 1996). Perhaps because lymphatic vessels are more leaky in their design than blood vessels-owing to the lack of tight intercellular junctions between lymphatic endothelial cells- the presence of lymph-node metastasis often represents an early prognostic indicator of tumor invasiveness and metastatic dissemination in several types of carcinoma and in melanoma (Alitalo et al, 2005). However other metastatic malignancies such as sarcomas, are notorious for metastasizing to distant sites without any prior evidence of local spread to regional lymph nodes. Regardless, it is thought that access to all organs of the body (lymph nodes excluded) is predominantly through the hematogenous circulation.

The molecular mechanisms controlling intravasation remain to be defined. It is possible that once cancer cells become highly motile with in primary tumors, they are naturally attracted to blood vessels due to chemo-attractive gradients and extracellular matrix tracks emanating from (or terminating) there. Indeed, this was observed in the intravital imaging studies of experimental mammary carcinomas (Condeelis and Segal, 2003).

1.4.2.4. Life in transit

Once malignant cells have invaded this circulatory compartment, they attain ready access to virtually all organ of the body. However they must be able to survive several stresses, including physical damage from hemodynamic shear forces and immune mediated killing. Recent advances have begun to make headway into the mechanisms that allow metastatic cells to evade these perils.

Circulating tumor cells may promote their survival by co-opting blood platelets, using them as shields. Clinically observed for well over a century,
malignancies have a tendency to induce a hypercoagulable state in their hosts (Nash et al, 2002). Histopathological analysis of early-stage hematogenous metastases in humans frequently reveals the coexistence of thrombosis, with abundant fibrin deposition (Ruiter et al, 2001). Disrupting the interaction between tumor cells and platelets in experimental models has validated this relationship as causal for metastasis to multiple target organs (Nash et al, 2002). Consequently, tumor emboli are believed to possess greater metastatic potential than naked tumor cells, owing at least in part to their resistance to immune-mediated mechanisms of clearance and to physical hemodynamic forces (Nash et al; 2002).

1.4.2.5. Extravasation

Having invaded and endured the circulation, metastatic cell must at some point escape once again, but this time out of the endothelial vasculature and into a target tissue in process called extravasation. Exactly when this event occurs in the cascade of metastasis may vary from tumor to tumor. In some cases, considerable growth within the intravascular space may occur until the lesion physically bursts through the limiting surrounding vasculature. (Al-Mehdi et al, 2000). The activation of Src family kinases in endothelial cells exposed to VEGF induces disruption in endothelial cell junctions, which can facilitate metastatic extravasation. Consistent with this, Src knock out mice were protected from lung metastatic colonization by VEGF-secreting cancer cells (Criscuoli et al, 2005).

1.4.3. Patterns of colonization

The organ distribution of full-blown metastases from a primary tumor is not random. After analyzing secondary cancer outgrowths in a series of autopsies for breast cancer victims, Stephen Paget proposed that disseminated cancer cells, or “seeds” would only colonize organ microenvironments, or “soils”, that were compatible with their growth (Paget, 1989). Clinical observation of cancer patients supports the notion that circulatory patterns alone provide only a partial explanation for preferred site of metastasis (Fidler, 2003). It is now appreciated that at least two classes of determinants affect site specific metastatic outgrowth. First, there must be an initiation of a viable premetastatic niche with in the target organ- one that facilitates the initial survival of extravasated tumor cells in a nonreceptive target organ.
Subsequently, the invading metastatic cell must display the appropriate functions to effectively colonize the new site.

**1.4.4. In a state of Dormancy**

The vast majority of tumor cells that have undergone extravasation still are not able to effectively colonize the new site. For reasons that are unclear, disseminated tumor cells might enter a state of cell cycle arrest or dormancy, possibly induced by incompatibilities between the cancer cells and the secondary soil(s). Recall that, to a wandering cancer cell, no soil is really friendly but only tolerant at best. To escape dormancy or to colonize a new organ outright, disseminated tumor cells must have the capacity to productively interact with the new microenvironment in order to extract growth and survival advantages.

**1.5. ANGIOGENESIS**

In each tissue, expansion of the blood supply is required for the growth of metastases beyond the limits of diffusion and provides oxygen, growth factors, nutrients and metabolites. Angiogenesis is the formation of a new blood supply from preexisting vasculature and is stimulated by an angiogenic ‘switch’ that occurs when the ratio of inducers to inhibitors tips in favor of inducers (Steeg, 2006). Like normal tissues, tumors require an adequate supply of oxygen, metabolites and an effective way to remove waste products (Papetti et al, 2002) These requirements may vary, however, among tumor types and change over the course of tumor progression (Hlatky et al, 2002). But gaining access to the host vascular system and generation of tumor blood supply are rate-limiting steps in tumor progression. The angiogenic switch can occur at different stages of the tumor-progression pathway, depending on the tumor type and the environment. The fact that tumors are dependent on blood supply has inspired many researchers to search for anti-angiogenic molecules and to design anti-angiogenic strategies for cancer treatment.

**1.5.1. Process of angiogenesis**

During embryonic vasculogenesis, blood vessels are formed de novo, from endothelial cell-precursors (angioblasts) that assemble into a primary capillary plexus. This primitive network then differentiates and new blood vessels sprout and branch
from preexisting capillaries – the process of angiogenesis. (Carmelie., 2000). Nonsprouting angiogenesis occurs by the formation of transcapillary posts of ECM and the proliferation of endothelial cells within an existing vessel, thereby splitting the vessel into two or more capillaries. This type of angiogenesis predominates in the lung during organogenesis (Risau, 1997). Sprouting angiogenesis involves the proteolytic degradation of the basement membrane surrounding endothelial cells in a vessel, followed by migration and proliferation of endothelial cells into the adjacent stroma. Differentiation and maturation of the endothelial cells, lumen formation, recruitment of pericytes and coalescence of tubes into loops completes the process of new blood vessel formation. Sprouting angiogenesis occurs in the yolk sac and in the embryo during later organogenesis, especially in the brain (Risau et al, 1997).

1.5.2. Factors inducing angiogenesis

The signals that initiate and sustain angiogenesis are multiple and complex. Proangiogenic cytokines and growth factors include vascular endothelial growth factors (VEGFs), fibroblast growth factors (FGFs), angiopoietins, transforming growth factor- (TGF β), platelet-derived growth factors (PDGFs), tumor necrosis factor-α (TNF α), epidermal growth factor (EGF), interleukin-8 (IL-8) and angiogenin, which are secreted by inflammatory cells (e.g., mast cells and macrophages), pericytes, keratinocytes (during epidermal wound healing), or tumor cells. Some of these factors act directly by binding to their respective receptors on endothelial cells to induce proliferation and/or migration, while others act on local stromal or inflammatory cells to stimulate angiogenesis (Li et al, 2003; Weinstat-Saslow and Steeg, 1994). ECM and basement membrane components also transduce both proangiogenic and antiangiogenic signals by binding to integrins on endothelial cells. The ECM in addition acts as a sequestration/storage compartment for angiogenic growth factors such as VEGF, bFGF and TGF 1, which can be released by proteolytic degradation of the ECM (Kalluri, 2003).

1.5.3. Tumor angiogenesis

Tumor angiogenesis involves an "angiogenic switch" that shifts the balance to more pro- than antiangiogenic signals and often occurs at an early, premalignant stage (Hanahan and Folkman, 1996; Carmeliet and Jain, 2000; Bergers and Benjamin,
Signals that trigger the angiogenic switch in tumors include metabolic stress, such as hypoxia and acidosis, genetic mutations that activate certain oncogenes or inactivate/delete tumor suppressor genes and the presence of an immune/inflammatory response within the tumor/lesion (Carmeliet and Jain, 2000). Without adequate vascular perfusion, the high proliferation rate in hyperplastic/dysplastic lesions is balanced by increased differentiation, apoptosis and/or necrosis and so tumor volume is limited. Developing hypoxia in a growing tumor mass upregulates VEGF expression by tumor cells and when VEGF levels become high enough to overcome endogenous antiangiogenic signals, angiogenesis is initiated (Hanahan and Folkman, 1996; Bergers and Benjamin, 2003). Hypoxia-activated HIF-1α also induces transcription of other angiogenic genes including NOS, PDGF-B and Ang-2 (Carmeliet and Jain, 2000). Many tumors have been found to express various pro-angiogenic growth factors, such as FGFs and VEGF (Bergers and Benjamin, 2000). Transformation of nontumorigenic epithelial cells and fibroblasts with mutant ras oncogenes has been shown to up-regulate VEGF expression, which correlates with acquisition of a tumorigenic and angiogenic phenotype (Petit et al, 2000; Grunstein, 1999). Loss of the tumor suppressor gene, p53, which frequently occurs in human cancers, can also contribute to the angiogenic switch by enhancing HIF-1α levels and thereby induction of VEGF expression, as well as by down-regulating the expression of the angiogenesis inhibitor, TSP-1 (Bergers and Benjamin, 2003; Ravi et al, 2000).

Expression of VEGF by tumor cells also induces vascular permeability, which leads to extravasation of plasma proteins providing a provisional matrix for migrating endothelial cells (Bergers and Benjamin, 2003). In addition to inducing angiogenesis from existing blood vessels, angiogenic factors released from tumor cells can recruit bone marrow-derived circulating endothelial progenitor cells, which express VEGFR-2 (Flk-1), as well as VEGFR-1-expressing hematopoietic stem and progenitor cells that facilitate the incorporation of endothelial progenitor cells into tumor vessels (Rafii et al, 2002). Another mechanism tumor cells use to enhance perfusion in solid tumors such as melanoma is the capacity of certain cancer cells to mimic endothelial cells to form ECM-rich, fluidconducting networks, called vasculogenic mimicry (Hendrix et al, 2003). Tumor blood vessels are different from normal blood vessels in that the
tumor vasculature is chaotic with tortuous, leaky and dilated or uneven in diameter. (Carmeliet and Jain, 2000; Ruoslahti, 2002).

The point at which these ‘normal’ processes differ from pathological angiogenesis is in the tightly regulated balance of pro- and anti-angiogenic signals. During normal physiological angiogenesis, new vessels rapidly mature and become stable. By contrast, tumors — described as “wounds that never heal” (Dvorak, 1986) — have lost the appropriate balances between positive and negative controls. One characteristic feature of tumor blood vessels is that they fail to become quiescent, enabling the constant growth of new tumor blood vessels. Consequently, the tumor vasculature develops unique characteristics and becomes quite distinct from the normal blood supply system. Tumor blood vessels are architecturally different from their normal counterparts (they are irregularly shaped, dilated, tortuous and can have dead ends). They are not organized into definitive venules, arterioles and capillaries like their normal counterparts, but rather share chaotic features of all of them. The vascular network that forms in tumors is often leaky and haemorrhagic, partly due to the overproduction of vascular endothelial growth factor (VEGF; also known as vascular permeability factor, VPF). Perivascular cells, which are usually in close contact with the endothelium, often become more loosely associated or less abundant (Benjamin, et al, 1999; Morikawa et al, 2002). Tumor vessels have also been reported to have cancer cells integrated into the vessel wall (Folberg et al, 2000; McDonald et al, 2000) and some tumors rely heavily on vasculogenesis, recruiting endothelial precursor cells from the bone marrow (Lyden et al, 2001). Blood flows irregularly in tumor vessels, moving more slowly and sometimes even oscillating. This leads to dysfunctional capillaries. Tumors can be quite heterogeneous in their vascular patterns and are able to overproduce their capillary networks. In normal tissues, by contrast, vessel density is dynamically controlled by the metabolic needs of nutrients and oxygen. So, the structural and functional abnormalities in tumor vessels reflect the pathological nature of their induction. Although we do not fully understand the molecular controls of all of these abnormalities, we can surmise that they are the result of the imbalanced expression and function of angiogenic factors.
1.6. CELL CYCLE REGULATION AND CANCER

Cancer cells are characterized by a failure of cell cycle control which results in their over proliferation (Griffiths et al, 2002). The process of replicating DNA and dividing a cell can be described as a series of coordinated events that compose a “cell division cycle”, illustrated for mammalian cells. At least two types of cell cycle control mechanism are recognized. The first type control involves highly regulated kinase family (Morgan, 1995). Second type of cell cycle regulation, checkpoint control, is more supervisory. Cell cycle checkpoints sense flaws in critical event such as DNA replication and chromosome segregation (Eledge, 1996). When checkpoints are activated, for example by underreplicated or damaged DNA, signals are relayed to the cell-cycle progression machinery. These signal cause a delay in cell cycle progression, until the danger of mutation has been averted and if the damage is not repairable the cell will lead to Programmed cell death (PCD).

According to the nuclear morphology, PCD can be divided into three subclasses: 1) classic apoptosis characterized by chromatin condensed to compact and almost geometric figures (stage 2 chromatin condensation), 2) apoptosis –like PCD with less compact, lumpy chromatin masses (stage 1 chromatin condensation) and 3) necrosis-like PCD that occurs either in the complete absence of chromatin condensation or at best with chromatin clustering to form loose speckles (Leist and Jäättela, 2001). Normally, only the fastest and most effective death pathway in evident, but one cell may also display characteristics of several death programs simultaneously (Bursch et al, 1996).

1.7. APOPTOSIS

Apoptosis is a process of programmed cell death and is regulated by various proteins, including membrane-associated proteins, such as Fas (CD95), Fas ligand (CD95L), tumor necrosis factor (TNF) α, TNF receptor and cytoplasmic/ nuclear proteins, such as Bcl-2, Bak, Bax and caspases (Selam et al, 2001; Nagata and Golstein, 1995; Daikoku et al, 1998; Tao XJ et al, 1997; Chittenden et al, 1995). Abnormalities in cell death control can contribute to a variety of diseases, including cancer, autoimmunity and degenerative disorders.
The most evident morphological signs of apoptosis are cellular shrinkage, membrane blebbing, nuclear condensation and fragmentation, which are the final steps of consequential signaling cascades (Huppertz et al, 1999). The swelling of the outer mitochondrial membrane (Vander Heiden et al, 1997) and release of cytochrome c (Kluck et al, 1997; Yang et al, 1997) and apoptosis inducing factor, an oxidoreductase-related flavoprotein (Susin et al, 1999) from the mitochondrial intermembrane space are also reported during apoptosis. Molecular changes during apoptosis include internucleosomal DNA cleavage (Willie 1980) and randomization of the distribution of phosphatidyl serine (PS) between the inner and outer leaflets of the plasma membrane (Fadok and Henson, 1998).

There are two different signaling pathways in cell leading to apoptosis, the death receptor pathway and mitochondrial pathway (Figure 1.2). Death receptor pathway is initiated at the cell surface through the Fas/TNF-R1 family protein (Ashkenazi and Dixit, 1998). Ligation of Fas either by its ligand, FasL or by its agonistic antibodies triggers the homotrimeric association of the receptors. The clustering of the death domains in the intracellular portion of the receptors recruits the adaptor molecule, FADD which then recruits pro-caspase 8. Activation of pro-caspase 8 through self cleavage leads to a series of downstream events, including activation of pro-caspase 3, cleavage of multiple caspase substrates and final cell death. There are however, a large number of death stimuli that do not seem dependent on the death receptor pathway. Instead, the death signals are transmitted to mitochondria through a bcl-2 family death agonist Bid, where release of cytochrome c is induced (Xiao-Ming, 2000). Cytochrome c activates Apaf-1, in the presence of dATP, which in turn activates pro-caspase 9 which cleave down stream effector caspase, caspase-3.

1.7.1. Caspases

The morphological changes that we recognize as apoptosis and associated biochemical changes seen in a eukaryoric cell are caused by proteases. Specifically activation of a family of intracellular cysteine proteases which cleave their substrates at aspartic acid residues, known as caspases for Cysteine Aspartyl-specific Proteases (Alnemri et al, 1996) These proteases are present as inactive zymogens in essentially all animal cells, but can be triggered to assume active states, generally involving their
Figure 1.2 Extrinsic and Intrinsic pathways leading to apoptosis (Werner et al, 2004)
proteolytic processing at conserved aspartic acid (Asp) residues. Currently, 11 human caspases have been identified: caspase-1–10 and caspase-14 (Alnemri et al, 1996; Pistritto et al, 2002). The protein initially named caspase-13 was later found to represent a bovine homologue of caspase-4 (Koenig et al, 2001) and caspase-11 and -12 are murine enzymes that are most likely the homologues of human caspase-4 and -5. There is a clear evolutionary tendency to increase the number of caspases over phylogenetic time, from four in C. elegans to seven in Drosophila and 11 in mice and humans (Lamkanfi et al, 2002).

From a functional perceptive, it is useful to view the caspases as either upstream (initiator) caspases or downstream (effector) caspases (Salvesen and Dixit, 1997). The proforms of up-stream initiator caspases possess large N-terminal prodomains, which function as protein interaction modules, allowing them to interact with various proteins that trigger caspase activation. Downstream caspases are largely dependent on upstream caspases for their proteolytic processing and activation. Initiator caspases possess long prodomains containing one of two characteristic protein–protein interaction motifs: the death effector domain (DED) (caspase-8 and -10) and the caspase activation and recruitment domain (CARD) (caspase-1, -2, -4, -5, -9, -11 and -12), providing the basis for interaction with upstream adaptor molecules. Among the initiator caspases, caspase-1, -11 and -5 form a subclass of caspases that controls both apoptosis and certain inflammatory responses. On the other hand, caspases that perform the downstream execution steps of apoptosis by cleaving multiple cellular substrates are typically processed and activated by upstream caspases. The downstream caspases form an ‘executioner’ class (caspase-3, -6, -7) and are characterized by the presence of a short prodomain. Due to the strict substrate recognition specificity, caspases cleave targets at one or a few highly selective sites without degrading other proteins.

1.7.2. p53

The p53 protein plays a central role in the cell. Cells that are insulted by oncogene expression, DNA damage or other forms of stress stabilize the p53 protein by phosphorylation or other modifications (Vogelstein et al, 2000; Xu, 2003). Once activated, p53 acts as a transcription factor and activates or represses a variety of
genes involved in, for example, cell-cycle regulation, the induction of apoptosis, or senescence (e.g., BAX, NOXA, PUMA, BID, CD95, APAF-1, DR5, p53AIP1). (Miyashita and Reed, 1995; Oda et al, 2000; Nakano and Vousden, 2001; Moroni et al, 2001; Oda et al. 2000; Wu et al, 1997). Each of these genes when silenced or removed from a particular model system produced partial resistance to p53-induced apoptosis. Most likely, these genes govern the decision to live or die based on the cell type investigated and the applied death stimulus (meaning, each gene contributes to certain death pathways, but not all). The p53-dependent apoptosis pathway is of particular interest in cancer therapy since it can be used to eliminate the tumors.

The involvement of p53 in multiple biological pathways suggests that its loss may have dramatic consequence for the cell and that it may be an important event in cancer development. The study of p53 gene knockout mice (Donehower et al, 1992) and the analysis of the status of the p53 gene in human tumors show that the loss of p53 activity is a key event in cancer development. In humans, p53 is found to be mutated in about 50% of cancers (Hollstein et al, 1999; Soussi et al, 2000).

1.7.3. The Bcl-2 family

A central player in the cellular genetic program and the link between apoptosis and cancer, emerged when B-cell lymphoma 2 (BCL-2), the gene that is linked to an immunoglobulin locus by chromosome translocation in the follicular lymphoma, was found to inhibit cell death, rather than promote proliferation (Vaux et al, 1988). Now the bcl-2 family of intracellular proteins is considered as the central regulator of caspase activation and its opposing factions of anti- and pro- apoptotic member arbitrate the life- or- death decision. A better understanding of how the Bcl-2 family controls caspase activation should result in new, more effective therapeutic approaches.

In mammals, Bcl-2 has at least 20 relatives associated with mitochondrial outer membrane and the endoplasmic reticulum/ nuclear membrane, all of which share at least one conserved bcl-2 homology (BH) domain. The class includes four other anti-apoptotic proteins: Bcl-xL, Bcl-w, A1 and Mcl1 and two groups of proteins that promote cell death: the Bax and BH3-only families. Bcl-2 is an integral membrane protein, even in healthy cells (Janiak et al, 1994), whereas Bcl-w and Bcl-xL only
become tightly associated with the membrane after a cytotoxic signal by an induced conformational change. These prosurvival Bcl-2 family proteins can prevent cytochrome c release and hence activation of caspases. It is becoming increasingly evident that every nucleated cell requires protection by at least one Bcl-2 homologue and that the abundance of these ‘guardians’ regulate tissue homoeostasis. Bcl-2 overexpression in hematopoietic lineages yield excess B,T and myeloid cells that are refractory to diverse cytotoxic insults (McDonnell et al, 1989; Strasser et al, 1991; Strasser et al, 1991; Sentman et al, 1991; Ogilvy et al, 1999). Conversely, inactivation of Bcl-2 homologous genes augments apoptosis in specific cell types, presumably because the concentrations of other guardians are too low to compensate.

The BH3-only proteins seem to be sentinels that are charged with triggering apoptosis in response to developmental cues or intracellular damage (Huang and Strasser, 2000). Initial knockout studies indicate that individual BH3-only proteins could have specialized physiological roles. Bid seems to promote death by activating Bax and Bak and it might also inactivate pro-survival relatives (Wang et al, 1996). Bax and Bak are widely distributed, whereas little-studied protein Bok is more prevalent in reproductive tissues. The presence of either Bax or Bak seems to be essential for apoptosis in many cell types which contribute to the permeabilization of outer mitochondrial membrane, allowing efflux of apoptogenic proteins (Gross et al, 1999).

1.8. INFLAMMATION

Cancer is fundamentally a chronic inflammatory disease, representing a state of continuous angiogenic and stromagenic support for the established neoplasm. (Sallusto and Lanzavecchia, 1999; Lotze, 1997). The inflammatory process involves a protective influx of white cells, complement, antibody and other plasma proteins into a site of infection or injury. It can nonetheless cause a range of uncomfortable symptoms. The typical effects of inflammation were identified by the Roman physician Aulus Cornelius Celsus, they are rubor (redness), tumor (swelling), calor (heat) and dolor (pain). A vigorous inflammatory response can also cause shock and death. Dilation and increased permeability of the blood vessels lead to increased local
blood flow and leakage of the fluid and account for the heat, redness and swelling. The migration of cells into the tissue and their local actions account for the pain. The main cell types seen in an inflammatory response in its initial phases are neutrophils, followed by macrophages, which mature from their precursor monocytes; these are therefore known as inflammatory cells.

1.8.1. Proinflammatory cytokines.

Cytokines are small, nonstructural proteins with molecular weights ranging from 8,000 to 40,000 D. There is no amino acid sequence motif or three dimensional structures that link cytokines; rather their biological activities allow them to group into different classes. They play important physiological roles in cell-to-cell communication, development and differentiation. For most part, cytokines are primarily involved in host response to disease or infection. Based on their biological activities, cytokines are often grouped as lymphocyte growth factors, mesenchymal growth factors, interferons, chemokines and colony stimulating factors. Some have been given the name ‘interleukin’ to indicate a product of leukocyte and a target of leukocytes.

Another way to look at some cytokines is their role in inflammation and this is particularly relevant to the importance to pain. Hence, some cytokines clearly promote inflammation and are called pro-inflammatory cytokines, whereas other suppresses the activity or the production of pro-inflammatory cytokines and are called anti-inflammatory cytokines. The important pro-inflammatory cytokines include TNF, IL-1, IL-6 and IL-12.

1.8.1.1. TNF-α

In 1962, O’Malley reported the production of a factor in the serum of normal mice challenged with endotoxin; since this serum could induce necrosis when administered to tumor-bearing animals, it was named tumor necrosis serum (TNS) (O’Malley et al, 1962). When Carswell et al. confirmed the activity in serum of mice injected with endotoxin, they renamed it tumor necrosis factor (TNF) (Carswell, 1975). Almost two decades ago, tumor necrosis factor (TNF) was identified as a protein produced by the immune system that played a major role in suppression of tumor cell proliferation. Extensive research since then has revealed that TNF is a...
major mediator of inflammation, viral replication, tumor metastasis, transplant rejection, rheumatoid arthritis and septic shock.

TNF and its family members represent a double-edged sword. TNF can contribute to tumorigenesis by mediating the proliferation, invasion and metastasis of tumor cells. Whereas physiologically they are important cytokines and required for normal responses, their inappropriate expression is harmful. TNF has been shown to be an autocrine growth factor for a wide variety of tumors. Through the activation of NF-κB, TNF-α induces the expression of various genes that are involved in invasion and metastasis, including adhesion molecules, urokinase plasminogen activator (UPA), MMP-9, COX-2 and VEGF. Several reports indicate that TNF-α is highly carcinogenic and mice deficient for TNF-α are resistant to skin carcinogenesis (Moore, et al, 1999).

1.8.1.2. IL-1β

IL-1 is a highly inflammatory molecule which showed overproduction in almost all inflammatory diseases. The reduction in its production or activity in a variety of disease states is likely a sensible therapeutic strategy. IL-1 is a cytokine with diverse immunologic, physiologic and hematopoietic effects produced mainly by macrophages and monocytes. Two forms of IL-1 (α and β) exist. Although these two glycoproteins of 17-kd molecular weight are distinct gene products and despite only a 26% homology and have same biological activities (Dinarello, 1988; Dinarello, 1991; March et al, 1985).

1.8.1.3. IL-6

IL-6 is a proinflammatory cytokine with a wide variety of biologic effects. It is produced by a range of cells, including T cells, monocytes and macrophages, fibroblasts, keratinocytes and endothelial cells and its variety of biologic effects led to its initial, independent characterizations as a B-cell growth factor and T-cell differentiation factor, a plasmacytoma growth factor and a hepatocyte stimulating factor. (Bajorin et al, 1990; Gauldie et al, 1987; Tosato et al, 1988.). IL-6 is a 21 to 30 kd glycoprotein of 212 amino acids that binds to a specific receptor that requires the same 130 kd membrane glycoprotein for mediation of signal transduction, as has been described for several cytokines (Tagga et al, 1989; Yanasaki et al, 1988). It is
involved in the pathogenesis of many diseases, including multiple myeloma, Castleman’s disease, glomerulonephritis, autoimmune diseases and certain neurological disorders. In addition, patients with certain leukemias and autoimmune disorders have improved after administration of heterologous in vitro neutralizing IL-6 antibody. IL-6 has been demonstrated to promote growth of multiple myeloma, Kaposi’s sarcoma and prostate cancer cells (Wei et al, 2003). And along with IL-1 and TNF, IL-6 can stimulate tumor cell growth and also can promote resistance to therapy (Tricot, 2000).

1.8.1.4. GM-CSF

Granulocyte monocyte-colony stimulating factor (GM-CSF) is a pleiotropic cytokine produced by a number of different cell types. Besides granulocyte-macrophage progenitors, GM-CSF is also a growth factor for erythroid, megakaryocyte and eosinophil progenitors. GM-CSF also modulates the function of differentiated white blood cells. For example, GM-CSF stimulates macrophages for antimicrobial and antitumor effects. The cytokine further enhances healing and repair by its actions on endothelial cells, fibroblasts and epidermal cells. GM-CSF is the pivotal mediator of the maturation and function of dendritic cells, the most important cell type for the induction of primary T-cell immune responses.

1.9. NITRIC OXIDE

Nitric Oxide (NO) is a gaseous signaling molecule that regulates various physiological and pathophysiological responses in the human body, including circulation and blood pressure, platelet function, host defense and neurotransmission in central nervous system and in peripheral nerves. High levels of NO are produced in response to inflammatory stimuli and mediate proinflammatory and destructive effects. However, like other inflammatory mediators, NO has also protective effect in some inflammatory responses. Since its discovery in 1987 (Palmer, et al, 1987; Ignarro et al, 1987) NO has been a target of intensive research and drug development.

1.9.1. Molecular mechanisms of action

NO is a reactive molecule that has a variety of effects depending on the relative concentrations of NO and the surrounding milieu in which NO is produced.
The molecular mechanisms that mediate the biological activities of NO can be divided into three categories. Firstly, NO reacts readily with transition metals, such as iron, copper and zinc. These metals are abundantly present in prosthetic groups of enzymes and other proteins and by that mechanism, NO regulates the activity of various enzymes. Secondly, NO is able to induce the formation of S-nitrosothiols from cysteine residues in a reaction called S-nitrosylation. Nitrosylation has been shown to modify the activity of several proteins involved in cellular regulatory mechanisms (Stamler et al, 2001). Thirdly, NO reacts very quickly with superoxide anion (O$_2^-$), resulting in the formation of peroxynitrite (ONOO$^-$). Peroxynitrite is a nitrating agent and a powerful oxidant that is able to modify proteins, lipids and nucleic acids.

1.9.2. Nitric oxide synthase

NO is synthesized from L-arginine in a reaction catalyzed by a family of nitric oxide synthase (NOS) enzymes. Conversion of L-arginine to NO and L-citrulline requires also NADPH and O$_2$ as cosubstrates and (6R)-tetrahydrobiopterin (BH4), FAD, FMN and iron protoporphyrin IX (haem) as co-factors (Knowles and Moncada, 1994; Marletta, 1994; Alderton et al, 2001). Three different NOS isoforms have been characterized. The neuronal (nNOS, NOS I) is predominantly expressed in neurons in brain and peripheral nervous system (Boissel et al, 1998). Endothelial NOS (eNOS, NOS III) is mainly expressed in endothelial cells (Shaul, 2002). Both nNOS and eNOS are constitutively expressed and are inactive in resting cells. Increase in free intracellular calcium concentration ([Ca$^{2+}$]i) stabilizes the binding of calmodulin to eNOS and nNOS and activates the enzyme to produce NO. Stimuli that increase the [Ca$^{2+}$]i (eg. acetylcholine in endothelial cells) trigger the production of NO and when the [Ca$^{2+}$]i decreases, the NO production ceases. That regulation makes NO production by constitutively expressed NOSs transient and short lasting (Knowles and Moncada, 1994; Marletta, 1994; Alderton et al, 2001).

The third isoform of the NOS family is the inducible NOS (iNOS, NOS II). No iNOS expression is found in most resting cells. Exposure to microbial products, such as lipopolysaccharide (LPS) and dsRNA or proinflammatory cytokines such as interleukin-1 (IL-1), tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) induces the expression of iNOS gene in various inflammatory and tissue cells. Binding of
calmodulin to iNOS is tight even at low [Ca2+]i and therefore, iNOS is also called as a calcium-independent NOS and it can constantly produce high levels of NO for prolonged periods (Alderton et al, 2001; Bogdan, 2001; Kleinert et al, 2003).

1.9.3. Regulation of iNOS expression

Although iNOS expression has shown in various mouse and human cells, there are marked cell type and species specific differences in the responsiveness of iNOS expression to different stimuli (Bogdan, 2001; Kleinert et al, 2003; Moilanen et al, 1999). For instance, responses in human cells seem to be quite different from those in mouse cells, which have been widely used in the studies on iNOS expression.

Transcription of mouse iNOS gene is regulated by ~1 kb promoter (Xie et al, 1993; Lowenstein et al, 1993), which contains putative binding sites for a number of transcription factors. In vivo footprinting assay revealed LPS-induced binding to octamer (Oct), E-box, nuclear factor kappa B (NF-κB) and interferon stimulated response element (ISRE) sites (Goldring et al, 1996). The mouse iNOS promoter contains two NF-κB elements, which are critical for iNOS expression as site directed mutagenesis of either of these sites significantly reduces promoter activity and chemical inhibitors of NF-κB prevent iNOS expression and NO production (Kim et al, 1997; Xie et al, 1994). IFN-γ is an efficient enhancer of iNOS expression in most cells. iNOS expression is seriously impaired in macrophages from mice deficient of signal transducer and activator of transcription 1 (Stat1) (Meraz et al, 1996), IFN regulatory factor-1 (IRF-1) (Kamijo et al, 1994) or IFN consensus sequence binding protein (ICSBP) (Contursi et al, 2000), demonstrating the importance of IFN-responsive transcription factors in iNOS expression. The role of activator protein 1 (AP-1) family of transcription factors in the regulation of murine iNOS transcription is unclear. Existence of both positive and negative regulatory AP-1 sites has been reported (Kizaki et al, 2001; Okada et al, 2003), but the composition of AP-1 which binds to these sites is not known. Overexpression of c-fos, fosB and c-jun suppresses iNOS promoter activity. Stat3, Elk-3 and upstream stimulatory factors 1 and 2 (USF-1 and USF-2) (Okada et al, 2003; Yu et al, 2002; Chen et al, 2003; Gupta et al, 2002) have been shown to negatively regulate iNOS transcription.
1.9.4. Signal transduction pathways linked to iNOS

Various signal transduction pathways have been suggested to regulate iNOS expression (Figure 1.3). The importance of pathways leading to the activation of transcription factors NF-κB and Stat1 have been discussed above. cAMP activating compounds can both enhance (Kunz et al, 1994; Pahan et al, 1997) and inhibit (Mustafa and Olson, 1998) cytokine induced iNOS expression. Use of PKC activating phorbol esters or PKC inhibitors have mainly suggested a positive role for PKC in cytokine induced iNOS expression (Chen et al, 1998; Paul et al, 1997) but also a negative role has been reported (Muhl and Pfeilschifter, 1994; Banan et al, 2002). This may reflect the observations that different PKC isoforms may have opposite effects. Although iNOS activity is independent of the [Ca2+]i, changes in [Ca2+]i regulate iNOS expression. Both stimulation (Park et al, 1995) and inhibition (Bereta et al, 1994) of iNOS expression by [Ca2+]i elevating agents have been reported. Interestingly, the effect of [Ca2+]i seems to depend on the extent of inducing stimulus. At low LPS concentrations, increase in [Ca2+]i stimulates iNOS expression, whereas at high LPS concentrations, increase in [Ca2+]i inhibits iNOS expression (Korhonen et al, 2001). The role of the mitogen-activated protein kinases in the regulation of iNOS expression has been investigated intensively. Extracellular signal regulated kinase 1 and 2 have been shown to up-regulate (Lahti et al, 2000; Kristof et al, 2001) iNOS expression.

1.9.5. Regulation of Inflammatory transcription factors by NO

Cellular responses to oxidative and nitrosative stress are often regulated at the level of transcription (Marshall et al, 2000). Both prokaryotic and eukaryotic cells have transcription factors that are regulated by NO and major pathways include NF-κB pathway, AP-1 pathway and Jak-Stat pathway.

1.10. NF-κB

Stimulus-induced nuclear factor-κB (NF-κB) activity, the central mediator of inflammatory responses and immune function, comprises a family of dimeric transcription factors that regulate diverse gene expression programs consisting of hundreds of genes. NF-κB, a nuclear transcription factor, was first identified in 1986 by Sen and Baltimore (Sen and Baltimore, 1986). As its name implies, it is a nuclear
Figure 1.3 Production of nitric oxide in tumors. (Fukumura et al, 2006)
factor bound to an enhancer element of the immunoglobulin kappa light chain gene in B cells (Sen and Baltimore, 1986). First considered a B-cell transcription factor, NF-κB is now known to comprise a family of ubiquitous proteins.

1.10.1. Molecular components

In vertebrates, NF-κB connotes not a single protein, but a family of more than a dozen transcription factors that are comprised of homo- and heterodimers of five proteins: p50, p52, c-Rel, RelA/p65 and RelB, encoded by the nfkb1, nfkb2, rel, rela and relb genes (Gilmore, 2006). These proteins share an approximately 300 residue long homologous domain near their N termini (Baldwin, 1996; Ghosh et al, 1998).

1.10.2. DNA recognition by NF-κB dimers

Extensive studies following diverse strategies over the past 20 years have helped to identify a large number of kB sequences as regulators of gene expression. The DNA sequences that specifically bind NF-κB dimers are collectively known as kB sites. Most kB sites appear to be 10 bp in length with the consensus sequence 5’-GGGRN W YYCC-3’ (where R denotes a purine base, N denotes any base, W denotes an adenine or thymine and Y denotes a pyrimidine base) (Sen and Baltimore, 1986; Chen and Ghosh, 1999). Solution-based DNA-binding data indicate that the NF-κB dimers bind kB sites with affinities ranging from 10–300 nM.

1.10.3. IkB

A primary mechanism by which NF-κB activity is controlled is through regulation of its ability to bind DNA. Inhibitor of kB (IkB) proteins, a subfamily of the large Ankyrin Repeat Domain (ARD) containing superfamily, binds NF-κB dimers in a manner that prevents DNA binding and nuclear accumulation. In most cell types, NF-κB dimers are located in the cytoplasm in an inactive form through association with any of several IkB inhibitor proteins (IkBa, -b, -e, -g, p105 and p100) (Gilmore, 2006). In response to a wide array of stimuli (Pahl, 1999), many of which are involved in intercellular communication such as proinflammatory molecules, IkB is rapidly phosphorylated, ubiquitinlated and degraded by the proteasome. The freed NF-κB dimer then translocates to the nucleus where it can modulate specific gene expression.
1.10.4. NF-κB activation pathways

There are multiple pathways for activation of NF-κB. The two most common pathways are the canonical (or classical) and the non-canonical (or alternative) pathways (Gilmore, 2006; Scheidereit, 2006)(Figure 1.4). In the canonical pathway, a complex such as p50-RelA/IKBα is activated by an IKK complex containing IKKa/IKKβ/NEMO, with IKKβ being the primary kinase for IkBα. In the canonical pathway proceeding from TNF stimulation to NF-κB, the IKK complex is brought to the TNF-R by binding of NEMO to a K63-ubiquitinated RIP1 adaptor molecule on the TNF-R. The activity of the IKKβ kinase is then enhanced by two phosphorylations in its activation loop (Ser 177, 181) and the downstream events of NF-κB signaling ensue.

1.10.5. NF-κB and diseases

NF-κB activation has been implicated in a wide variety of diseases, including cancers, diabetes mellitus, cardiovascular diseases, autoimmune diseases, viral replication, septic shock, neurodegenerative disorders, ataxia telangiectasia (AT), arthritis, asthma, inflammatory bowel disease and several other inflammatory conditions.

1.10.6. NF-κB Mediates Carcinogenesis

NF-κB has been implicated in carcinogenesis because it plays a critical role in cell survival, cell adhesion, inflammation, differentiation and cell growth. Cancer is a hyperproliferative disorder that results from tumor initiation and tumor promotion and ultimately produces tumor metastasis. Notably, several genes involved in cellular transformation, proliferation, invasion and angiogenesis are regulated by NF-κB. Constitutive expression of NF-κB has been shown in cell lines derived from breast, ovarian, colon, pancreatic, thyroid, prostate, lung, head and neck, bladder and skin tumors (Rayet and Gelin, 1999). This has also been seen for B-cell lymphoma, Hodgkin’s disease, T-cell lymphoma, adult T-cell leukemia, acute lymphoblastic leukemia, multiple myeloma, chronic lymphocytic leukemia and acute myelogenous leukemia. Various carcinogens including 7,12-dimethylbenz(a)anthracene (DMBA) (Banerjee et al, 2002), benzo(a)pyrene diol epoxide, 4- (methylnitrosamino)-1-(3-pyridyl)-1-butanol and nicotine (Shishodia et al, 2003) have been shown to activate
Figure 1.4 NF-κB activation pathway

**Diagram Description:**
- **CD40L** binds **CD40**, leading to LTαβ, BAFF, LPS, IL-1, and TNF activation.
- **NIK** is activated, leading to IKKα and IKKβ activation.
- IKKβ phosphorylates **IkBα**, promoting its degradation via ubiquitination and proteasomal degradation.
- The degradation of IkBα releases **NF-κB** (p65, p50, RelB, RelA) into the nucleus.
- **NF-κB** activates transcription of genes involved in lymphoid organogenesis (PNA4, GlyCAM-1), chemokines (BLC, SLC, ELC, SDF-1), cytokines (BAFF/Blrs), and others.
- **NF-κB** activates transcription of cytokines/chemokines (IL-1β, TNF, GM-CSF, IL-8, RANTES, MIP-1α, MCP-1), enzymes (NOS, COX-2), and adhesion molecules (VCAM-1, ICAM-1, E-Selectin).
NF-kB. For example, DMBA-induced NF-kB activation was shown to occur not only in vitro but also when DMBA was administered in vivo to animals (Zhong et al, 1997). Besides tumor initiators, tumor promoters such as phorbol ester and okadaic acid have also been shown to activate NF-kB (Shishodia et al, 2003). Additionally, NF-kB is activated by hypoxia (Koong et al, 1994) and an acidic pH (Schutze et al, 1992), both characteristics of the tumor microenvironment.

Besides inducing apoptosis, almost all chemotherapeutic agents also activate NF-kB. These include DNA-damaging agents such as doxorubicin (DoxR) (Ashikawa et al, 2004), camptothecin (Singh et al, 1998), gemcitabine (Arlt et al, 2003) and cisplatin; microtubule depolymerizing agents such as taxol (Hwang and Ding, 1995) alkylating agents such as melphalan (Donepudi et al, 2001) and the glutathione reductase inhibitor 1, 3-bis (2-chloroethyl)-1-nitrosourea (Galter et al, 1994). Similarly, γ-radiation, x-rays and UV radiation have also been shown to activate NF-kB (Brach et al. 1991).

1.10.7. NF-kB and oncogenes

Several oncogene products that can activate NF-kB have been identified, including ras (Finco and Baldwin, 1993), bcr-abl (Hamdane et al, 1997) and myc (Duyao et al, 1992). Oncogenic Ha-Ras–induced signaling also activates NF-kB transcriptional activity, which is required for cellular transformation (Finco et al, 1997). How these genes induce NF-kB activation, however, is poorly understood. Oncogenic Ras enhances NF-kB transcriptional activity through Raf dependent and Raf-independent mitogen-activated protein kinase signaling pathways (Norris and Baldwin, 1999).

1.10.8. NF-κB Activation inhibits apoptosis and enhances Cell Proliferation

Several gene products that negatively regulate apoptosis in tumor cells, including inhibitor of apoptosis proteins (IAPs) 1 and 2, X-linked IAP, cellular Fas-associated death domain-like interleukin-1β-converting enzyme (FLICE)-like inhibitory protein (cFLIP), were shown to be controlled by NF-kB activation. For example, Bcl-xL suppressed cytochrome C release from the mitochondria, the IAPs inhibited caspase-3 and caspase-9 (Kawamura et al, 2003) and FLIP inhibited
caspase-8 (Matta et al, 2002). An antiapoptotic role of NF-kB has been alleged in T-cell lymphoma, osteoclasts, melanoma, pancreatic cancer, bladder cancer and breast cancer.

Several genes that mediate cell proliferation are also regulated by NF-kB, including the growth factors TNF, IL-1β and IL-6 (Libermann and Baltimore, 1990). For instance, TNF was shown to be a growth factor for glioblastoma cells (Aggarwal et al, 1996) and cutaneous T cell lymphomas (Giri and Aggarwal, 1998), IL-1β for acute myelogenous leukemia (Estrov et al, 1998) and IL-6 for multiple myeloma (Bharti et al. 2003) and head and neck squamous cell carcinoma (Kato et al, 2000). Suppression of NF-kB in these tumors downregulates the cytokine expression and inhibits tumor cell proliferation. Besides growth factors, certain cell cycle regulatory proteins such as cyclin D1, which is required for transition of cells from the G1 to S phase, are also regulated by NF-kB (Mukhopadhyay et al, 2002). Additionally, in some cells, prostaglandin E2 (PGE2) was shown to induce proliferation of tumor cells and the synthesis of cyclooxygenase-2, which controls PGE2 production, was also shown to be regulated by NF-kB activation (Yamamoto et al, 1995).

1.10.9. NF-kB and Metastasis

Helbig et al. (Helbig et al, 2003) demonstrated that NF-kB regulates the motility of breast cancer cells by directly upregulating the expression of CXCR 4 and he could show the positive effect of activated NF-kB in the migration and organ specific homing of metastatic breast cancer cells. In addition, Fujioka et al. (Fujioka et al, 2003) showed that inhibiting constitutive NF-kB activity by expressing IκBαM suppressed liver metastasis, but not tumorigenesis, in the metastatic human pancreatic tumor cell line AsPc-1 in an orthotopic nude mouse model. Several proteases (e.g., matrix metalloproteinases [MMPs] and the serine protease urokinase-type plasminogen activator [uPA]) that influence the invasive characteristics of tumors are regulated by NF-kB (Farina et al, 1999; Novak et al, 1991; Bond et al, 1998). uPA is another critical protease involved in tumor invasion and metastasis. Novak et al. (Novak et al, 1991) reported that the transcriptional activation of the uPA gene by phorbol myristate, IL-1 and TNFα requires the induction of NF-kB activity and the decay of its short-lived repressor protein IκB α.
1.10.10. NF-kB and Angiogenesis

It is now well recognized that the induction of the tumor vasculature (i.e., angiogenesis) is critical for the progression of tumors and metastasis. Tumor vascularization has been shown to be dependent on several chemokines (e.g., monocyte chemoattractant protein-1, IL-8) and growth factors (e.g., TNF, vascular EGF [VEGF]) produced by tumor cells itself or by macrophages, neutrophils and other inflammatory cells (Loch et al, 2001). The production of these angiogenic factors has been shown to be regulated by NF-kB activation (Chilov et al, 1997; Yu et al, 2003) demonstrated that NF-kB and VEGF were significantly overexpressed and associated with increased microvessel density in the colorectal cancer specimens suggesting increased expression of NF-kB contributes to tumor angiogenesis in colorectal cancer.

Highly metastatic melanoma cells were found to express high levels of constitutive NF-kB activity that was suppressed by transfection with IkBaM. Suppression of constitutive NF-kB activity inhibited tumor growth, prevented lung metastasis and decreased microvessel density (angiogenesis), which correlated with a decrease in the level of IL-8 expression (Huang et al, 2000). Also, inhibition of NF-kB activity blocked basic fibroblast growth factor-induced angiogenesis. These findings further underscore the role of NF-kB activation in mediating angiogenesis.

NF-kB is an ideal target for anticancer drugs. Given the hyperproliferative nature of cancer, which involves transformation, initiation, promotion, angiogenesis, invasion and metastasis and the diversity of its clinical presentation, aggressiveness and current treatment strategies, the implication is that an equally diverse number of potential targets exist in the molecular pathways leading to its formation. In this regard, several strategies have been used to block the activation of NF-kB and a wide range of compounds, such as IKK inhibitors, inhibitory peptides, antisense RNA and proteasome inhibitors, have been found to block various steps leading to NF-kB activation.

1.11. IMMUNOMODULATION BY NATURAL PRODUCTS

Immunomodulation is defined as changes in the body's immune system, caused by agents that activate or suppress its functions, including both innate and adaptive
arm of immunity. Immune system is increasingly found to be involved each and every step of tumorigenesis. In this context it appears worthwhile to target the modulation of immune system to reduce the risk of developing cancer. This may be antigen independent and may directly induce production of mediators and effector molecules by the immunocompetent cells. This type of antigen independent immunity is thus distinct from one achieved by conventional immunization or by passive immunization using antibodies (Upadhyay, 1997). Immunomodulators can regulate the cytokine production such as tumor necrosis factor, interleukins and interferons and these cytokines may, in turn activate T-cells or NK cells.

Use of plants and plant products as immunomodulators is still in a developing stage. There are few plants reported with known immunomodulatory activity. Viscum album a semiparasitic plant has shown to stimulate both humoral as well as cell mediated immune response (Kuttan and Kuttan, 1992). Similarly an extract from the plant Withania somnifera has shown to stimulate the immune system (Davis and Kuttan, 2000) reduce leukocytopenia during chemotherapy (Davis and Kuttan, 2000), and radiation therapy (Kuttan, 1993) and inhibit urotoxicity induced by chemotherapeutic drug cyclophosphamide (Davis and Kuttan, 2000). It has been reported that Piper longum, an immunopotentiating plant, enhances the total bone marrow cells (Singh et al, 1984). Tinospora cordifolia which is widely used in Indian system of medicine has been reported for its immunomodulatory and antitumour activities (Mathew and Kuttan, 1974). Curcumin which is present in the plant Curcuma longa has shown to stimulate the immune system in animals (Antony et al, 1999). It has also been reported to reduce the leukocytopenia in radiation (Thressiamma et al, 1985) and chemotherapeutic drug treated animals (Soudhamini and Kuttan, 1991).

1.12. CHEMOPREVENTION BY NATURAL PRODUCTS

Like majority of other human disorders, cancer is basically preventable. One of the promising approaches to reduce the risk of cancer is chemoprevention (Greenwald, 2001). According to both clinical observations and experimental models, cancer develops in a stepwise fashion starting with a single oncogenic mutation in a single
cell. Chemoprevention is the attempt to use natural and synthetic compounds to intervene in the early precancerous stages of carcinogenesis, before malignancy manifests. Recently, there have been considerable efforts to search for naturally occurring substances for the intervention of carcinogenesis. Many components derived from dietary or medicinal plants have been found to possess substantial chemopreventive properties (Surh, 2003). However, a delicate balance must be struck between a compound’s cancer-fighting capabilities and its toxicological profile for it to progress from a lead to a clinically useful agent.

The use of natural products begins with the use of vincristin and vinblastin from *Catharanthus roseus* and in late 1960s to cure Hodgkins lymphoma (Mann, 2002). Development of the structurally and mechanistically novel taxane and camptothecin represented a landmark in cancer research because of their significant anti-solid tumor efficacy (Wall et al, 1966). Curcumin isolated from *Curcuma longa* was found to cytotoxic against several tumor cell lines. It is also proved as antimetastatic against B16F-10 melanoma cells in C57BL/6 mice model (Menon et al, 1999). Iscador, an extract of the plant *Viscum album* was found to inhibit 20-methylcholanthrin induced carcinogenesis in mice (Kuttan et al, 1996) and also inhibited lung metastasis induced by B16F-10 melanoma cells (Antony et al, 1997). Several clinical trials on the use of nutritional supplements and modified diets to prevent cancer are ongoing. Sulforaphane, an isothiocyanate rich in broccoli, is a potent inhibitor of lung metastasis in C57BL/c mice (Thejass and Kuttan, 2006). Plants extract such as *Momordica charantia*, *Camellia sinensis*, *Polygonium caspidatum* are effective on highly metastatic PC-3M prostate cancer cell line (Rao et al, 2004). MNNG induced glandular sarcoma development was inhibited by the use of *Phyllathus amarus* (Raphael et al, 2006). *In vivo* administration of crude extract of *Thuja occidentalis* retarded the development of highly metastatic B16F-10 cells in mice and increased the life span of tumor bearing mice (Sunila and Kuttan, 2006). Several natural occurring compounds were also studied for its antimetastatic potential (Thejass et al, 2006; Guruvayoorappan et al, 2007).

Several compounds have been screened as antiangiogenic agents which include plant extract such as *Tinospora cordifolia*, *Piper longum*, isolated compound diallyl
sulfide andrographalide and Ametoflavone (Leyon and Kuttan, 2004; Sunila and Kuttan, 2006; Thejass and Kuttan, 2007; Sheeja et al, 2006; Guruvayoorappan et al, 2007). Piperine, a phenolic component of black pepper could effectively inhibit transcription factor NF-kB which offer its role in cancer therapy (Pradeep and Kuttan; 2004).

1.13. COMPOUNDS USED IN THE PRESENT STUDY

1.13.1. Punarnavine

Punarnavine is an alkaloid found in the plant Boerhaavia diffusa. Boerhaavia diffusa (family-Nyctaginaceae) is a perennial herb and it is ascribed the name punarnava, (Punah punarnava bhawati iti, in Sanskrit, translates as “that which becomes fresh again and again . . .”) a drug known since long in the indigenous system of medicine in India. It is widely distributed in the tropics and subtropics (CSIR, 1988.). It has a long history of uses by indigenous and tribal people and in Ayurvedic or natural herbal medicines (Dhar et al, 1968). Pharmacological studies have demonstrated that B. diffusa possesses anti-inflammatory (Bhalla et al, 1968), antifibrinolytic (Jain and Khanna, 1989), anticonvulsant (Adesina, 1979), antistress (Mungantiwar et al, 1997) and antibacterial properties (Olukoya et al, 1993), which makes it a very useful medicinal plant. The alkaloidal fractions of Boerhaavia diffusa also found to possess immunmodulatory activity (Mungantiwar et al, 1999).

The chemical examination of the plant Boerhaavia diffusa was first undertaken by Ghosal in 1910, who found the presence of an alkaloidal component, a waxy amorphous mass and sulphide, chloride and nitrate anions in the ash (Chopra et al, 1923) found the presence of a water soluble alkaloid, which they designated as ‘Punarnavine’, along with large amounts of potassium nitrate and other potassium salts. Agarwal and Dutt (1934) could not isolate any alkaloid from their samples of the plant and isolated an acid designated as boerhaavic acid. Later on, Agarwal and Dutt (1935) and Basu and Sharma (1947) described the isolation of the alkaloid Punarnavine in a crystalline form.

The chemical formula of Punarnavine is C_{17}H_{22}N_{2}O and having a melting point of 236–237°C. (Agarwal and Dutt, 1935; Basu and Sharma, 1947; Surange and
Pendse, 1972). It is also considered as the active principle in the plant extract (Sethi and Zafar, 2003).

1.13.2. Glycyrrhizic acid

Although terpenoids are widely used for medicinal purpose in many Asian countries, their biogenesis and pleiotropic actions has not impacted on the practice of western medicines (Suh et al, 1998). Glycyrrhizic acid (GA) is the main bioactive ingredient of licorice (*Glycyrrhiza glabra*). Glycyrrhizic acid is glycoside of glycyrrhetenic acid (Figure 1) found in *Glycyrrhiza glabra*, an important medicinal plant in Ayurvedic system of medicine. Our laboratory had already reported the Immunomodulatory effect of Glycyrrhizic acid (Raphael and Kuttan, 2003, 2008). It is already reported that Glycyrrhizic acid induces apoptosis in human stomach cancer KATO III and human promyelotic leukemia HL-60 cells (Hibasami et al, 2005) and prostate cancer cell lines DU-145 and LNCaP (Thirugnanam et al, 2008). Glycyrrhizic acid also reported to inhibit TNF mediated apoptosis in the human hepatoblastoma line HepG2 (Yoshikawa et al, 1999). GA was found to modulate critical end points of oxidative stress induced apoptosis and could be beneficial against liver diseases where oxidative stress is known to play a crucial role (Tripathi et al, 2008).

1.13.3. Ursolic acid

Ursolic acid (3ß-hydroxy-urs-12-en-28-oic acid) is a pentacyclic triterpenoid derived from berries, leaves, flowers and fruits of medicinal plants, such as *Boerhaavia diffusa, Rosemarinus officinalis, Eriobotrya japonica, Calluna vulgaris, Ocimum sanctum* and *Eugenia jumbolana* (Liu, 1995). It has been reported to posess a wide range of pharmacological properties and is one of the most promising chemopreventive agent (Shih et al, 2004). Ursolic acid has been shown to suppress tumorigenesis (Huang et al., 1994) and inhibit tumor promotion (Tokuda et al, 1986; Ohigashi et al, 1986; Nishino et al, 1988). Many of these effects of ursolic acid are mediated through suppression of the expression of lipoxigenase, COX-2, MMP-9 and iNOS (Simon et al, 1992; Najid et al, 1992; Ringbom et al, 1998; Subbaramaiah et al, 2000; Cha et al, 1996; Cha et al, 1998; Suh et al, 1998) all of which are genes regulated by NF-κB. In addition, ursolic acid and its derivatives have been shown to induce apoptosis in a wide variety of cancer cells including breast carcinoma,
Ursolic acid inhibits the cell proliferation of human lung cancer cell line A549 showed that the blocked cell cycle progression in the G1 phase (Hsu et al, 2004). It also decreased the protein expression of cyclin D1, D2 and E and their activating partner cdk2, 4 and 6 with concomitant induction of p21/WAF1. Ursolic acid is able to inhibit key steps of angiogenesis in vitro, including endothelial cell proliferation, migration and differentiation (Cardenas et al, 2004).

1.13.4. Limonene

In citrus fruits (Chayet et al, 1977), peppermint and other plants, d-limonene is formed by the cyclization of geranylpyrophosphate in a reaction catalyzed by limonene synthase (Alonso et al, 1992; Kjonaas et al, 1983). Limonene then serves as a precursor to a host of other oxygenated monocyclic monoterpenes such as carveol, carvone, menthol, perillyl alcohol and perillaldehyde (Karp et al, 1990; McGarvey and Croteau, 1995). D-limonene is a prevalent flavoring agent for fruit juices, soft drinks, baked goods, ice cream and pudding. Orange oil, naturally consisting of 90–95% d-limonene, is a commercially available food flavoring agent. Furthermore, because of its pleasant citrus fragrance, d-limonene is commonly added to cosmetics, soaps and other cleaning products.

Limonene has well established chemopreventive activity against many cancer types. Dietary limonene reduces the incidence of spontaneous lymphomas in p532/2 mice (Hursting et al, 1995). Furthermore, when administered either in pure form or as orange peel oil (95% d-limonene), limonene inhibits the development of chemically induced rodent mammary (Elegbede et al, 1984; Elson et al, 1988; Maltzman et al, 1989; Wattenberg and Coccia, 1991), skin (Elegbede et al, 1986), liver (Dietrich and Swenberg, 1991), lung and forestomach (Wattenberg et al, 1989; Wattenberg and Coccia, 1991) cancers. In rat mammary carcinogenesis models, the chemopreventive effects of limonene are evident during the initiation phase of 7-12-dimethylbenz[a]anthracene (DMBA)2- induced cancer (Elson et al, 1988) and during the promotion
phase of both DMBA- and nitrosomethylurea (NMU)-induced cancers (Elson et al, 1988; Maltzman et al, 1989). Dietary limonene also inhibits the development of ras oncogene–induced mammary carcinomas in rats (Gould et al, 1994). There are many reports (Kawamori et al, 1996) that the development of azoxymethane-induced aberrant crypt foci in the colon of rats was significantly reduced when they were given 0.5% limonene in the drinking water.

The present study is trying to assess the effect of the natural products Punarnavine, Glycyrrhizic acid, Ursolic acid and Limonene on the activation of NF-κB and expression of iNOS gene during tumor specific angiogenesis and metastasis.