Review of Literature

Chapter 1

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Cancer is a disease of misguided cells, which have high potential of excessive proliferation without apparent relation to the physiological demand of the organ involved (Hanahan and Weinberg, 2000). Cancer is a multi-factorial multi-staged and multi-mechanistic complex process. It involves the interaction of the environmental and host factors in the inception, progression of manifestation (Seth and Ana, 2001). Inherited genetic dispositions contribute significantly to 5-10 percent of breast cancer and 5-13 percent of colon cancer incidences (Emory et al, 2001). In the industrialized nations, roughly 7 percent of cancer deaths are attributable to viral infections; 4 percent to occupational hazards; 2 percent to sunlight; 2 percent to pollutions of air, water, and soil; and less than 1 percent to food additives and industrial products (Balmain et al, 2003).

**Etiology of cancer**

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. Carcinogens are extremely diverse structures and include both natural and synthetic products. Some are direct acting carcinogens (require no chemical transformations to induce carcinogenisity) eg, alkylating agents. The indirect acting carcinogens are referred as procarcinogens, eg, Benzo(a)anthracene, Benzo(a)pyrene etc. All chemical carcinogens are highly reacting electrophiles that react with the electron rich atoms like RNA, DNA and protein.

Metals are known to be carcinogenic to humans. Some of the important
metals such as arsenic and arsenic compounds, chromium, nickel, cadmium and beryllium can induce the development of lung cancer and prostate cancer (Sabine et al, 1987). Physical carcinogens such as x-ray, y-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 (Darcel, 1994).

The most life-threatening aspects of the oncogenic process is metastasis. Even though the clinical significance of such expression of the malignant phenotype has been well appreciated, advances in understanding the molecular mechanisms involved in metastasis have lagged behind other developments in the cancer field.

**Metastasis**

The most deviating aspect of cancer is the probability of malignant cells to spread from primary site to distant target organs. Metastatic dissemination of a neoplasia to secondary sites is the primary cause of death among cancer patients (Fiddler, 1997). The appearance of metastases in latter stages of neoplastic disease results in unfavorable clinical prognostics; metastatic cells become resistant to the majority of known drugs and treatment strategies (Condeelis et al, 2000; Engers et al, 2000). Formation of metastases is a multi-step event that may be arbitrarily divided into four stages.
a) Migratory and adhesive properties of tumor cells

Neoplastic cells that are capable of migration appear at the primary site (primary colony) (Malcolm et al, 2004). These cells exhibit altered cell-to-cell interactions (they lack homotype relations via E-cadherins) as well as altered interactions with the extracellular matrix (ECM) (Engers et al, 2000). An important role in this altered relation between neoplastic and normal cells as well as the extracellular matrix is ascribed to adhesion molecules such as integrins, selectins, ICAM-1, VCAM-1, NCAM, mucins, glycosphingolipids, CD 44 molecules, Lu- ECAM-1 and others (Peter et al, 2005). The migrating cells are also able to secrete and/or activate proteolytic enzymes (matrix metalloproteases, MMP) involved in cell locomotion across the ECM (Engers et al, 2000).

b) Intravasation of tumor cells

Migrating metastatic cells penetrate into the lumen of blood vessels or, rarely, lymphatic vessels (intravasation). The cells are passively carried with the bloodstream and become arrested in micro vessels of diameter smaller than their own (Chambers et al, 2000). The arrested cells would frequently become deformed. They might attach to blood vessel walls, most likely by adhesive interactions. Alternatively, single metastatic cells could be arrested in vessels with a diameter larger than their own (Stanis et al, 2002). In that case interactions of adhesive nature would definitely be responsible for the cells sticking on to the blood vessel walls.
c) Extravasation of tumor cells

During extravasation cancer cells leave the blood or lymph vessels. Their penetration through the vessel wall does not cause mechanical damage to the latter, 24 hours from the arrest of metastatic cells in microcirculation 80% of them undergo extravasation (Chambers et al, 2000). Metastatic cells adhering to the walls pulmonary vessels do not actually extravasate but begin to proliferate and form micro colonies entirely within the blood vessels, and the vessel wall is destroyed after sometime resulting is the colony growth outside of it (metastasis) (Stanis et al, 2002).

d) Formation of metastases.

The last stage of the metastatic process involves formation of secondary colonies called metastases. Importance of this stage of metastasis may be the chemotactic interactions. Due to the presence of specific receptors cancer cells are believed to migrate towards the source of specific chemokines. These effector molecules are believed to be responsible for the observed preferences in metastatic spread to specific organs; for eg, melanoma metastasizes foremost into lungs while breast cancer into bones, etc. (Muller et al, 2001).

Matrix metalloproteinases (MMPs) in tumor progression

In considering the steps in the process of tumor growth and metastasis tumor cell population must not only be able to invade into surrounding tissues as the tumor mass expands in size, but must also be able to cross two, and in most cases three, basement membranes to successfully complete its journey to a secondary site. The main component of basement membrane is type 1V collagen.
with additional components of laminin, entactin and fibronectin. Proteases of the aspartic, cysteine and serine classes as well as matrix degrading metalloproteinases are candidate proteolytic facilitators of tumor cell invasion and metastasis. Several agents have been developed that block the synthesis of MMPs, prevent them from interacting with the molecules that direct their activities to the cell surface or inhibit their enzymatic activity.

MMPs can be divided into four categories based on substrate preference: collagenases, gelatinases, stromelysins and membrane–associated MMPs. Gelatinase A (MMP-2) and gelatinase–B (MMP-9) are key enzymes for degrading type IV collagen, which is a major component of the basement membrane (Gijbels et al, 2004). Expression levels of MMP–2 and MMP–9 are associated with tumor metastasis for various human cancers (Hiro-omi et al, 2000). Metastatic tumor expresses high levels of type IV collagenase activity than the non–metastatic tumors (Stefan et al, 1993). MMPs are also largely involved in the process of neoangiogenesis. During angiogenesis, endothelial cells also produce MMP-1, MMP-2, MMP-3 and membrane type–MMP (MT-MMP) (Kleiner, 1999). The role of tumor associated MMPs seems to be in almost all facets of tumor progression; tumor growth, invasion and metastasis. The matrix metalloproteinases, as the name implies, depend upon a metal ion (Zn+) for activity and have several interesting features that implicate them in the process of metastasis. Common salient features of MMP include a conserved metal binding site in the catalytic domain consisting of the amino acid sequence HEXGHXXGXXHS and a conserved sequence in the pro–region comprised of

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the amino acids PRCGVPDV. The latter sequence, responsible for the latency of the enzyme, introduces an additional level of proteolytic activation. The activity of these molecules is thought to be controlled by a mechanism known as cysteine swich (Fu et al, 2001) in which a conserved unpaired cysteine residue in the pro-domain forms a coordinate bond with the zinc ion in the active site, an association which effectively ‘close the door’ of the enzyme and renders it inactive. MMPs are synthesized as inactive precursors and are activated by proteolytic cleavage (Nagase et al, 1999; Brew et al, 2000). Therefore, the regulation of MMPs occurs at three levels: gene expression, proenzyme processing, and inhibition of enzymatic activity. There are classes of molecules present endogenously known as tissue inhibitors of metalloproteinases which could inhibit the activity of MMPs (TIMPs) (Rainer et al, 2001).

**Tissue inhibitors of metalloproteinases (TIMPs)**

Tissue inhibitors of metalloproteinases (TIMPs) are the main physiologic inhibitors of the MMPs (Jiang et al, 2002; Baker et al, 2002). TIMPS are secreted proteins that complex with individual MMPs and regulate the activity of individual MMPs. Together, the MMPs and TIMPs form a complex biological system strictly controlling degradation of extracellular matrix. The MMPs and TIMPs have a significant role in facilitating tumor invasion and metastasis, not only through their direct role in degrading extracellular matrix but also by interaction with other biological systems implicated in tumor invasion, including cell adhesion molecules, cytoskeletal proteins and growth factors (Leeman et al, 2003; Egeblad et al, 2002).
TIMP-1

TIMP-1 mRNA expression is up-regulated in many human cancer types and in some cases correlates with more severe clinical outcome eg, colorectal carcinoma, non-small cell lung carcinoma and breast carcinoma (Sternlicht et al, 1999). Studies in experimental mouse models have revealed paradoxically that TIMP-1 can exhibit proneoplastic and antineoplastic effects during primary and metastatic tumor development (Noritake et al, 1999).

TIMP-2

TIMP-2 is a multifunctional inhibitor of angiogenesis, tumor growth and tumor invasion (Hoegy et al, 2001). These processes involve not only tumor cells themselves but also the modulation of complex tumor-host interactions. Because the host response to the tumor microenvironment can act either to facilitate or to inhibit tumor invasion and spread, manipulating these host response elements has become a major focus of novel anticancer strategies (Feldman et al, 2003; Liotta et al, 2001). Although TIMP-2 can block the action of MMPs (Hoegy et al, 2001), it may also rely on MMP-independent mechanisms that modulate tumor-host interactions (Seo et al, 2003). TIMP-2 has a direct role in regulating tyrosine kinase-type growth factor receptor activation.

Angiogenesis

Angiogenesis, the formation of new capillaries, is among the key events in various destructive pathologic processes, such as tumor growth, metastasis, arthritis etc as well as in physiologic processes, like organ growth and development, wound healing and reproduction (Folkman, 1995). Blood vessels
constitute the first organ in the embryo and form the largest network in our body but sadly are also often deadly. When disregulated, the formation of new blood vessels contributes to numerous malignant, ischemic, inflammatory, infectious and immune disorders. Molecular insights into these processes are being generated at rapidly increasing pace, offering new therapeutic opportunities that are currently being evaluated.

**Angiogenesis in tumor growth and metastasis.**

Angiogenesis is required for invasive tumor growth and metastasis and constitutes an important point in the control of cancer progression. For tumors to develop in size and reach metastatic potential they must make an angiogenic switch through perturbing the local balance of proangiogenic and antiangiogenic factors.

Tumors that have become neovascularized often express increased levels of proangiogenic proteins, such as vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF). The expression of proangiogenic proteins can be induced by several factors, including hypoxia, activation of oncogenes or inactivation of tumor suppressor genes (Laughner et al, 2001; Ravi et al, 2000; Semenza, 2000). In some tumors, the angiogenic switch is the result of down regulation of antiangiogenic factors (Arbiser et al, 1997). In most adult tissues, the balance between proangiogenic and antiangiogenic signaling favors vasculature. In some cases, however, proangiogenic activities prevail, resulting in the tumor vascularization and metastatic growth.

Two general approaches have been used in the development of
antiangiogenic agents: inhibition of proangiogenic factor and therapy with endogenous inhibitors of angiogenesis (eg: angiostatin, endostatin).

**Vascular endothelial growth factor**

Solid tumors are multicompartamentalized structures, consisting of three major compartments: cancer and stromal cells, the extracellular matrix (ECM), and the vasculature (Jain et al; 2002). The volumes of each of these components vary depending on the origin and size of the tumor and the organ in which primary tumor develops (Jain et al; 2002). Tumors require vasculature to gain access to oxygen and other nutrients, allowing growth and metastasis (Carmeliet et al, 2000). VEGF (vascular endothelial growth factor) has been shown to be one of the most potent angiogenic factors produced by tumor cells (Ferrara et al, 1997 and, Nicosia; 1998). It binds to endothelial cell surface receptors and activates various functions of the cell, including angiogenesis (Gera et al, 1999; Veikkoln et al, 2000). VEGF, also known as vascular permeability factor (VPF or VEGF-A) is the critical and central regulator of angiogenesis. The other members of the VEGF family, VEGF-B, VEGF-C, VEGF-D and PIGF also play a role in angiogenesis. It can up-regulate expression of adhesion molecules on vascular endothelium (Melder et al, 1996).

**Inhibitors of angiogenesis**

Purified angiostatin inhibited angiogenesis in both *in vitro* and *in vivo* assay systems and blocked growth of metastases. Sequence analysis of angiostatin revealed that it is a proteolytic fragment of plasminogen. The inhibitory activity of angiostatin is specific to this proteolytic fragment because the intact
plasminogen lacked this activity.

Another endogenous inhibitor of angiogenesis is endostatin, which is also a proteolytic fragment of another protein, collagen XVIII (Reilly et al, 1997). Endostatin was shown to be a more potent inhibitory factor than angiostatin. Systemic application of recombinant endostatin was capable of inhibiting angiogenesis as well as blocking growth of several primary tumors.

**Role of hypoxia in angiogenesis**

Beyond a certain size, simple diffusion of oxygen to metabolizing tissues becomes inadequate. Tumor development forms, the increasing metabolic demands of the growing mass of cells. Many tumors develop a severely hypoxic microenvironment (Christopher et al, 2003) and secrete angiogenesis-stimulating factors (Daniela et al; 2005) such as induce platelet-derived growth factor (Eunice et al, 2001) and VEGF (Shwciki et al, 1992). In tumors, VEGF expression is enhanced in zones surrounding necrotic foci, suggesting a mechanism by which a hypoxic microenvironment might stimulate tumor angiogenesis (Jin et al, 2006).

By activation of the hypoxia-inducible factor (HIF) family of genes, which cod for heterodimeric basic helix-loop-helix proteins composed of α and β subunits. HIF-1α is manufactured in the cytoplasm of cells but is rapidly degraded under normoxia, however, the intracellular content of HIF-1α increases immediately after a decrease in oxygen tension. HIF-1α is a transcription factor that mediates hypoxic induced responses, including apoptosis and VEGF gene regulation (Carmeliet et al, 1998).

Hence; the oxygen availability is an important regulator of tumor angiogenesis.
Role of immune system in tumor development - immune surveillance

Host provides both humoral and cell mediated immune responses to tumor antigens and proven to be effective in the immune destruction of tumors. A number of tumors have been shown to induce tumor-specific cytotoxic-T lymphocytes (CTLs). The important effectors include natural killer cells, macrophages and tumors specific antibodies.

T-Lymphocytes

CTLs provide effective antitumor immunity in host. CTLs may perform a surveillance function by recognizing and killing potentially malignant cells that express peptides which are derived from mutant cellular or oncogenic viral proteins which are presented in association with class I MHC molecules.

Role of NK cells and macrophages

NK cells can be activated by direct recognition of tumor or as a consequence of cytokines produced by tumor-specific T lymphocytes. Recognition of tumor cells by NK cells is not MHC restricted. In some cases, Fc receptors on NK cells can bind to antibody-coated tumor cells leading to antibody dependent cellular cytotoxicity (ADCC). Numerous observations indicate that activated macrophages also play a significant role in the immune responses to tumors by releasing lysosomal enzymes, reactive oxygen metabolites or by producing TNF-α. Macrophages also express Fc receptors enabling them to mediate ADCC. Activated macrophages secrete TNF-α that has potent antitumor activity.
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Antitumor antibodies

Antibodies are probably less important than T cells in mediating effective antitumor immune responses. But antitumor antibodies of the appropriate subclass are effective in suppressing small number of tumor cells. Binding of antibody to the tumor target cell does not by itself result in suppression or destruction. It serves as a recognition signal for cytotoxic effectors such as complement, macrophages, or K cells to perform the cytotoxic event.

Complement

The complement system comprises a group of more than 30 serum and cell surface proteins most of which are beta globulins with protease activity. The complement system plays a significant role in humoral immune responses. The binding of complement components to the appropriate immunoglobulin subclass initiates a cascade of complement activation and macro nuclear aggregation that results in the release of anaphylatoxins, which cause neutrophil chemotaxis, neutrophil activation, increased vascular permeability. Assembly of the membrane attack complex, which inserts in the lipid bilayer of the target cell membrane forming a ‘doughnut’ and thus providing for the free exchange of water and electrolytes and consequent osmotic lysis of the cell.

Antibody-Dependent Cell-Mediated Cytotoxicity (ADCC)

In ADCC, the target tumor cells, which are coated with IgG antibodies, are selectively lysed by killer cells, a special type of lymphomonocytic cell (Schulz et al, 1983). Several different leukocyte populations like neutrophils, eosinophils, mononuclear phagocytes and NK cells are capable of lysing the
target cells. Recognition of bound antibody occurs through a low affinity receptor for Fcγ on the leukocyte, called FcγRIII or CD16. 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.

**Tumor escape mechanism**

Malignant tumors may express protein antigens, which are recognized as foreign by the tumor host, and although immunosurveillance may limit the outgrowth of some tumors, it is clear that the immune system often does not prevent the occurrence of human lethal cancers. It may be due to the rapid growth and spread of a tumor overwhelms the effector mechanism of the immune responses. The inability of the host to develop an effective immune response has also been shown in several classes (Dean et al, 2001). The process of tumor escape may be a result of several mechanisms as given below.

a). Class I MHC expression can be down regulated on tumor cells, which is required for CTL recognition.

b). Tumor products may suppress antitumor immune responses (eg, TGF-β).

c). Loss of surface expression of tumor antigens.

d). Tumor surface antigens can be hidden from the immune system.

**Cytokines**

Cytokines are small secreted proteins which mediate and regulate immunity, inflammation, and hematopoiesis. They are small, structural proteins
with molecular weights ranging from 8 KD to 40 KD. They act by binding to specific membrane receptors, which then signal the cell via second messengers, often tyrosine kinases, to alter its behavior (gene expression). Responses to cytokines include increasing or decreasing expression of membrane proteins (including cytokine receptors), proliferation, and secretion of effector molecules. Cytokines are endogenous immunostimulatory proteins (Guillot et al, 2002). Cytokines play an important role in tumor metastasis. Some of the cytokines may inhibit tumor growth by interfering with host tumor relationship for example by inhibiting tumor angiogenesis and modulation of extra cellular matrix.

**Interleukin-1 (IL-1)**

IL-1 is one of the most important immune response-modifying interleukin. The predominant function of IL-1 is to enhance the activation of T-cells in response to antigen. Activation of T-cells, by IL-1, leads to increased T-cell production of IL-2 and of the IL-2 receptor, which in turn augments the activation of the T-cells in an autocrine loop. This effect of T-cell activation by IL-1 is mimicked by TNF-α which is another cytokine secreted by activated macrophages. The most salient and relevant properties of IL-1 in inflammation are the initiation of cyclooxygenase type 2 (COX-2), type 2 phospholipase A and inducible nitric oxide synthase (iNOS). Production of IL-1 by different cell types occurs only in response to cellular stimulation. IL-1 is also an angiogenic factor and plays a role in tumor metastasis and blood vessel supply (Kaoru et al, 2006). IL-1 also stimulates the production of other proinflammatory cytokines like IL-6. In addition to its effects on T-cells, IL-1 can induce proliferation in non-lymphoid
IL-2, produced and secreted by activated T-cells, is the major interleukin responsible for clonal T-cell proliferation. IL-2 also exerts effects on B-cells, macrophages, and natural killer (NK) cells. The production of IL-2 occurs primarily by CD4+ T-helper cells. Indeed, the IL-2 receptor is not expressed on the surface of resting T-cells and is present only transiently on the surface of T-cells. In contrast to T-helper cells, NK cells constitutively express IL-2 receptors and will secrete TNF-α, IFN-γ and GM-CSF in response to IL-2, which in turn activate macrophages. IL-2 has been used clinically in several ways.

Interleukin-6 (IL-6)

IL-6 is produced by macrophages, fibroblasts, endothelial cells and activated T-helper cells. It is a key inflammatory mediator produced by many cell types. In particular, IL-6 is the primary inducer of the acute-phase response in liver. IL-6 also enhances the differentiation of B-cells and their consequent production of immunoglobulin. Unlike IL-1, IL-2 and TNF-α, IL-6 does not induce cytokine expression; its main effects, therefore, are to augment the responses of immune cells to other cytokines. In humans, IL-6 is a growth factor for myelomas, (Elizabeth et al, 2000) suggesting further applications of IL-6 blockers.

Tumor Necrosis Factor (TNF)

TNF-α was originally identified as a cytokine responsible for endotoxin
induced necrosis (Bazzoni and Beutler, 1995). Several independent groups reported that therapy with recombinant TNF-α was effective against several types of murine models of hepatic and pulmonary metastasis (Jan et al, 2001). TNF-α and TNF-β have been shown to exhibit direct antitumor activity, killing some tumor cells and reducing the rate of proliferation of others while sparing normal cells. In the presence of TNF-α or TNF-β, a tumor undergoes visible hemorrhagic necrosis and tumor regression. TNF-α has also been shown to inhibit tumor-induced vascularization (angiogenesis) by damaging the vascular endothelial cells in the vicinity of a tumor, thereby decreasing the flow of blood and oxygen that is necessary for progressive tumor growth (Anita et al., 2004). TNF has potent antitumor activity against large tumor burdens in some murine models. (Flavio et al, 2000). However, Humans can only tolerate 2% of the systemic TNF dose (by weight) required in mice, due to dose limiting hypotension (Dong et al, 2001). High doses of TNF, administered locally via direct tumor injection (Kramer et al, 2001) or isolated limb perfusion can result in dramatic tumor regression in some cancer patients.

**Interferon - (INF-γ)**

Interferons are a family of proteins that are produced by the T-cells in response to viral infections or stimulations with double stranded RNA, antigens, or mitogens (Maryam et al, 2000). IFN-γ is secreted primarily by CD8+ T-cells. Nearly all cells express receptors for IFN-γ and respond to IFN-γ binding by increasing the surface expression of class I MHC proteins, thereby promoting the presentation of antigen to T-helper (CD4+) cells. Interferons have a variety of
biologic properties which include immunomodulatory activities, antiviral activities, the ability to interfere with cell proliferation, inhibition of angiogenesis, regulation of differentiation, and enhancement of the expression of a variety of cell-surface antigens (Kenji et al, 2001). Interferons have antitumor activity against a variety of tumor types, including hairy cell leukemia, chronic myelogenous leukemia, cutaneous T-cell lymphoma, and Kaposi’s Sarcoma (Jiang et al, 1983).

**GM-CSF**

Colony stimulating growth factors (CSFs) are cytokines that stimulate the proliferation of specific pluripotent stem cells of the bone marrow in adults. GM-CSF is a pleotropic cytokine produced by a number of different cell types. GM-CSF is a growth factor for erythroid, megakaryocyte and eosinophil progenitors. IL-3 (secreted primarily from T-cells) is also known as multi-CSF, since it stimulates stem cells to produce all forms of hematopoietic cells. GM-CSF stimulates macrophages for antimicrobial and antitumor effects. 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. GM-CSF may enhance Ab-dependent cellular cytotoxicity and the generation and cytotoxicity of NK cells. (Jay et al, 2000). GM-CSF is a macrophage activating factor and promotes the differentiation of Langerhans cells into dendritic cells. Recombinant GM-CSF and G-CSF are increasingly used to speed bone marrow recovery after cancer chemotherapy.
**Nuclear factor-κB (NF-κB)**

Nuclear factor κB (NF-κB) designates a group of transcription factors defined by their ability to bind a specific DNA sequence first identified in the enhancer of immunoglobulin κ light chain gene. NF-κB factors are dimers of Rel family of proteins. There are five members of the NF-κB family of transcription factors: Rel (c-Rel), Rel A (p65), Rel B, NF-κB 1 (p105/p50) and NF-κB 2 (p100/p52). Together, these proteins regulate the expression of genes encoding cytokines, chemokines, adhesion molecules and antimicrobial peptides, thereby orchestrating both innate and adaptive immune responses (Gosh et al, 2002). NF-κB/Rel proteins exist as homo or hetero dimers and possess a conserved N-terminal Rel homology domain (RHD) that mediates dimerization as well as DNA binding.

In most cell types, inactive NF-κB complexes are sequestered in the cytoplasm via their interaction with inhibitory proteins known as Inhibitory kappa B (IκBs). In response to multiple stimuli, including cytokines, viral and bacterial pathogens and stress-inducing agents the latent cytoplasmic NF-κB/IκBa complex is activated by phosphorylation on conserved series residues at the N-terminal portion of IκB; this modification occurs at Ser 32 and Ser 36 in the case of IκBa (Karin et al, 2002; Kumar et al, 2003). Phosphorylation targets IκBa for ubiquitination by the SCF-ubiquitin ligase complex, which leads to degradation of the inhibitory subunit by the 26S proteosome (Karin et al, 2002, Wilkinson, 2003). This process activates NF-κB, which then translocates to the nucleus and binds to its cognate DNA-binding site (5'-GGGRNNYYCC-3') in the promoter or
enhancer regions of specific genes.

The ability of NF-κB to suppress apoptosis and to induce expression of proto-oncogenes such as C-myc and cyclin D1, which directly stimulate proliferation, suggest that NF-κB may stimulate in many aspects of oncogenesis (Pahl, 1999; Guttridge et al; 1999) NF-κB also regulates the expression of various molecules such as cell adhesion proteins, matrix metalloproteinases, cyclooxygenase-2 (cox-2), iNos, chemokines, and inflammatory cytokines, all of which promote tumor cell invasion and angiogenesis (Bharti et al, 2004). Inhibition of NF-κB abrogates tumor cell proliferation (Younes et al, 2003; Bharti et al, 2003; Mukhopadhyay et al, 2001).

Although it is widely accepted that inhibition of NF-κB triggers apoptosis in many tumor cell types (Yamamoto et al, 2001), there are a few exception in which NF-κB activation blocks malignant growth. NF-κB and oncogenic Ras both induce cell-cycle arrest in normal human epidermal cells. The cell cycle arrest in normal human epidermal cells. The cell cycle arrest by oncogenic Ras can be bypassed by inhibition of NF-κB through the over expression of IκB a protein, which results in malignant epidermal tissues resembling squamous cell carcinoma (Van et al, 1999; Seitz et al, 1998). These findings thus suggest that NF-κB can play a different role in the regulation of cell growth in tissue-context-dependent manner.

**Apoptosis**

Cell death is a physiological process which is required for normal development and existence of multicellular organisms. In most cases,
physiological cell death occurs by apoptosis as opposed to necrosis. Abnormalities in this process are implicated as cause or contributing factor in a variety of diseases. Inhibition of apoptosis can promote neoplastic transformation, particularly in combination with disregulated cell cycle control, and can influence the response to tumor cells to anti-cancer therapy. A family of intracellular proteases, the caspases, is responsible directly or indirectly for the morphological and biochemical changes that characterize the phenomenon of apoptosis. Diverse regulators of the caspases, including activators and inhibitors of cell death proteases are also discovered. It is an essential process in controlling tissue homeostasis in multicellular organisms. Apoptosis is sometimes referred to as programmed cell death (PCD) because it is an integral part of the developmental program and is frequently the end result of temporal course of cellular events.

Apoptosis can be induced by a variety of stimuli such as ionizing radiations, gluco-corticoids chemotherapeutic agents, lymphokines deprivation and various oxidants (Rajeev Goel 1998). Although the stimuli which induce apoptosis vary markedly, the morphological features of the process are however conserved in different cell types. It includes chromatin condensation, nuclear fragmentation, Plasma membrane blebbing, cell shrinkage and formation of apoptotic bodies.

Caspases

A family of intracellular cysteine proteases which cleave their substrates at aspartic acid residues, known as caspases (Cysteine Aspartyl-specific proteases) (Alnemri et al, 1996). These proteases are present as inactive zymogens in
essentially all animal cells. In humans and mice, approximately 14 caspases have been identified. They can be sub grouped according to either their amino acid sequence similarities or their protease specificities. Though most caspases are directly involved in cell death, a few are not, atleast in mammals and higher eukaryotes. A subgroup of caspases, including caspase 1, 4 and 5 in humans, is involved in processing of proinflammatory cytokines such as pro-interleukin-1β (pro-IL-1β), pro-IL-18. Many pathways for activating caspases are exist, but only two have been elucidated in detail. One of these centers on tumor necrosis factor (TNF) family receptors, which use caspase activation as a signaling mechanism, thus connecting ligand binding at the cell surface to apoptosis induction (Ferry et al, 2005). The other involves the participation of mitochondria, which release caspase activating proteins into the cytosol, thereby triggering apoptosis (Tomomi et al, 1998). The death receptor and mitochondrial pathways for caspase activation are sometimes referred to as the extrinsic and intrinsic apoptosis pathway respectively. Caspase-8 represents the apical caspase in the TNF family death receptor pathway, whereas caspase-9 serves as the apical caspase of the mitochondrial pathway (Alakananda and Ayako, 2002). In the case of intrinsic pathway, release of cytochrome c from mitochondria triggers caspase activation by binding to the caspase-activating protein Apaf-1 (Newmeyer et al, 2000). The Apaf-1 protein normally resides is an inactive conformation in the cytosol, but on binding cytochrome c, an ATP/dATP-binding oligomerization domain within this protein mediates Apaf-1 aggregation (Zhou et al, 1998). The oligomerized complex then binds Pro-caspase-9, and facilitates trans-processing of caspase-9
zymogens via the induced proximity mechanism (Li et al, 1998).

**Bcl-2**

Apoptosis is an evolutionarily conserved cell suicide process executed by cysteine proteases (caspases) and regulated by the opposing factions of the Bcl-2 protein family (Suzanne et al, 2003). They are a family of homologous proteins, where some members are proapoptotic and some are antiapoptotic. In humans, 20 members of the Bcl-2 family genes are actively participating in apoptosis. These genes encode the anti-apoptotic proteins, Bcl-2, Bcl-XL, Mcl-1, Bfl-1(A1), Bcl-W and Boo (Diva) as well as the pro-apoptotic proteins Bax, Bak, Bok (Mtd), Bad, Bid, Bim, Bik, Hrk etc. Some of these proteins may display anti-apoptotic activity in some cellular backgrounds and have pro-apoptotic functions in other cellular contexts (e.g., Boo/Diva, Bcl-2, Bax) (Chen et al, 1996). Many Bcl-2 family proteins are constitutively localized to the membranes of mitochondria, whereas others are induced to target these organelles in response to specific stimuli. Caspase-8 mediated activation of Bid represents an important mechanism accounting for cross-talk between the death receptor (extrinsic) and mitochondrial (intrinsic) pathway (Yin et al, 1999). When the Bcl-2 family proteins reach the mitochondria, they regulate the release of cytochrome c from mitochondria, with pro-apoptotic Bcl-2 family proteins inducing or making it easier to induce release of this caspase activating protein and antiapoptotic members of the family suppressing cytochrome c release.

Bcl-2 family proteins have been reported to control the release of other proteins from mitochondria. The proteins include [i] certain caspases (caspase-2,
3 and 9) which reportedly are sequestered inside mitochondria in some types of cells (Susin et al, 1999) [ii] apoptosis inducing factor (AIF), a flavoprotein implicated in nuclear manifestations of apoptosis via caspase-independent mechanisms (Susin et al, 1999) and [iii] Smac/Diablo, the inhibitor of IAP family proteins (Du et al, 2000; Verhagen et al, 2000). All of these proteins are encoded within the nuclear genome, transported into mitochondria, and stored in the space between the inner and outer membranes and awaiting to release into the cytosol upon breakdown of the outer membrane.

**MAP Kinase**

The mitogen-activated protein kinase (MAPK) Pathway is one of the primordial signaling systems that nature has used in several permutations to accomplish an amazing variety of tasks. It exists in all eukaryotes, and controls such fundamental cellular processes as proliferation, differentiation, survival and apoptosis. The basic arrangement includes a G-protein working upstream of a core module consisting of three kinases: a MAPK kinase kinase (MAPKKK) that phosphorylates and activates a MAPK (Robinson et al, 1997; Schaeffer et al, 1999; Dhanasekaran et al, 1998). Two components of this pathway, Ras and Raf, are proto-oncogenes. The major function of this pathway pertains to growth control in all its facets, including cell proliferation, transformation, differentiation and apoptosis.

A wide variety of hormones, growth factors and differentiation factors as well as tumor promoting substances, employ this pathway. Most of these stimuli activate Ras proteins by inducing the exchange of GDP with GTP, which converts
RAS into its active conformation. The Ras exchange factor, sos (son of sevenless), is towed to the membrane by the growth–factor–receptor–bound protein 2 adapter protein. Activated Ras functions as an adapter that binds to Raf kinases with high affinity and causes their translocation to the cell membrane, where Raf activation takes place. The Raf family of serine/threonine. Specific kinases comprise three members in higher vertebrates, A-Raf, B-Raf and C-Raf or Raf-1, which play a vital role in regulating cell growth, differentiation and apoptosis. They lie at the apex of a highly conserved protein kinase module which relays extracellular signals to the nucleus (Pearson et al, 2001; Kolch, 2000). In this module Raf kinases phosphorylates and activates MEK-1/2 which in turn phosphorylates and activates ERK-1/2. Activated ERK-1/2 can then translocate to the nucleus and activate transcription factors by phosphorylation, thus altering the expression of specific genes.

In addition, ERK-1/2 has a number of cytosolic substrates which influence gene expression directly or indirectly. Active ERK may also allow the tumor to develop its own angiogenic support system by inducing the expression of angiogenic factors such as vascular endothelial growth factor (VEGF) (Eliceiri et al, 1998). Activation of ERK in tumors may allow evasion of apoptosis by inducing cell survival. ERKs are known to play a role in cell survival in many cell systems. In fibroblasts, ERK activation by Raf leads to a selective reduction in expression of the Bim pro-apoptotic member of the Bcl-2 family and B-Raf over expression in fibroblasts has also been shown provide a protection against apoptosis by inactivating caspases after cytochrome C release (Erhardt et al,
Conventional cancer therapies.

Designing a proper treatment plan for a patient with malignant disease depends upon determining extend of disease spread, together with a knowledge of the natural history and the available therapeutic alternatives or the particular type of cancer. Accurate diagnosis is a critical step in planning appropriate cancer therapy. The application of current treatment techniques (surgery radiation therapy chemotherapy and biological therapy) results in the cure of > 50% of patients diagnosed with cancer

Surgical therapy

Surgery is an effective method to cure patients whose tumors are confined to particular anatomical sites. Surgery has been used as means of cytoreduction when complete excision has not been possible. However, unless such surgical debunking is combined with additional therapy, such as chemotherapy or radiation therapy, it will not much effective.

The selection of patients who will benefit from this approach requires considerable judgment and skill. Overly aggressive surgical intervention in palliative setting may lead to prolonged hospitalizations, unnecessary discomfort and additional financial burden to the patient or family.

Radiation therapy.

Radiation therapy is a local modality used in the treatment of cancer. This word depends to a large extent on the inherent radio sensitivity to the tumor and adjacent normal tissues. Ideally radiation therapy should destroy cancerous tissue
while causing minimal destruction to surrounding normal structures. Isotopes such as $\text{Cs}^{313}$, $\text{H}^{192}$, $\text{Co}^{60}$ are used in radiation treatment.

Radiation therapy is dependent on the application of ionizing electromagnetic radiation to tumor site. The term x-ray denotes high-energy electromagnetic radiation produced by instruments such as linear acceleration. γ-rays are also electromagnetic radiation but are produced by radioactive isotope decay. Both are used in radiation therapy and there is no inherent difference in their physical characteristics or biologic effects. A given dose of radiation kills a constant percentage of cells. Ionizing radiation generates free radicals and reactive oxygen intermediates that damage cellular substituents including DNA.

Radiation therapy combined with chemotherapy has largely replaced surgery as a curative treatment for carcinoma of the anus. Radiation therapy is also used in the palliative management of many tumors.

Radiation therapy is associated with both acute toxicity and long-term sequel. Common manifestations include skin reaction with erythematous, desquamation, gastrointestinal toxicity, hair loss with nausea, vomiting, dysphagia or diarrhea and myelosuppression with leukopenia, thrombocytopenia, and anemia. Rapidly proliferating normal tissues such as intestinal mucosa, bone marrow and skin are particularly susceptible to the radiation-induced cytotoxicity. Radiation therapy associated with an increased risk of developing solid tumors in previously irradiated fields. In view of extreme radio sensitivity of lymphocytes, the immune response is generally depressed following radiotherapy and patients are rendered more susceptible to infection (Yamagata and Green, 1976).
Chemotherapy

Cancer chemotherapy had its roots in the work of Paul Ehrlich, who coined the word chemotherapy. There are four ways chemotherapy is generally used as an induction treatment for advanced disease, as an adjunct to the local methods of treatments, as the primary treatment for patients who present with localized cancer and by direct installation into sanctuaries or by site directed perfusion of specific regions of the body most affected by the cancer.

One of the most important and still evolving roles for systemic chemotherapy is its use in the adjuvant setting. Its purpose is to eliminate undetectable micrometastatic disease. Chemotherapy whether given with curative or palliative intent, usually requires multiple cycles of treatment. Some of the commonly used important chemotherapeutic agents are of different types such as alkylating agents, antimetabolites, natural products, hormone antagonists, miscellaneous agents etc.

Every chemotherapeutic regimen administered in adequate doses will have some deleterious side effects on normal host tissues. Myelosuppression, nausea, vomiting, stomatitis and alopecia are most frequently observed complications associated with chemotherapy (Rivera, 2003). Alkylating agents such as cyclophosphamide are potential carcinogens. Another major problem in chemotherapy is the tumor cell resistance to drugs or chemotherapeutic agents because the cancer cell presents a variable and moving target for anticancer drugs.

Biologic or immunotherapy

Biologic therapy is cancer treatment that produces antitumor effects
primarily through the action of natural host defense mechanism. Biologic therapy has emerged as an important fourth modality for the treatment of cancer (Mitchell, 1988). Most applications of biologic therapy for cancer have attempted to stimulate immune defense mechanisms. Many immuno therapies attempted to cause the tumor to appear more 'foreign' compared with normal tissues or tried to magnify relatively weak host immune reaction to growing tumors.

Strategies for the immunotherapy of cancer can be divided into active and passive approaches. Active immunotherapy refers to the immunization of the tumor-bearing host with materials designed to elicit an immune reaction capable of eliminating or retarding tumor growth. Active immunotherapy can be subdivided into nonspecific or specific immunization.

The advent of recombinant cytokines provided a more selective means for stimulating the immune system. Treatment with the interferones (Maier et al, 2003) or with IL-2 is a form of nonspecific active immunotherapy. Cytokines comprise the largest group of biologic therapeutics in clinical trials and include interferons, interleukins and hematopoietic growth factors. Lymphoid cells incubated with IL-2 develop a capacity to lyse fresh tumor cells (Wang et al, 2003).

The development of techniques for generating monoclonal antibodies has greatly improved the ability to obtain preparations with specific reactivity to human tumor-associated antigens. These antibodies are being employed alone or conjugated with toxins or radiolabel in cancer treatment.

Quite often the presence of the cancer itself will associate with
immunosuppression, also the treatment given for the cancer (radiotherapy and chemotherapy) is immunosuppressive. Infections are therefore not uncommon in patients with widespread cancer undergoing therapy. The organism responsible may cause mild symptom in normal individuals but can be severe or fatal in the immunodepressed patients.

**Use of immunomodulators.**

Immunomodulation is any procedure, which can alter the immune system of an organism by interfering with its functions; if it results in an enhancement of immune reaction is named as immuno stimulation and primarily implies stimulation of non-specific system that is stimulation of the function and efficiency of granulocytes, macrophages, complement, certain T-lymphocytes and different effector substances. Immunosuppression implies mainly to reduce resistance against infections, stress and may be because of environmental or chemotherapeutic factors. Apart from specific stimulative or suppressive activity certain agents have been shown to posses activity to normalize or modulate the pathophysiological processes in the underlying immune response and hence the terms Immunomodulation or immunomodulatory agents are now used.

**Chemical agents as immunomodulators**

Most of the chemical agents, which are fairly known to have effect on immune system, are immunosuppressants and cytotoxic agents (Nasrollah, 2004). The immunostimulants are mainly reported from the natural resources.

A wide variety of compounds are capable of potentiating immune
responses. Many of the drugs and hormones particularly the biogenic amines, cholinergic agents, prostaglandin appear to be linked to change in cyclin nucleotide metabolism in lymphocyte and thus it can enhance or suppress various aspects of immune response. Other nonspecific stimulants include BCG, Corynbacterium parvum and levamisol. Interleukins-1 is produced by macrophages and can stimulate the growth of thymocytes and T-cells. IL-2 is a protein produced by T-lymphocytes in response to antigenic or mitogenic stimuli. It is useful as a general restorative of immune competence in immuno suppressed individuals (Hamer et al 2004). Among the synthetic, semisynthetic or isolated pharmacological agents azothioprine and penicillamine have been extensively studied. Azathio prine inhibits DNA synthesis and has anti-inflammatory activity by virtue of it effects on PMN and monocyte production. Penicillamine is a currently accepted second line disease modifying agents used in advanced unresponsive rheumatoid arthritis. Despite the beneficial effects obtained with this drug, its usefulness is limited by its toxic side effects, which include rash, taste abnormalities and hematological toxicities (Israeli 1981). It can be assumed that the chemotherapeutic agents available today have mainly immunosuppressant activity. Most of them are cytotoxic and exert variety of side effects. Number of plant extracts have been shown to be immunomodulators and are briefly reviewed here.

Immunomodulators from natural products

A large number of chemotherapeutic agents used in cancer treatment have been discovered from natural products. Similarly, several laboratories through out
the world have directed considerable effort towards discovering new therapeutic
agents from natural products. Because listing the entire spectrum of compounds
that have been discovered by various groups could be overly cumbersome, we
describe some important contributions but not in an exhaustive manner.
Traditionally, plants are selected based on its known efficacy for treatment of
inflammatory mediated diseases including cancer or related diseases. In our
laboratory, interesting studies have been carried out using Iscador, which is an
extract of the plant *Viscum album* and we have reported its antimetastatic activity
(Antony and Kuttan, 1996), We also reported the antimetastatic as well as
antitumor activity of naturally occurring sulfur compounds such as Dialyl sulfide,
Diallyl disulfide, Allyl Methyl Sulfide (Manesh and Kuttan, 2003) and
polyphenolic compounds such as Curcumin, Catechin and Piperine, an alkaloid
(Menon et al, 1999; Pradeep and Kuttan, 2002). It has been reported that the
anticarcinogenic activity of *Curcuma longa* and *Embilica officinalis* (Ruby et
al 1995; Rajeshkumar et al 2003). It is interesting to note that many of the clinically
used antineoplastic drugs such as camptothecin, taxol, vincristine, vinblastine are
plant derived products and interestingly several clinical trials on the use of
nutritional supplements and phytochemicals to prevent cancer are going on now.

Curcumin from turmeric, capsaicin from chilli-peppers, 6-gingerol from
ginger, epigallocatechin-3 gallate from green tea, genistein from soyabean,
lyopene from tomatoes, sulforaphane from broccoli, diallyl sulphide from garlic,
resveratol from grapes, caffeic acid phenyl esters from honey, indol-3-carbinol
from cabbage etc are well studied immunomodulative agents that target different
cellular signaling molecules which regulate proliferation and differentiation. The past 10 years studies of signal transduction pathways which has resulted in the development of important new molecular therapeutics for cancer, particularly the use of many plant derived agents are seem to be relatively free of the undesirable toxicities of classical chemotherapeutic agents may have very well effect in the control of tumorigenesis.

**Plants and their active principles Included in this Study**

*Piper longum*

Piper longum is an important medicinal plant used in traditional medicine by many people in Asia and Pacific islands especially in Indian medicine (James, 1999). *Piper longum* is a component of medicines reported as good remedy for treating gonorrhea, menstrual pain, tuberculosis, sleeping problems, respiratory tract infections, chronic gut related pain and arthritic conditions (Singh et al, 1992). Other reported beneficial effects of *Piper longum* include analgesic and diuretic effects, relaxation of muscle tension and alleviation of anxiety (Singh and Blue Menthal, 1997). Piperine was the first amide isolated from piper species and was reported to display central nervous system depression, antipyretic and anti-inflammatory activity (Virinder et al, 1997).

**Piperine**

Piperine is the active phenolic component of black pepper (*Piper nigrum*) and long pepper (*Piper longum*). Black pepper and long pepper is being used as a spice or component of indigenous system of medicines (Virinder et al, 1997).
Piperine is a potent inhibitor of mixed function oxygenase and it is a nonspecific inhibitor of p450 isoenzymes (Atal, 1985). Constituents of piper species have inhibitory activity on prostaglandin and leukotriene biosynthesis in vitro (Stohr et al, 2001). We have reported its antimetastatic activity and its inhibitory potential of nitric oxide (NO) and tumor necrosis factor-α (TNF-α) production. It has been reported that Piperine could inhibit the lung metastasis of B16F-10 melanoma cells by the inhibition of the activation of NF-κB and the proinflammatory cytokine gene expression. (Pradeep and Kuttan, 2004)

**Thuja occidentalis L**

Arbor vita (*Thuja occidentalis* L.) is a native European tree widely used in homeopathy and evidence-based phytotherapy. *Thuja occidentalis*, commonly known as Arbor vitae or white cedar, is indigenous to eastern North America and is grown in Europe as an ornamental tree. Today, it is mainly used in homeopathy as mother tincture or dilution (Deutscher et al 1985; Deutscher et al 2003). The fresh plant (related to the dry substance) contains essential oil, coumarins, water-soluble polysaccharides, flavanoids, and tannic agents (Harnischfeger and Stolze, 1983). A critical factor for *Thuja* use as a medicinal herb is its content of essential oil. The immunopharmacological potential of *Thuja* has been investigated in various *in vitro* and *in vivo* test models (Höld et al, 2000; Teuscher et al, 2004).

**Thuja polysaccharide**

The fresh plant (related to the dry substance) contains 4.9% water-soluble polysaccharides. High molecular weight glycoproteins/polysaccharides are highly relevant for the activity of the plant (Neth et al, 1995) *Thuja* polysaccharides
(TPS) inhibited human immunodeficiency virus (HIV)-dependent cell death. The *Thuja* polysaccharides fraction caused an increase in the proliferation rates of spleen cells from mice. High molecular weight subfractions of TPS proved to be highly mitogenic on peripheral blood leukocytes. It was demonstrated that the mitogenic and cluster-forming activity of TPS causes T-cell induction particularly of the CD4-positive T-helper/inducer cells in connection with an increased production of interleukin-2 (IL-2). This indicates that not only proliferation but also a differentiation to fully functional T-helper cells takes place. Only T cells, not B cells, were stimulated by TPS. *Thuja occidentalis herba* led to an increased secretion of the cytokines IL-1, IL-6 and tumor necrosis factor-α (TNF-α) in the cell culture supernatant, with regard to TNF-α (Bodinet et al, 1999). Because of the proven immunomodulatory efficacy of the preparation, it was assumed that the positive effect of this extract exerted on infected mice is mediated first of all by its immunostimulating activity and that the direct antiviral activity is rather of secondary importance (Bodinet et al, 2002). *Thuja occidentalis* is widely used in homeopathy and evidencebased phytotherapy. Its immunopharmacological potential has been demonstrated in numerous *in vitro* and *in vivo* test models showing its immunostimulating and antiviral activities.