CHAPTER 1

REVIEW OF LITERATURE
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1.1. INTRODUCTION

Cancer is a group of diseases of higher multicellular organisms characterized by alterations in the expression of multiple genes, leading to deregulation of the normal cellular program for cell division and cell differentiation. This results in an imbalance of cell replication and cell death that favours growth of a tumour cell population.

Cancer is the leading cause of death in economically developed countries and the second leading cause of death in developing countries. Based on the GLOBOCAN 2008 estimates, about 12.7 million cancer cases and 7.6 million cancer deaths are estimated to have occurred in 2008; of these, 56% of the cases and 64% of the deaths occurred in the economically developing world (Jemal et al., 2011). In India, the total cancer cases are likely to be 979,786 cases in the year 2010 which will be increasing to 1,148,757 cases in the year 2020, according to the incidence data generated by population-based cancer registries at Bangalore, Barshi, Bhopal, Chennai, Delhi and Mumbai for the years 2001-2005 (Takiar et al., 2010).

Cancer arises when a cell, for a variety of reasons, escapes the normal constraints placed on its growth and begins to divide in an unregulated fashion. In tumour cells, molecules that regulate signalling pathways which stimulate growth are often mutated, resulting in a constant ‘on’ signal to the cell. In addition, tumour cells often develop their own autocrine loops for growth, wherein a growth factor required for the activation of a pro-growth signalling pathway is constantly produced and secreted into the extracellular milieu. Cancer cells can grow by escaping from the attack of immune cells, thus disrupting the host immune system, which is progressively suppressed as a result of tumour progression and metastasis. The molecular mechanisms by which cancer cells evade the host immune system have been investigated in mouse models and clinical samples.

The characteristics that delineate a malignant cancer from a benign tumour are the abilities to invade locally, to spread to regional lymph nodes, and to metastasize to distant organs in the body (Ruddon, 2007). The metastatic characteristic, which may be predisposed or acquired during the development of
the disease, is governed by a number of genetic mechanisms, or metastasis-related genes.

1.2. IMMUNE SYSTEM

The principal function of the immune system is to provide protection against foreign invading pathogens, which can gain access to the body by different routes. Targeted immune response is generated by the combined interaction of the cellular and humoral responses, both coordinated by the production of active substances—the cytokines. Cellular immunity refers to the immune mechanisms mediated by T lymphocytes, regardless of immunoglobulin molecules, whereas humoral immunity denotes secretion of antibodies by B lymphocytes (Nossal, 1987). The humoral and cellular arms of the immune system always act in a synergistic manner to provide protection to the host.

B lymphocytes are derived from bone marrow precursors and are responsible for the production of antibodies after differentiation into plasma cells. They are also involved in antigen presentation to T-cells, secretion of immunoregulating cytokines and establishment of 'memory' towards antigenic determinants (Hamaoka and Ono, 1986).

T lymphocytes are initiated in hematopoietic stem cells and differentiating in the thymus. T-cells are heterogeneous by virtue of their different functions such as lysis of foreign cells, modulation of the interaction between B and T cells, regulation of monocyte functions. T-cells carrying CD4 molecules (T-helpers) interact with antigen associated with MHC class II on the surface of the antigen presenting cell, and T-cells expressing CD8 molecules (cytotoxic T-cells) engage in suppression of the immune response (Acuto and Reinherz, 1985).

Cytokines are soluble mediators secreted by macrophages or monocytes (monokines) or lymphocytes (lymphokines). These mediators act as stimulatory or inhibitory signals between cells; those between cells of the immune system are known as interleukines. Cytokines that induce chemotaxis of leukocytes are referred to as chemokines. Amongst the array of cytokines produced by macrophages and T cells, IL-2 is of particular interest due to its pivotal role in amplifying immune responses. IL-2 can promotes growth of T cells and IL-2 receptor bearing B cells and natural killer (NK) cells.
Certain non-specific effector mechanisms can augment the effects of antibody and some of these factors are older, in evolutionary terms, than antibody production itself. The major factors are phagocytic cells (macrophages and polymorphonuclear leucocytes), which remove antigens including bacteria, and complement, which can either directly destroy an organism or facilitate its phagocytosis.

The complement system consists of a series of heat-labile serum proteins which are activated in turn. The components normally exist as inactive precursors; once activated, a complement component may then act as an enzyme which cleaves several molecules of the next component in the sequence.

Macrophages are the tissue equivalent of monocytes and represent the mononuclear phagocytic system. Mature macrophages differentiate principally in the tissues. Monocytes circulate for only a few hours before entering the tissues where they may live for weeks or months as mature macrophages. A major function of the mononuclear phagocyte system is to phagocytose invading organisms and other antigens. Macrophages have prominent lysosomal granules containing acid hydrolases and other degradative enzymes with which to destroy phagocytosed material.

Neutrophils are phagocytic cells that play a major role in the body's defence against acute infection. They synthesize and express adhesion receptors so they can adhere to, and migrate out of, blood vessels into the tissues in response to chemotactic agents produced at the site of inflammation.

Antibody-dependent cell mediated cytotoxicity (ADCC) is a mechanism by which antibody-coated target cells are destroyed by cells bearing Fc receptors (NK cells, monocytes, neutrophils), with no involvement of the MHC. The mechanism of target-cell destruction includes the release of cytoplasmic components such as perforin and granzymes (as with cytotoxic T cells). ADCC represents an additional mechanism by which bacteria and viruses can be eliminated.

NK cells look like large granular lymphocytes. They can kill target cells, even in the absence of any antibody or antigenic stimulation. They are non-specifically activated by agents such as mitogens, interferon and IL-12. NK cells form an integral part of the early host response to viral infection and altered self
cells. Animals and rare patients with deficient NK cell function are found to have an increased incidence of certain tumours and viral infections. NK cells are therefore thought to be important in ‘immune’ surveillance against tumours.

Compounds that are capable of interacting with the immune system to upregulate or downregulate specific aspects of the host response can be classified as immunomodulators or biologic response modifiers (Tzianabos, 2000). Investigators have focused on discovering compounds that can positively modulate the biologic response of immune cells thus enhancing the host’s ability to resist infections and diseases like cancer.

1.3. IMMUNE SYSTEM IN CANCER

The role of immune system in surveillance against malignant transformations was suggested from the experiments of early 20th century showing strong immune-mediated rejection of transplantable tumours in mice. The concept of “immune surveillance” was proposed by Jones and Burnet (Burnet, 1970) in the 1970's. The immune surveillance hypothesis originally describes the ability of the immune system to recognize and destroy malignant cells before they develop into detectable cancers. A revised version of it, the immunoediting hypothesis, proposes that tumour cells that are not destroyed by the immune system can evolve and modify the immune system to avoid destruction (Tham and Abastado, 2011).

Schreiber and colleagues (Dighe et al., 1994; Ikeda et al., 2002) demonstrated that early tumour growth is comprised mostly of transformed cells that undergo apoptosis when they bind interferon (IFN) -γ and chemokines produced by cells of the innate immune response including NK cells, γδ-T lymphocytes and macrophages. This effectively eliminates most of the tumour cells, however it also selects for a minority of malignant cells that have mutations or alterations that make them resistant to an immune induced apoptosis. The absence of one or more chains of the IFNy receptor, or mutations in the tyrosine kinases associated with this receptor (JAK-1/2 or STAT-1), prevent the triggering of the apoptosis cascade making these cells resistant to the immune surveillance mechanism. These resistant clones then develop into tumours of clinical significance unimpeded by the immune response. Therefore,
the innate immune response may eliminate most transformed cells during the early stages of tumour growth, however it may also result in the selection of a resistant population of malignant cells.

Immune surveillance is supported by the high incidence of tumours in immunosuppressed patients following stem cell or organ-transplantation (Malmberg and Ljunggren, 2006). Although many of the malignancies that arise in these individuals are virally induced, there is also evidence of solid tumours, including melanomas, in patients receiving immune suppressive treatment (Penn, 1996; Pham et al., 1995).

**Immunoediting**

A consequence of immune-mediated tumour recognition is a continuous “sculpturing” of the tumour phenotype, more recently referred to as immunoediting. The immune selection pressure favours the development of less immunogenic tumour cell clones, which escape recognition by a functioning immune system and results in tumour progression (Dunn et al., 2004).

Immunoeediting is broadly divided into three stages, elimination, equilibrium and escape. During the elimination phase, tumour-specific antigens trigger an immune response, which leads to the destruction of neoplastic cells, preventing development of cancer. This elimination is an integrated action of innate and adaptive immune mechanisms. The equilibrium phase of immunoeediting describes a dynamic balance between the immune system’s containment of the tumour and acquisition of immune evasive strategies by subsets of tumour cells. Random mutations in the genetically unstable tumour give rise to tumour cell variants that are less immunogenic. The equilibrium phase is probably the longest of the three phases of immunoeediting, extending over a period of several years in humans (Dunn et al., 2004). Tumour cell escape from immune surveillance may be achieved via a number of ways. Tumour cells can modify the antigen processing and presentation process leading to down-regulation of MHC molecules, loss of expression of antigens recognized by the immune system and therefore reduced immunogenicity. Tumour cells also suppress the immune system through a complex network, leading to specific or generalized tolerance.
**Tumour cell immune escape**

The mechanisms involved in the tumour cell escape from immune surveillance can be discussed under three principles, (a) escape by loss of recognition, (b) escape by loss of susceptibility and (c) escape by induction of immune suppression (Figure 1.1).

Immune recognition of tumours by T cells (TCR engagement by MHC class I peptide complex) and NK cells depends on multiple receptor–ligand interactions. Lack of immune recognition may depend on an altered balance in signalling through activating and inhibitory receptors, favouring inhibition. This includes loss of HLA-class I, defective antigen processing and presentation (Seliger *et al.*, 2002), loss of tumour antigen expression (Yee *et al.*, 2002), shedding of MHC molecules, down-modulation of activating receptors including NKG2D and NCR (Doubrovina *et al.*, 2003), expression of ligands for inhibitory receptors (HLA-G, HLA-E, CD48), counterattack on FasL, B7-H1, soluble HLA class I (Fournel *et al.*, 2000) and suppression by antitumour responses by T<sub>reg</sub> (Liyanage *et al.*, 2002).


Tumours can suppress immunity both systemically and in the microenvironment of the tumour (Finn, 2008). Immunosuppressive molecules such as TGF-β (Teicher, 2007), soluble Fas ligand (Houston *et al.*, 2003), and immunosuppressive enzyme indolamine-2,3-dioxygenase (IDO) (Muller and Prendergast, 2007) mediate inhibition of T-cell activation. The tumour microenvironment can be dominated by regulatory T cells that suppress antitumor effector T cells by producing the immunosuppressive cytokines TGF-β and interleukin-10 (Zou, 2006). Studies by Woo *et al.*, (2001) and Liu *et al.*, (2007) shows that murine tumours that produce TGF-β can convert antitumor
Figure 1.1

Supression of immune system by tumour cells
effector T cells into regulatory T cells, thereby escaping their own destruction by immune cells.

The immunosuppressive effects of a tumour can also be systemic. An increase in regulatory T cells has been observed in the peripheral blood of patients with head and neck cancer (Chikamatsu et al., 2007) or melanoma (Fecci et al., 2006). Patients with colorectal cancer or pancreatic tumours have increased numbers of activated granulocytes (Schmielau and Finn, 2001) and myeloid-derived suppressor cells (Nagaraj and Gabrilovich, 2007), both of which suppress tumour-specific T cells in mice (Kusmartsev et al., 2005).

1.4. INFLAMMATION AND CANCER

Inflammation is defined as increased vascular permeability accompanied by an infiltration of 'inflammatory' cells, namely neutrophil polymorphonuclear leucocytes and later macrophages, lymphocytes and plasma cells. Vascular permeability may be increased by a number of agents, which include complement fragments. Mobilization and neutrophils attraction to inflammatory site from the bone marrow by cytokines generated by activated T cells. The triggering of mast cells via IgE leading to release of histamine and leukotrienes

Chronic inflammation represents a major pathologic basis for the majority of human malignancies. In a persistently inflamed tissue, excessive generation of reactive oxygen species (ROS) can cause genomic instability which leads to initiation of cancer (Lu et al., 2006). Although inflammation acts as an adaptive host defence against infection or injury and is primarily a self-limiting process, inadequate resolution of inflammatory responses often leads to various chronic ailments including cancer (Schottenfeld and Beebe-Dimmer, 2006). The role of inflammation in development and progression of cancer has first been proposed by Rudolf Virchow in 1863 (Kundu and Surh, 2008). About 25% of all cancers occurrences are somehow associated with chronic infection and inflammation (Pervez Hussain and Harris, 2007).

The possible mechanisms by which inflammation can contribute to cancer development include induction of genomic instability, alterations in epigenetic events and subsequent inappropriate gene expression, enhanced proliferation of
initiated cells, resistance to apoptosis, aggressive tumour neovascularization, invasion through tumour-associated basement membrane and metastasis, etc.

Within the tumour microenvironment, various pro inflammatory mediators participate in a complex inflammatory signalling that facilitates extravasation of tumour cells through the stroma, thereby fostering tumour progression (Ariztia et al., 2006)

**Cytokines**

Cytokines are secreted or membrane bound, nonstructural proteins with molecular weights ranging from 8 to 40 KDa that regulate diverse physiological processes, such as growth, development, differentiation, wound healing and immune response. Tumour necrosis factor (TNF)-α, IL-6 and IL-1 are among the most prominent inflammatory cytokines in the tumour microenvironment. These cytokines activate proinflammatory signalling by activation of intracellular kinases (Janus- activated kinase, phosphatidylinositol-3-kinase/Akt, IKK, and MAP kinases) with subsequent activation of transcription factors, (STAT, NF-κB, and AP-1) (Yoshimura, 2006) and help to maintain inflammatory tumour microenvironment.

TNF is detected in malignant and/or stromal cells in human ovarian, breast, prostate, bladder, and colorectal cancer, lymphomas, and leukaemia, often in association with IL-1 and IL-6 and macrophage colony stimulating factor (Balkwill and Mantovani, 2001). TNF-α and IL-1 activate endothelial cells and trigger a cascade of proinflammatory small molecule mediators. Increased gene expression for phospholipase A2 type II, Cyclooxygenase (COX)-2, and nitric oxide synthase (iNOS) results in elevated production of their products, PAF, PGE₂, and nitric oxide (NO). Alone or in combination, these mediators decrease the tone of vascular smooth muscle. The upregulation of endothelial leukocyte adhesion molecules results in adherence of circulating neutrophils to the endothelium, and increased production of chemokines such as IL-8 facilitates the emigration of neutrophils into the tissues (Dinarello and Fantuzzi, 2001). Chemokines also activate degranulation of neutrophils leading to tissue destruction and more inflammation. IL-10 inhibits T helper activity with resultant suppression of cytotoxic T cell generation and activity, as well as inhibition of NK and lymphokine-activated killer (LAK) cell cytotoxicity
IL-6 participates in inflammation-associated carcinogenesis by modulating the expression of genes involved in cell cycle progression and by inhibiting of apoptosis (Lin and Karin, 2007). An elevated level of IL-6 has been implicated in the pathogenesis of various cancers including Hodgkin lymphoma (Cozen et al., 2004), gastric carcinoma (Kai et al., 2005) and colon carcinoma (Schneider et al., 2000). Studies by Borg et al., (2005) reveals a correlation between VEGF production and secretion of IL-1 and IL-6 in human pituitary tumour cells suggesting the role of these cytokines in promoting angiogenesis.

**Chemokines**

Chemokines comprise a family of approximately 50 low molecular weight chemotactic cytokines, which are involved in the development of lymphocytes, recruitment and migration of leukocytes, angiogenesis, haematopoiesis, atherosclerosis, tumour growth and metastasis, and HIV-infection (Vandercappellen et al., 2008). The main stimuli for chemokine production are lipopolysaccharide (LPS), pro inflammatory cytokines, such as IL-1β and TNF-α.

Most tumours produce chemokines of the two major groups; CXC (α) and CC (β). CC chemokines are major determinants of macrophage and lymphocyte infiltration in melanoma, carcinoma of the ovary, breast, and cervix, and in sarcomas and gliomas (Balkwill and Mantovani, 2001). Some chemokines (CCL17, CCL22) contribute to tumour cell escape from the immune system by recruiting Th2 effectors and T_{reg} cells. CCL2 is produced at the tumour site by tumour cells and level of CCL2 expression correlates significantly with tumour associated macrophage (TAM) accumulation in primary tumours (Sica et al., 2002). Several studies have reported the involvement of chemokines and chemokine receptors in cell proliferation, migration, invasion and metastasis of different types of tumours (Kundu and Surh, 2008). Chemokines secreted by tumour cells are indirectly involved in angiogenesis by activating stromal cells to produce CCL2 or CXCL8 that can act on endothelial cells to indirectly promote angiogenesis (Somasundaram and Herlyn, 2009). Chemokines was found to mediate metastasis of epithelial ovarian cancer through up-regulation of MMP-2 and MMP-9 (Yuecheng and Xiaoyan, 2007).
Cyclooxygenase-2 and prostaglandins

Cyclooxygenase-2 (COX-2), the inducible form of cyclooxygenase, serves as an interface between inflammation and cancer. It is overexpressed in various inflammation-associated cancers and helps to maintain a persistent inflammatory state in the premalignant and malignant lesion. Abnormally elevated COX-2 causes promotion of cellular proliferation, suppression of apoptosis, enhancement of angiogenesis and invasiveness, etc., which account for its oncogenic function (Surh et al., 2001). Stable transfection of COX-2 in normal MCF-10A cells was found to increase proliferation and resistance to apoptosis, decreased differentiation and enhanced cell transformation (Lu et al., 2005).

COX-2 and some of its products also participate in inflammatory angiogenesis via mechanisms involving increased expression of VEGF, promotion of vascular sprouting, migration and tube formation, induction of MMPs, and activation of EGFR-mediated angiogenesis. Role of VEGF as a key mediator in the COX-2 angiogenic pathway was demonstrated by Gallo et al., (2001). LPS activation of human epidermoid carcinoma (A-431) and squamous cell carcinoma (SCC-9) cells resulted in increased COX-2 mRNA expression and PGE$_2$ production as well as increased VEGF mRNA and protein expression. HIF-1α is considered to function as a molecular link between COX-2 and VEGF in the course of angiogenesis (Fukuda et al., 2003). Studies by Huang et al., (2005) also suggests that the COX-2/PGE$_2$/HIF-1/VEGF pathway contributes to tumour angiogenesis associated with gastric cancer.

COX-2 promotes the breakdown of arachidonic acid to produce a series of prostaglandins, such as PGE$_2$, PGF$_{2\alpha}$, and 15d-PGJ$_2$. Studies by Narisawa et al., (1987) and Rigas et al., (1993) demonstrated that PGE$_2$ is capable of promoting mouse skin and colon carcinogenesis. Elevated levels of PGE$_2$ have been observed in various types of human cancers (Bennett, 1986); colon (Rigas et al., 1993), basal cell carcinomas and squamous cell carcinomas of the skin (Vanderveen et al., 1986). PGE$_2$ promotes cell proliferation and tumour-associated neovascularization, and inhibits cell death, thereby favouring tumour growth (Mann and DuBois, 2004).
Nitric oxide and nitric oxide synthase

Nitric oxide, a multifunctional gaseous molecule and a highly reactive free radical, is produced endogenously during arginine metabolism by different isoforms of NOS. There are three isoforms of NOS: neuronal NOS (nNOS, also known as NOS1), inducible NOS (iNOS or NOS2) and endothelial NOS (eNOS or NOS3) (Moncada et al., 1991). nNOS and eNOS are constitutively expressed predominantly in neuronal cells and vascular endothelial cells, respectively. Tumour-associated stromal fibroblasts and immune cells commonly express iNOS (Fukumura et al., 2006). During inflammation, cytokines such as TNF-α, IL-1β and IFNγ induce iNOS expression in macrophages and epithelial cells through NF-κB (Surh et al., 2001) activation, leading to increased levels of NO. The expression of iNOS and the level of NO have been shown to be elevated in various precancerous lesions and carcinomas (Chen and Stoner, 2004; Jaiswal et al., 2001).

Nitric oxide promotes tumour progression and metastasis by maintaining tumour blood flow through angiogenesis, vessel maturation and vessel dilation. NO-induced cGMP activates the Ras–Raf–MEK–ERK pathway, increasing the DNA binding of activator protein 1 (AP1), which results in increased cell proliferation and migration (Jones et al., 2004). Decreased leukocyte adhesion and the cytotoxic effects of NO allow tumour cells to evade immune-cell attack.

Tumour associated macrophages

Tumour associated macrophages represent the major inflammatory component of the stroma of many tumours. TAMs derive from circulating monocytic precursors, and are directed into the tumour by chemokines released by tumour cells. The presence of TAM at the tumour site represents one of the hallmarks of cancer-associated inflammation (Sica et al., 2008). TAM modulates the adaptive immunity by the production of PGE2, IL-10 and TGFβ which suppresses T cell activation and proliferation (Sica et al., 2000). IL-10, alone or in concert with IL-6 is responsible for the up-regulation of macrophage B7-H4 expression, a molecule implicated in the suppression of tumour-associated antigen-specific T cell immunity (Allavena et al., 2008). Clinical studies reveal a correlation between the high TAM content of tumours and poor patient prognosis.
TAM promote angiogenesis by the production of growth factors (e.g. acidic fibroblasts growth factor, basic fibroblasts growth factor, VEGF, GM-CSF, TGF-α, IGF-I, PDGF), monokines (e.g. TNF-α, IL-1, IL-6, CXCL8, prostaglandins, interferons) and angiogenic factors as well as protease enzymes which degrade the extracellular matrix (Sica et al., 2002). TAM can also stimulate tumour-cell proliferation and favour invasion and metastasis.

NF-κB

Nuclear transcription factor kappa B protein family consists of five different members, which are namely p65 (RelA), c-Rel, RelB, p50 and p52. These proteins are all characterized by a structurally conserved N-terminal 300 amino acid region containing specific domains, which allow dimerization, nuclear localization and DNA-binding (Li and Verma, 2002; Siebenlist et al., 1994). Only p65, c-Rel and RelB are directly able to activate the transcription of target genes. The transcriptional capacity of p50 and p52 are dependent on dimerization with p65, c-Rel or RelB (Atreya et al., 2008). Bacterial cell wall components like lipopolysaccharide, pro-inflammatory cytokines like TNF-α or IL-1, viruses and DNA damaging agents act as stimuli for activation of NF-κB, resulting in removal of the inhibitory IκB subunit and subsequent translocation to nucleus (Karin and Greten, 2005).

Improper activation of NF-κB contributes to tumour development and progression either by transactivating several target genes that have inflammatory (e.g., COX-2, iNOS, and TNF-α), anti-apoptotic (e.g., clAP1, clAP2, XIAP, Bcl-2, Bcl-3 and Bcl-XL0), cell cycle regulatory (e.g., cyclin D1) and proangiogenic (e.g., VEGF and angiopoietin) functions or by down-regulating apoptosis-inducing genes (e.g., p53, Bax, and Bad) (Chen et al., 2001). Up-regulated or constitutive expression of NF-κB has been identified in many forms of cancer such as pancreatic cancer (Chandler et al., 2004), hepatocellular carcinoma (Guo et al., 2005) and colorectal cancer (Rayet and Gélinas, 1999). The NF-κB-dependent activation of cell adhesion molecules, such as vascular cell adhesion molecule (VCAM) and intercellular adhesion molecule (ICAM), which have been found to increase in various cancers, are involved in leukocyte adhesion and migration within the inflammatory tumour microenvironment.
1.5. **TUMOUR METASTASIS**

Despite improvements in diagnosis, surgical techniques, general patient care, and local and systemic adjuvant therapies, most deaths from cancer result from the progressive growth of metastases that are resistant to conventional therapies. In a large number of patients, metastasis can occur before diagnosis of the primary disease. Metastasis is a multistep process in which cancer cells derived from the primary tumour migrate to regional or distant sites where they reinitiate their development (Chiang and Massague, 2008). It is a highly selective process dependent on the interplay of intrinsic tumour cell properties and host factors, so the localization of secondary tumour as well as metastatic efficiency of various tumours differ significantly.

Study using radiolabeled tumour cells demonstrates that after 24h in the circulation, less than 0.1% of the tumour cells remain viable. Moreover, less than 0.01% of tumour cells entering the circulation eventually survived to generate lung metastases (Fidler, 1970). These results emphasize that metastasis is a highly inefficient pathological process and the mere presence of tumour cells in the circulation does not predict that metastasis will occur.

**Metastatic cascade**

A distinguishing feature of malignant cells is their capacity to invade surrounding normal tissues and metastasise through the blood and lymphatic system to distant organs. The term invasiveness describes the ability of cells to cross anatomic barriers, for example basement membrane, interstitial stroma and intercellular junctions that separate tissue compartments (Mignatti and Rifkin, 1993).

The process of metastasis consists of sequential and interlinked steps as follows (Figure 1.2);

1) The growth of neoplastic cells in the primary tumour is progressive with nutrients for the expanding tumour mass initially supplied by simple diffusion.
2) After the tumour mass exceed 1–2 mm in diameter, extensive neo-capillary network from the surrounding vasculature must occur which is mediated by the synthesis and secretion of proangiogenic factors.
Figure 1.2

Formation of a cancer metastasis
3) Local invasion of the host stroma occurs with the help of protease enzymes and adhesion molecules.

4) Thin-walled capillaries offer low resistance to penetration by tumour cells, providing entry into the circulation.

5) Tumour cell aggregates are detached and embolized, to protect against shear haemodynamic forces and the immune system. Few tumour cells that can aggregate with host cells will survive.

6) Surviving cells must arrest in the capillary beds of organs, either by adhering to capillary endothelial cells or by adhering to subendothelial basement membrane.

7) Tumour cells will extravasate, probably by the same mechanisms that influence initial invasion.

8) Proliferation of cells within the organ parenchyma to form the initial colonies completes the metastatic process.

9) To continue growing, the micrometastases must develop a vascular network and continue to evade the host immune system.

   Each step involved in this complex process can be rate limiting since a failure at any of the steps eliminates the cell from the process (Langley and Fidler, 2007). The immune system regulates many aspects of this process.

   Disruption of cell–cell adhesion is an important step in the metastatic process, where the metastatic cell detaches from the primary tumour. Epithelial tumours are found to lose E-cadherin mediated adhesions as they progress toward malignancy. Integrins are also important mediators of the malignant phenotype during oncogenic transformation. In particular, the \( \alpha_6\beta_4 \) integrin, binds to the ECM component, laminin, forming signalling complexes with oncogenic receptor tyrosine kinases, EGFR, and Her2. \( \alpha_v\beta_3 \) and \( \alpha_5\beta_1 \) integrins have also been implicated in later stages of metastasis, specifically during adhesion of circulating tumour cells to the vasculature (Gupta and Massagué, 2006).

   The first step is tumour cell attachment via cell surface receptors, which specifically bind to components of the matrix such as laminin and fibronectin. The anchored tumour cell next secretes proteolytic enzymes to degrade the ECM in a highly localized region close to the tumour cell surface. The third step is
tumour cell locomotion into the region of the matrix modified by proteolysis (Liotta, 1986).

**Extracellular matrix**

The extracellular matrix (ECM) consists of a supramolecular aggregate of connective tissue proteins including collagens, glycoproteins, glycosaminoglycans, and proteoglycans, interacting through covalent and non-covalent bonds to form highly insoluble materials. Due to the complexity of ECM structure, an array of lytic enzymes is necessary for its degradation (Mignatti and Rifkin, 1993).

Collagens consist of three polypeptide chains (α-chains) wound around one another in a triple helix. Types I, II and III are called interstitial or fibrillar collagens and are the main types found in connective tissue (Nimni and Harkness, 1988). Laminin and fibronectin, the major glycoproteins, binds to collagens and proteoglycans, and mediating their attachment to cells (Rosso et al., 2004). GAGs are linear polysaccharides formed by repeating disaccharide units that may contain sulphate groups. GAGs are strongly hydrophilic and form hydrated gels, filling most of the extracellular space. Some examples of GAGs in the ECM are hyaluronic acid, chondroitin, dermatan sulfate and heparan sulphate (Jackson et al., 1991). Basement membrane (BM) is the thin layer of ECM that separates epithelia or endothelia from underlying connective tissue. Type IV collagen is a major component of BM (McKinnell, 1998). BM becomes locally permeable to cell movement during remodelling and invasive processes such as wound healing, angiogenesis and neoplasia. Benign tumours do not usually disrupt the integrity of the BM and ECM, while malignant tumours invade the stroma (Mignatti and Rifkin, 1993).

ECM degradation by tumour-derived proteinases appears to be a prerequisite for cell invasion. So inhibiting the degradation of BM would impede invasion and metastases.

**Proteinases in cancer**

The invasive and metastatic potential of cancer has been correlated in a number of studies with the activity of various proteinases. The tumour cells along with adjacent stromal tissue and tumour-infiltrating immune cells secrete
and activate various proteinases to mediate ECM degradation. Four major classes of proteinases are involved in ECM degradation; Serine (eg: urokinase-type plasminogen activator, Cathepsin G), Cysteine (eg: Cathepsin B, K, L), Aspartyl (eg: Cathepsin D) and Matrix metalloproteinases (eg: MMP-2, MMP-9). In addition, endo and exoglycosidases contribute to ECM degradation by selectively hydrolysing GAGs and the amino sugar moieties of proteoglycans (Alexander and Werb, 1991).

Plasmin is secreted by the liver as inactive proenzyme, plasminogen (Johnsen et al., 1998). Plasminogen is activated to plasmin by tissue plasminogen activator (tPA), urokinase plasminogen activator (uPA) and factor XII (Hageman factor). tPA is of minor importance to tumour progression but uPA is involved in angiogenesis, tumour growth and metastasis. The binding of uPA to the receptor increases the effectiveness of the activation and localizes the proteolytic activity to the invading front of the tumour (Johnsen et al., 1998).

**Matrix metalloproteinases (MMPs)**

MMPs comprise a family of zinc-dependent endopeptidases, which can degrade virtually any component of ECM. There are eight distinct structural classes of MMPs; five are secreted forms and three are membrane-type (MT-MMPs). These enzymes are synthesized as zymogens and kept inactive by an interaction between a cysteine-sulphydryl group in the propeptide domain and the zinc ion bound to the catalytic domain; activation requires proteolytic removal of the propeptide prodomain. All have similar metal dependency (zinc and calcium), optimum pH (neutral pH) and inhibitor susceptibility (Sternlicht and Werb, 2001).

Various kinds of MMP inhibitors exist in tissues to limit the amount of ECM turnover. Most important are the tissue inhibitors of MMPs (TIMPs). The TIMPs appear to be members of a multigene family and currently four TIMPs have been identified: TIMPs -1, -2, -3, and -4 (Hayakawa, 2002). TIMP -1, -2 and -4 are secreted in soluble form, whereas TIMP-3 is associated with the ECM. TIMPs act to inhibit MMP activity by forming complexes with MMPs. Both TIMP-1 and -2 bind non-covalently to active MMPs in a 1:1 molar ratio and specifically inhibit their activity. Inactivation of MMPs is also caused by binding to plasma proteins, such as alpha -2 macroglobulin (Westermarck and Kähäri, 1999).
Metastasis suppressor genes

Non-metastatic clone-23 (nm23) gene was the first identified metastasis suppressor gene. It is known to be a family of eight proteins occurring in all cellular compartments. Enforced expression of nm23 in cell lines of diverse cellular origin was found to suppress metastasis without altering tumour growth. Expression of nm23 in vitro correlates with reduced invasion, motility, and soft agar colonization and induction of differentiation. NM23 was found to phosphorylate serine 392 of KSR (kinase suppressor of Ras), which is a scaffold protein for the mitogen activated protein kinase cascade. This interfere the signalling through the Ras-ERK-MAPK, which is important in metastasis (Shevde and Welch, 2003).

KAI-1 is an evolutionarily conserved member of the tetraspanin transmembrane protein family of leukocyte surface glycoproteins. KAI-1 directly associates with the EGF receptor and suppress metastasis by altering the proliferative and migratory signals delivered through EGFR (Odintsova et al., 2000). Downregulation of KAI-1 was also seen in cancer lines of urogenital, gynecological, and pulmonary origin (Lynch et al., 1998). KISS-1 was identified as a melanoma metastasis suppressor using subtractive hybridization to compare chromosome 6 metastasis-suppressed melanoma hybrids with metastatic parental cells (Shevde and Welch, 2003). Enforced expression of KISS-1 suppressed metastasis of melanoma and breast carcinoma (Lee and Welch, 1997). RhoGDI-2 (Rho GDP dissociation inhibitor) stabilize the GDP-bound form of RhoGTPase and sequester them in an inactive non-membrane localized, cytoplasmic compartment. In an earlier bladder carcinoma study, RNA expression of RhoGDI2 was associated with decreased metastatic potential (Shevde and Welch, 2003). Transfection and enforced expression of RhoGDI-2 suppressed metastasis of T24 human bladder carcinoma variants (Gildea et al., 2002).

1.6. TUMOUR ANGIOGENESIS

Angiogenesis is a physiological process of primary vascular plexus formation, involving the de novo differentiation of endothelial cells from in situ mesoderm-derived precursor cells via sprouting or non sprouting processes
(Makrilia et al., 2009). It is a vital component of many physiological and pathological processes including wound healing, maturation of corpus luteum, chronic inflammation, delayed hypersensitivity, as well as malignant disease.

Neovascularisation is a requirement for solid tumour growth beyond 1–2 mm in diameter. Blood vessels play an important role in tumorigenesis by supplying nutrients and allowing metastatic spread (Cox et al., 2000). The angiogenic process commences when endothelial cells in the vessel wall are exposed to angiogenic factors from the surrounding tissue (Rak et al., 1993). The endothelial cells respond by a series of characteristic morphological and functional changes in which the cells proliferate, enzymatically degrade the basement membrane, migrate toward the angiogenic stimulus, form and join capillary sprouts to create a capillary loop and a functional lumen (Figure 1.3), and finally deposit a new, initially fragmented and leaky basement membrane (Folkman, 1984). The microvessel density within the tumour mass, which is a measure of angiogenesis, confers a poor prognosis in many solid tumours including breast (Weidner et al., 1991), prostate (Weidner et al., 1993), ovarian (Hollingsworth et al., 1995), colorectal (Saclarides et al., 1994) and gastric carcinoma (Tanigawa et al., 1996).

While the actual angiogenic process is similar in normal and tumour tissue, there seem to be some generic differences in regulation of the angiogenic process, as well as both the architecture and function of the resulting vessels. In physiological situations, angiogenesis is turned off once the process is complete. In contrast, angiogenesis in cancer is not self-limiting - once tumour-induced angiogenesis is turned on it continues indefinitely until the tumour is eradicated or the host succumbs to the disease (Rak et al., 1995). Second, tumour angiogenesis results in a wide variety of structural and functional anomalies, which are not found in normal blood vessels.

**Pathophysiological characteristics of tumour vasculature**

The principal function of blood vessels arising during development, hair growth and pregnancy in normal physiological conditions is to provide adequate levels of nutrients and oxygen to the parenchymal cells and to remove waste products. Due to their abnormal structure tumour vessels fail to do so. The normal vasculature is characterized by dichotomous branching while tumour
Figure 1.3

The process of angiogenesis

1. Angiogenic factor production
2. Angiogenic factor binding
3. EC proliferation
4. EC migration
5. Degradation & Invasion of ECM
6. Tube formation
7. Loop Formation & Vessel wall maturation
vasculature is unorganized and has trifurcations and branches with uneven diameters (McDonald and Choyke, 2003). The structure of vessel wall is also abnormal in tumours, characterized by large inter-endothelial junctions and increased number of fenestrations, vesicles and vesico-vacuolar channels and the lack of normal basement membrane and an organized coat of perivascular cells which normally protect endothelial-lined vessels against changes in oxygen or hormonal balance (Dvorak et al., 2002). These abnormalities in blood vessel architecture result in increase in resistance to blood flow leading to hypoxic and acidic regions in tumours, conditions that have negative implications for drug delivery and lower the efficacy of anti-cancer treatments.

Hydrostatic and oncotic pressures in tumours become almost equal between the intra- and extravascular spaces, due to high vascular permeability (Jain, 1988). During tumour development, the high rate of tumour cell proliferation in a confined space creates mechanical stress, which compresses the intra-tumour vessels. Consequently, the interstitial fluid pressure in tumours is increased and the interstitial fluid oozes out from the tumour periphery into the surrounding normal tissue, carrying angiogenic and lymphangiogenic growth factors and even metastasizing tumour cells (Makrilia et al., 2009).

**The angiogenic balance**

Small tumours, unable to induce angiogenesis, stop expanding and reach a steady state –dormancy- in which the number of dying cells counterbalances the number of proliferating cells. The switch of a tumour from an avascular to a vascular phenotype is referred to as the ‘angiogenic switch’, after which the tumour becomes invasive and malignant. The switch to the angiogenic phenotype depends on a net balance between angiogenesis stimulators and inhibitors exported by the tumour cells, mobilized from the extracellular matrix, or released by infiltrating inflammatory cells such as macrophages (Bouck et al., 1996). Mouse tumour models in which lymphangiogenic growth factors are overexpressed have shown that this causes increased growth of lymphatic vessels inside the tumour as well as metastatic spread of the tumours, and blocking these factors reduced metastasis (Luttun et al, 2004).
**Regulators of angiogenesis**

A large number of growth factors and cytokines have been shown to play a crucial role in angiogenesis. Positive modulators of angiogenesis include VEGF, FGF, hyaluronic acid fragments, TGF-β, TNF-α, EGF, angiogenin okadaic acid, IL-8, IL-6, haptoglobin, HGF and gangliosides (Cockerill *et al.*, 1995). Serum levels of angiogenic factors are higher in tumour patients than controls and could be used as a prognostic tool or as markers of angiogenesis (Kondo *et al.*, 1994).

VEGF, one of the most potent angiogenic factors, is also known as vascular permeability factor. VEGF is a highly conserved, 34-42 kDa homodimeric glycoprotein, which shares homology with platelet derived growth factor. The VEGF family includes the VEGF-A, -B, -C, -D, -E factors and the placenta growth factor (Ferrara *et al.*, 2003). VEGF acts selectively on vascular endothelial cells through receptors on the cell surface that includes flt-1 (VEGFR-1), and flk-1/KDR (VEGFR-2). The VEGF-A–VEGFR-2 interaction plays a crucial role in angiogenesis, through the coordinate signalling of endothelial cell proliferation, migration and recruitment of endothelial cell progenitor cells (Gerber *et al.*, 1998).

Basic fibroblastic growth factor (bFGF) was the first discovered angiogenesis stimulator in 1982, followed by the discovery of acidic fibroblastic growth factor (aFGF) (Brower, 2003). Both proteins are members of a family of growth factors, which are characterized by their high affinity binding to heparin, and both have been found to be strongly chemotactic and mitogenic for endothelial cells. FGFs stimulate endothelial cell proliferation (Gospodarowicz *et al.*, 1989) and migration (Terranova *et al.*, 1985), as well as production of collagenase and plasminogen activator (Millauer *et al.*, 1993).

Platelet-derived growth factor (PDGF) is a potent mitogen for cells of mesenchymal origin. PDGF-B and platelet-derived growth factor β receptor (PDGFR-β) have important roles in the development and differentiation of the vessel wall (Lindahl *et al.*, 1997). PDGF has been found to stimulate angiogenesis *in vivo* and experiments with knockout mice have suggested a role for PDGF in the recruitment of pericytes that are needed for the development of capillaries in tumours (Andrae *et al.*, 2008).
TGF-β family of proteins consists of at least three members TGF-β 1,2,3. Paradoxically, although TGF-β shows an inhibitory effect on endothelial cell proliferation, it can induce angiogenesis in vivo (Roberts et al., 1986). TGF-β and its receptors are highly expressed in malignant gliomas, especially in areas of vascular hyperplasia and around necrotic regions (Wong et al., 2009).

Angiopoietins is a family of endothelial growth factors that signal via the Tie2 RTK and play an important role in vessel maintenance, growth and stabilization. There are four types of angiopoietins known: Ang-1, -2, -3 and -4. The biological effect of Ang-2 depends on VEGF level. In the presence of endogenous VEGF, Ang-2 promotes vessel dilatation, remodelling of the basal lamina, proliferation and migration of endothelial cells, and stimulates sprouting of new blood vessels (Thomas and Augustin, 2009).

**Endogenous angiogenesis inhibitors**

Endogenous angiogenesis inhibitors have been broadly categorised into 4 groups: interferons, interleukins, TIMP, and proteolytic fragments (Feldman and Libutti, 2000). Natural inhibitors of angiogenesis can be produced by cancers and normal tissues and are lost during tumour development. Tumour suppressors induce the expression of these anti-angiogenic molecules, such as thrombospondin-1, and oncogenes reduce their expression (Harris, 2003).

Tissue inhibitor metalloproteinases inhibits the activities of MMP-1, MMP-2 and MMP-9, which leads to reduced ECM remodelling and suppression of endothelial cell migration and invasion (Gomez et al., 1997). Cancer metastasis occurs as a result of an imbalance between MMPs, and their inhibitors. Giannelli et al., (2004) found that pro-MMP-9 and TIMP-1 serum concentrations are inversely correlated in breast cancer patients. Their results show that after surgery, when the breast cancer tissue was removed, pro-MMP-9 concentrations dramatically decreased and TIMP-1 concentrations strongly increased. TIMP-2 can also downregulate bFGF-induced endothelial cell proliferation (Murphy et al., 1993).

Angiostatin, an internal fragment of plasminogen, is a specific inhibitor of endothelial cell proliferation. Angiostatin induces apoptosis in endothelial cells and tumour cells and functions as an inhibitor of t-PA catalyzed plasminogen activation (Kirsch et al., 1998). Endostatin, the C terminal fragment of collagen
XVIII, is a powerful cytokine that inhibits endothelial cell migration and induces endothelial cell apoptosis and cell cycle arrest. Endostatin directly interacts with VEGFR-2 and inhibits VEGF binding (Kim et al., 2002). Endostatin also inhibits integrin function, bFGF binding, and MMP-2 activity in endothelial cells (Wong et al., 2009). Thrombospondin-1 belongs to a family of extracellular matrix proteins. The interaction between TSP-1 and its receptor, CD36, activates a sequence of intracellular events finally resulting in endothelial cell apoptosis (Sargiannidou et al., 2001). Vasostatin and platelet-associated platelet factor-4 are N-terminal and C terminal fragments of calreticulin and are potent angiogenesis inhibitors (Li et al., 2007). Osteopontin is a phosphorylated glycoprotein involved in angiogenesis, tumour growth, invasion, metastasis and wound healing (Ribeiro-Silva and Oliveira da Costa, 2008).

Pharmacologic agents in inhibition of angiogenesis

The special features of the tumour associated vasculature make anti-angiogenic therapy an attractive anticancer therapeutic agent. Specific strategies to inhibit angiogenesis include; blocking growth factor production, neutralisation of circulating growth factors, inhibition of RTK activation and suppression of intracellular signalling cascades. Although no anti-angiogenic drug has been approved, many have undergone Phase I/II clinical trials.

The compounds under clinical trial includes; Inhibitors of matrix metalloproteases (solimastat, AG3340, neovastat, BMS 275291), Blockers of angiogenic growth factors (Anti-VEGF antibody, interferon alpha, angiozyme), Inhibitors of endothelial cell migration and proliferation (endostatin, angiostatin, squalamine, TNP-70, thalidomide, 2 methoxyoestradiol), Blockers of endothelial cell surface proteins, e.g. integrins (vitaxin, EMD 121974), Copper chelating agents (penicillamine, tetrathiomolybdate, captopril), Angiogenic blockers by unique/multiple mechanisms (CAI, IL-12, IM862) and drugs with other main mechanisms of action (methotrexate, taxanes, camptothecins and their analogues, vinca alkaloids and signal transduction inhibitors) (Harris, 2003).

1.7. APOPTOSIS

Important safeguards exist within the cells to prevent replication of cells bearing mutations. These are DNA repair mechanisms, redundant pathways to
produce an end result, check-points that provide time for repair, and proteasomes for the elimination of defective proteins. As the final safeguard, there is apoptosis, causing death of defective and dangerous cells and thus prevent mutations that cause cancer (Sellers and Fisher, 1999). Apoptosis is a terminal cell fate, a highly regulated “suicide” process that is distinct from necrosis. It is programmed, active, highly selective mechanism of cell death characterized by morphological features such as membrane blebbing, cell shrinkage, nuclear fragmentation, chromatin condensation, and chromosomal DNA fragmentation (Best et al., 1999).

After a cell is severely damaged the time of checkpoint arrest may be too brief to permit complete repair, and such cells are eliminated by apoptosis. Checkpoint genes including p53 are involved in activating apoptosis, and other proteins including NF-κB can prevent apoptosis. Apoptosis is performed by proteases named caspases and by nucleases, activated by a family that includes positively acting Bax and negatively acting Bcl-2 proteins (Pardee, 2004).

**Apoptotic pathways**

The apoptotic process is triggered off by two major signalling pathways; extrinsic, which responds mainly to extracellular stimuli, and intrinsic, activated by modulators within the cell itself. The two pathways are apparently separate from each other at the beginning, but they converge in a single crucial point at the end, i.e., activation of the effector caspase-3 (Russo et al., 2006). In addition to this, there is other pathways namely, granzyme B-mediated pathway, which is activated by immune cells. But the events regulating the apoptotic pathway are very often altered in tumour cells.

**Extrinsic pathway**

It is also called as the death receptor-mediated pathway and is important in the immune system. Death receptors are transmembrane proteins which binds with a death ligand (TNF-α, TNF-β, TRAIL, FasL, etc) and transmit the apoptotic signal to the interior of the cell. The mammalian death receptors belonging to the TNF family (TNFR1, CD95 [Fas/Apo1], TRAIL-R1/Apo2, TRAIL-R2, DR3/TRAMP/Apo3 and DR6/TR7) have intracellular death domain (Ashkenazi and Dixit, 1999).
The binding of ligand to their receptor induces trimerization, leading to conformational change that forms death-inducing signalling complex (DISC). Upon activation, the DD recruits a DD-containing adaptor such as Fas-associated death domain protein (Chinnaiyan et al., 1995). Interaction of FADD and Fas through their DD unmasks the NH$_2$ terminal death-effector domain (DED) of FADD, allowing it to recruit caspase-8 to the Fas signaling complex (Muzio et al., 1996). Caspase-8 in turn becomes autoactivated by oligomerization, and stimulates other caspases, including caspase-3, -6, and -7 (Enari et al., 1996). DR-4 and DR-5 have also been shown to recruit FADD and caspase-8 to activate the apoptotic machinery (Kischkel et al., 2000).

**Intrinsic pathway**

During apoptotic process mitochondria experience changes in the membrane potential, the cytoplasmic pH, and the cellular redox state. Various apoptotic stimuli, such as cytotoxic stress, oxidative stress, heat shock and DNA damage, lead to the release of cytochrome C from mitochondria to the cytosol (Finkel, 2001). Cytochrome C is a resident protein of the mitochondrial intermembrane space. In the cytosol, cytochrome C interacts with apoptotic protease-activating factor-1 and dATP, leading to the formation of a high-molecular mass cytoplasmic complex referred to as the apoptosome (Acehan et al., 2002).

Apaf-1, a 130 kDa protein, is composed of three functional domains: a short N-terminal caspase recruitment domain (CARD), a central CED-4 homology domain, and a long C-terminal ”WD-40” repeat domain (Zou et al., 1997). In the presence of cytochrome c and dATP, Apaf-1 can interact with procaspase 9 through their mutual CARDS, which results in its activation. Caspase 9 is thus able to recruit and activate caspase 3, which is the effector of both pathways.

**Perforin/granzyme pathway**

Granzyme B is a serine protease expressed by cytotoxic T lymphocytes and natural killer cells, having substrate specificity similar to caspases (Pinkoski et al., 2001). The granzyme B structure reveals an overall fold similar to that found in cathepsin G and human chymase with substrate specificity (Ruggles et
Granzyme B induces caspases through cytosolic proapoptotic caspase-3. However, granzyme B cannot fully process procaspase-3. The full activation may involve other proapoptotic mediators such as cytochrome C and Smac, which are released from mitochondria (Barry et al., 2000). Also granzyme B cleaves Bid at ASP-75 to generate a 14 kDa grB-truncated product (gtBid), which in turn translocates to mitochondria and begins to recruit Bax to mitochondria through a caspase-independent mechanism. Once this process completes cytochrome C is released and apoptosis proceeds (Heibein et al., 2000).

**Cell cycle and its regulation**

The cell cycle consists of four distinct phases, G1, S, G2 and M. Activation of each phase is dependent on the proper progression and completion of the previous one. Cells that have temporarily or reversibly stopped dividing are said to have entered a state of quiescence called G0 phase (Sherr, 1994). Progression of eukaryotic cells through these major transitions is mediated by sequential activation and inactivation of cyclin-dependent kinases (Figure 1.4). The active forms of these kinases occur as heterodimers that are composed of a regulatory subunit (cyclin) and its catalytic subunit (cyclin-dependent protein kinase). Cyclins facilitate cell cycle progression and are regarded as positive regulators of the cycle (Vidal and Koff, 2000). The expression of cyclin D, E and A, mediates G1 transition, while the appearance of cyclin A and B facilitates S and M progression, respectively. Cyclin D1 was found to be overexpressed in laryngeal squamous cell carcinoma (Dong et al., 2002), MNNG induced rat gastric adenocarcinoma (Takasu et al., 2007), squamous carcinomas and adenocarcinomas of the esophagus and in adenocarcinomas of the stomach (Arber et al., 1999)

A key component of the regulation of cell cycle entry in mammalian cells is the retinoblastoma protein, pRb. This molecule mediates the progression of cells from G1 to S phase of the cell cycle. The hyperphosphorylation of pRb by cyclin D-CDK4/CDK6 complexes during mid to late G1 phase results in the release of transcription factor, E2F1, from pRb, which then transcribes genes (eg. dihydrofolate reductase, cyclin A, and cyclin E) critical for cell cycle progression (Martin-Castellanos, 1997).

Cell cycle inhibitors have been classified into two different families; inhibitors of cdk4 (INK4) or the kinase inhibitor proteins (CIP/KIP), based on
Figure 1.4

Cell cycle and its regulation
their structural similarities (Vidal and Koff, 2000). The CIP/KIP family consists of three proteins: namely p21CIP1/WAF1, p27KIP1 and p57KIP2. The INK4 family group contains four proteins; p16INK4a, p15INK4b, p18INK4c and p19INK4d. The inhibitory activity of INK4 is restricted to CDK4 and CDK6 (Carneiro and Koff, 2004).

**Role of p53**

The p53 gene, called as “Guardian of the Genome” is the first tumour suppressor gene to be identified in 1979 (Vogelstein et al., 2000). It is one of the most highly mutated genes in human tumours and tumours with p53 mutations have been found to associate with a more aggressive phenotype and a worse prognosis in multiple tumour types (Royds and Iacopetta, 2006). p53 protein is 393 amino acids long and contain three functional domains typical of a transcription factor; an acidic amino-terminal transactivation (TA) domain; a central core DNA-binding domain (DBD); and a carboxyterminal oligomerization domain (OD) (Zamamiri-Davis and Zambetti, 2004).

The p53 is normally ‘off’ and the pathway is activated upon cellular stresses, such as DNA damage, hypoxia, loss of cell survival signals, telomere attrition and oncogene activation. Upon activation, p53 elicits cellular responses, such as cell cycle arrest, senescence and apoptosis by acting as a transcription factor or interacting with other proteins, such as the Bcl family, including Bcl-2. The p53 stress response has been shown to be crucial for the prevention of tumour formation in both mice and humans (Bond and Levine, 2007).

As a consequence of its functions, the transcription, translation, protein stabilization, subcellular localization and activation of p53 are tightly controlled by several mechanisms. The murine double minute-2 (mdm-2) is one of the enzymes involved in labelling p53 with ubiquitin to mediate the proteasomal degradation of p53 (Momand et al., 2000). In response to cellular damage, either p53 or mdm-2 is post-translationally modified to up-regulate the former molecule for nuclear accumulation. Ataxia telangiectasia mutated (ATM), checkpoint kinase 2 (CHK2) phosphokinases and ataxia telangiectasia-related (ATR) phosphokinase initiate the phosphorylation of p53 and prevent mdm-2 association (Carr, 2000).
**Bcl-2 family**

Bcl-2 family members are the key regulators of apoptosis and most members are potent anti-apoptotic. At least 15 Bcl-2 family members have been identified in mammalian cells and others in viruses, which are be divided into 3 subfamilies; Bcl-2, Bax and BH3 families (Gross et al., 1999). Bcl-2 subfamily includes all the anti-apoptotic molecules; Bcl-2, Bcl-xL, Bcl-w, Mcl-1, Al, NR-13, BHRF1, LMW5-HL, ORF16, KS-Bcl-2, E1B-19K and CED9. All members possess at least one of four conserved motifs known as Bcl-2 homology domains (BH1 to BH4). Bax subfamily (Bax, Bak and Bok) and BH3 subfamily (Bik, Blk, Hrk, BNIP3, BimL, Bad, Bid and EGL-1) are pro-apoptotic. All of them differ markedly in their relatedness to Bcl-2 (Adams and Cory, 1998).

The bcl-2 protein, a membrane-associated protein of the mitochondria, nuclear envelope, and endoplasmic reticulum, cause damage to these compartments and affect their behaviour by modifying the flux of small molecules or proteins (Kroemer, 1997). Overexpression of bcl-2 was found to suppress apoptosis induced by a wide variety of stimuli, including myc- induced death in fibroblasts (Fanidi et al., 1992), growth factor withdrawal, oxidative stress (Hockenbery et al., 1993), and calcium elevation (Zhong et al., 1993). Bcl-xL mediates anti-apoptotic activity by inhibiting the association of Apaf-1 with procaspase-9 and thereby preventing caspase-9 activation (Hu et al., 1998).

Bid exerts pro-apoptotic activity by activating the mitochondria pathway through permeabilization of the mitochondrial outer membranes. Bid is able to induce the release of multiple mitochondrial inter-membrane space proteins, including cytochrome c, Smac, Endonuclease G and AIF (Yin, 2006). Bax is a pro-apoptotic protein acting through the intrinsic pathway. Bax is present in viable cells and activated by pro-apoptotic stimuli. Activation implies structural changes, migration from cytosol to mitochondria and endoplasmic reticulum membranes and changes in the aggregation status. it release of mitochondrial factors (cytochrome c, SMAC), promotes Ca\(^2+\) leakage through ER membrane and cause bax-dependent membrane pore formation (Ghibelli and Diederich, 2010).

**Caspases – the executioners of apoptosis**

Caspases are aspartate-specific cysteine proteases that execute specific proteolytic activity upon activation. They are members of the interleukin-1 \(\beta\) -
converting enzyme family and consist of 15 mammalian members that are grouped into two major sub-families, namely inflammatory caspases and apoptotic caspases (Vaculova and Zhivotovsky, 2008). Caspases are synthesized as catalytically-dormant tripartite proenzyme, a single polypeptide chain of 32–55 kDa with 3 domains; a 17–21 kDa central large internal domain containing large catalytic subunit, a 10–13 kDa small C-terminal domain also called small catalytic subunit, and a 3–24 kDa NH2-terminus prodomain called death domain (Chowdhury et al., 2008). Activation involves the association of the large and small subunits to form a heterodimer by either auto-cleavage by self-aggregation, or by other caspases resulting in a 'caspase cascade' that permits a rapid amplification of caspase activation. Caspases-2, -8, -9 and -10 are the initiator caspases, whereas caspase-3, and to a lesser extent caspases-6 and -7, serve as effector caspases.

The active enzymes breakdown the cytoskeletal structure leading to loss of the cellular architectural integrity. Cleavage of ICAD (inhibitor of caspase-activated DNase) also called DNA fragmentation factor allows its binding partner CAD to migrate to the nucleus and execute inter-nucleosomal digestion, generating 180-bp DNA fragments referred to as "DNA laddering" (Liu et al., 1997). Cleavage of poly-ADP-ribose polymerase (PARP) resulting in a 84 kDa protein fragment (Lazebnik et al., 1994) or formation of DNA laddering are widely referred as the "gold standard" in apoptosis.

1.8. CURRENT THERAPIES

Surgery

Surgery is the oldest treatment for cancer and, until recently, was the only treatment that could cure patients with solid tumours when the tumour is confined to the anatomic site of origin. The extension of the surgical resection to include areas of regional spread can cure some patients, although regional spread often is an indication of undetectable distant micrometastases. Presence of metastases fails to provide a complete cure in most cancer patients (e.g., pulmonary metastases in sarcoma patients, hepatic metastases from colorectal cancer). The magnitude of surgical resection is improved in the treatment of many cancers by the use of adjuvant treatment modalities (Rosenberg, 2008).
**Radiation therapy**

Radiotherapy is defined as treatment of malignant disease by ionizing radiations. Radiation therapy alone is often used with curative intent for localized tumours, and is synergistic with chemotherapy in susceptible cancers. It is useful in cases where surgical removal of cancer is not possible or when surgery might debilitate the patients, such as advanced lung, head and neck cancers. Unfortunately, this therapy is not tumour specific and has other serious side effects, most important of which is myelosuppression and there is probability of developing metastatic disease in distant organ sites (Lawrence *et al.*, 2008).

**Chemotherapy**

Chemotherapy still remains the most effective modalities of managing metastasis of cancer cells. Most chemotherapeutic agents currently in use exert their effect primarily on the cell multiplication and tumour growth. It is also a non specific mode of treatment with serious adverse effects like bone marrow suppression, mucositis and hair loss. Some of them cause cumulative dose-dependent toxicities to slowly- proliferating or non-proliferating normal tissues like kidney, liver, nervous system and heart. Bone marrow myeloid cells are the first to be adversely affected by chemotherapy, leading to decline in the number of peripheral blood cells (DeVita, 2008).

**Immunotherapy**

Immunotherapy refers to the use of cells, molecules and genes of the immune system for the treatment of infectious diseases, autoimmune diseases, neoplastic diseases and graft versus host disease. It relies on the ability of the immune system to identify and destroy the tumour cells and elicit long lasting memory of this interaction. Cancer immunotherapy has 3 main approaches; (1) Nonspecific stimulation of immune reactions, to stimulate effector cells or to inhibit regulatory cells, (2) Active immunization to enhance anti-tumour reactions (cancer vaccines), and (3) Passively transfer activated immune cells with antitumour activity (adoptive immunotherapy) (Restifo *et al.*, 2008).
1.9. **IMMUNOMODULATION BY NATURAL PRODUCTS**

Natural plant products have played a pivotal role in the health care of many cultures, both ancient and modern. For centuries, drugs were entirely based on natural origin and composed of herbs, animal products and inorganic materials. Medicinal plants played a prominent role in traditional systems of medicine such as Chinese, Ayurveda, and Egyptian, which are still in common use today (Sarker *et al.*, 2005). According to the World Health Organization, 75% of people in developing countries still rely on plant-based traditional medicines for primary health care.

An immunomodulator is a substance that has an effect on the immune system; it induces either immunostimulation or immunosuppression. Immunostimulation via natural substances is considered to be a most promising way for the prevention and cure of neoplastic diseases. Immunomodulatory agents mediate their actions by activating cytotoxic effector cells such as cytotoxic T-lymphocytes and natural killer cells. Immunomodulators can play a significant role in the production of cytokines such as TNF, interleukins and interferons, which are involved in the regulation of immune responses.

*Thuja occidentalis*, commonly used in Homeopathic system, was found to augment the cell mediated immune responses in metastatic tumour bearing mice by augmenting ADCC, ACC and natural killer cell mediated tumour cell killing (Sunila *et al.*, 2011). *Viscum album*, a semi-parasitic plant can stimulate humoral and cell mediated immune responses (Kuttan and Kuttan, 1992) and inhibit solid tumour development in mice (Kuttan *et al.*, 1992). Similarly methanolic extract of the plant *Withania somnifera* has shown to stimulate the immune system (Davis and Kuttan, 2000b), reduce leukocytopenia during radiation therapy (Kuttan, 1996) and inhibit urotoxicity induced by chemotherapeutic drug cyclophosphamide (Davis and Kuttan, 2000a). Alcoholic extract of the fruits of *Piper longum* and its component piperine, has shown to have immunomodulatory and antitumor activity (Sunila and Kuttan, 2004). Curcumin which is present in the rhizome of *Curcuma longa* has shown to stimulate the immune system in animals (Antony *et al.*, 1999). Many of the clinically used antineoplastic drugs such as camptothecin, taxol, vincristine, vinblastine are
plant derived products and several clinical trials on the use of nutritional supplements and phytochemicals to prevent cancer are going on now.

1.10. COMPOUNDS USED IN THE PRESENT STUDY

*Aerva lanata*

*Aerva lanata* is a woody, prostrate, succulent, erect, hoary-tomentose, perennial herb belonging to the family *Amaranthaceae* available in India, Ceylon, tropical Africa, Java and Philippines (Figure 1.5.A.). It is an important medicinal plant growing throughout the plains of India. It is used in Indian folk medicine as a demulcent, diuretic, to clear uterus after delivery, to prevent lactation and in treatment of lithiasis, diabetes mellitus, urinary calculi, hematemesis, bronchitis, nasal bleeding, cough, scorpion stings, fractures and spermatorrhoea, (Chowdhury et al., 2002). Phytochemical analysis of *Aerva lanata* showed the presence of steroids, alkaloids, flavonoids, tannins, saponins and polysaccharides (Soundararajan et al., 2006).

The partially purified petroleum ether extractable fraction of the whole plant *Aerva lanata* was found to protect Sprague Dawley rats against liver damage induced by carbon tetra chloride (Nevin and Vijayammal, 2005). *Aerva lanata* was also found to be very effective on the urinary risk factors of calcium oxalate urolithiasis (Soundararajan et al., 2006). The partially TLC-purified fraction (PEF) of petroleum ether extract was proved to be cytotoxic to Dalton’s lymphoma ascites, Ehrlich ascites and B16F10 cell lines *in vitro* (Nevin and Vijayammal, 2003). Different solvent fractions of the whole plant of *Aerva lanata* were found to have antimicrobial and cytotoxic activity (Chowdhury et al., 2002). *Aerva lanata* was also successful in preventing cisplatin- and gentamicin-induced acute renal failure (Shirwaikar et al., 2004).

10-Methoxycanthin-6-one

Various alkaloids have been demonstrated to have anti-cancer capability via inhibition of inflammatory responses, prevention of metastasis, angiogenesis and induction of apoptosis. 10-Methoxycanthine-6-one is a β carboline alkaloid (Figure 1.5.B.) from the plant *Aerva lanata*. Structurally related compounds like 1-Methoxycanthin-6-one have been reported to induce apoptosis in human
Figure 1.5

A) Aerva lanata

B) Chemical structure of 10-Methoxycanthin-6-one

C) Chemical structure of Thujone

α Thujone

β Thujone
leukemia (Jurkat), thyroid carcinoma, and hepatocellular carcinoma cell lines via a JNK-dependent mechanism (Ammirante et al., 2006).

**Thujone**

Thujone, a monoterpenoid ketone, occurs in nature as a mixture of α-(−)- and β-(+) -diastereoisomers (Figure 1.5.C.), in the essential oils and parts of the plants of Artemisia (Lopes-Lutz et al., 2008), Salvia (Mathe et al., 2007), Thuja (Naser et al., 2005) and Juniperus species (Tunalier et al., 2002). The ratios of α to β-thujone vary with source. Thujone is the major constituent in the essential oil of Thuja occidentalis (Naser et al., 2005), which is proved to be having immunomodulatory (Sunila et al., 2011) and anti metastatic activity (Sunila and Kuttan, 2006). Thujone oil has been used in beverages and herbal medicine (Sirisoma et al., 2001). Essential oils containing Thujone are being used in traditional medicine for the following purposes; abortifacient, female hormone activity, emmenagogue; digestive problems, anthelmintic, and for corns, warts, acne, fever, cough, rheumatism, dropsy (Albert-Puleo, 1978; Millet et al., 1981).

Thujone is reported to have anti nociceptive activity in mice (Rice and Wilson, 1976). Thujone act as an antagonist on GABA and 5-HT3 receptors in the brain (Deiml et al., 2004). In mouse, intraperitoneal administration of α/β Thujone mixture at a concentration of 590 mg/kg body weight can cause muscle spasms and convulsions (Wenzel and Ross, 1957). In a series of coordination and behavioural tests in mice, Thujone at sub-convulsive dose of 3 mg/kg i.p., showed normal behaviour without any psychotropic action and a depression of activity and exploratory behaviour at 24mg/kg (Le Bourhis and Soenen, 1973).
OBJECTIVES OF THE PRESENT STUDY

1) To determine the anti inflammatory and anti tumour activities of *A. lanata*, 10-methoxycanthin-6-one and Thujone using *in vivo* and *in vitro* systems.

2) To determine the immunomodulatory effect of *A. lanata*, 10-methoxycanthin-6-one and Thujone.

3) To determine the effect of *A. lanata*, 10-methoxycanthin-6-one and Thujone on the inhibition of metastasis and to study the mechanism of its action using *in vivo* and *in vitro* systems.

4) To determine effect of *A. lanata*, 10-methoxycanthin-6-one and Thujone on the tumour specific angiogenesis using *in vitro* and *in vivo* models.

5) To determine the effect of *A. lanata*, 10-methoxycanthin-6-one and Thujone on the regulation of cell cycling and inducing apoptosis in B16F-10 melanoma cells.

6) To determine the effect of the isolated compound, 10-methoxycanthin-6-one on the reversal of toxicity of current therapies – Radiation and chemo therapy.