Chapter - I

Introduction &
Review of Literature
1.1 INTRODUCTION

Majority of cancer patients who succumb to the disease die from metastasis. In most cases, primary tumor can be removed by surgery, or local irradiation, but cells disseminated in the body may give rise, after variable time periods, to metastasis formation, unless they can be completely eradicated by treatments. In order to improve prevention, treatment and cure of metastasis, the basic principles and mechanisms of this process need to be elucidated. However, the metastatic cascade is extremely complex.

Although it was in 1889 that Paget's "Seed and soil" hypothesis of metastatic growths was published (264), it was only in the last three decades that metastasis research started to become one of the most rapidly growing fields in modern medical research, with progress in molecular and cell biology as well as immunology.

The metastatic process has many different facets. Malignant cells acquire the potential to leave the primary tumor, invade surrounding structures and tissues by an active process, disseminate in the vascular system, withstand adverse physical, cellular and humoral factors during the period, adhere to and emigrate by active motility from vessels and infiltrate a target organ to give rise to metastasis in a highly selective pattern (83). The basic requirements of tumor cells for being successful during this complicated cascade are being investigated. Basic concepts and methods of molecular, developmental and cell biology as well as immunology and pathology are being applied for this purpose. Although a real breakthrough in prevention and treatment of metastasis is still expected, the approach to this problem has considerably improved our knowledge.

In recent years important contributions have been made in the mechanism of invasion. Specific molecules which mediate cell adhesion,
motility regulating factors, the role of extracellular matrix, specific enzymes involved in this process, inhibiting or promoting factors that take part of metastasis, are the factors involved.

Invasive cancer cells degrade natural tissue barriers, i.e., the basement membrane and the connective tissue (269,289). Specific proteolytic enzymes produced by tumour cells, stromal cells and infiltrating leukocytes mediate this process. Matrix degrading proteases are of several types, of which matrix metalloproteinases play a major role in basement membrane degradation (10). Overexpression of specific MMPs is important in cancer cell invasion, and an overall balance between protease and protease inhibitors ultimately determine the extent of tissue degradation. MMP-2 and MMP-9 (type IV collagenases) are capable of degrading type IV collagen, gelatin and fibronectin (260,107,148). Overexpression of type IV collagenase increases the metastatic phenotype (61,62,101), decreasing type IV collagenase activity reduces metastatic capacity of tumour cells (154). MMP-9 expression plays a role in invasion and metastasis (148).

The mode of vascular dissemination and organotrophy of metastatic cells is better understood. Specific adhesion processes appear to be involved in organ specific lodging, some of which are similar or identical to those involved in physiological adherence of normal cells. Metastasis appears to develop from the specific interaction of the disseminated tumour cells with local positive and negative growth factors which suppress or, sometimes after long dormancy periods, promote proliferation at specific sites of the body. Final growth of metastasis is dependent on angiogenesis (168), in which a variety of factors are involved.

Relevant genes, their putative aberrant regulation and possibilities of their modification also reverted the metastatic phenotype. Detailed studies on the characterization of membrane alterations of tumour cell metastasis i.e. melanoma led to the production of monoclonal antibodies,
which have been used in experiments to interfere with organ-specific adherence and even growth of metastatic cells. Progress has been reported in attempts to increase antigenicity of tumour cells and immune competence of the host, improving the basis for clinical immunotherapy of metastasis (172). New highly sensitive methods in radiology and nuclear medicine have made early detection of metastases easy. Therefore theoretical, experimental and clinical metastasis research remains essential for future progress in the attempts to come closer to prevention and effective treatment of cancer metastases.

In the present study we have looked into the inhibition of metastasis by naturally occurring polyphenols and their mechanism of action. For this study, a highly metastatic cell line B16F-10 melanoma cells was selected and in vivo lung metastatic studies were carried out in C57BL/6 mice.

Polyphenolic compounds selected were mainly flavonoids, isoflavones and phenolic acids, which are non-toxic dietary constituents. Most of the compounds possess anticarcinogenic activity and were found to stabilize the basement membrane against the activity of proteolytic enzymes.

Curcumin, the yellow pigment of turmeric possesses antioxidant (316), anti-inflammatory (242), anticarcinogenic (324) and antimutagenic (251) activities. Catechin possesses antioxidant, anticarcinogenic (227) and antimutagenic (318) activities. Quercetin was reported as an antitumour promoter (243), rutin and morin possess anticarcinogenic (361) activity. Ellagic acid was reported to be an antimutagen and anticarcinogen (381,305,323). Genistein and daidzein, are soybean isoflavones. Genistein inhibits tyrosine specific protein kinases (4) and growth of a wide range of cancer cells (232). Daidzein inhibited proliferation of certain types of tumour cells such as breast cancer and HL-60 cells (69).

Chapter III : Cytotoxic and tumour reducing activity of polyphenolic compounds
Chapter IV : Effect of polyphenolic compounds on lung metastasis of B16F-10 melanoma cells in mice.
Rasayanas are non-toxic indigenous drug formulations prepared from several herbal materials, with known biological activities such as anticarcinogenic, antiinflammatory, chemopreventive, immunomodulatory and antioxidant (166,167,278,279,280). Since these herbal preparations contain several polyphenolic compounds, the antimetastatic and anticarcinogenic activity of some rasayanas were screened.

The present thesis has been divided into the following chapters. Review of literature is given in the following section of this chapter.

Chapter II  :  Materials and Methods
Chapter III :  Cytotoxic and tumour reducing activity of polyphenolic compounds
Chapter IV  :  Effect of polyphenolic compounds on lung metastasis of B16F-10 melanoma cells in mice.
Chapter V   :  Effect of polyphenolic compounds on the in vitro invasion of B16F-10 melanoma cells through collagen matrix.
Chapter VI  :  Studies on anticarcinogenic activity of polyphenolic compounds in mice.
Chapter VII :  Immunological mechanism of action of polyphenolic compounds in mice.
Chapter VIII:  Studies on the antimetastatic activities of anti-inflammatory agents, in mice.
Chapter IX  :  Anticarcinogenic and antimetastatic activity of Rasayanas.
Chapter X  :  Summary and Conclusion.

Bibliography and list of papers published are given in the last part of the thesis.
1.2 REVIEW OF LITERATURE

1.2.1 Metastasis

Metastasis, the spread of cells from the primary neoplasms to distant sites and their growth is the most crucial aspect in the prognosis of cancer. Despite significant improvements in early diagnosis, surgical techniques, general patient care, and local and systemic adjuvant therapies, most deaths from cancer are due to metastases (86,87,88). In a large number of patients with cancer, metastasis may well have occurred by the time of diagnosis (87,339,376). The metastases can be located in different lymph nodes and visceral organs and in various regions of the same organ, thus complicating their treatment. Furthermore, the specific organ environment can modify the response of a metastatic tumour cell to a systemic therapy and alter the efficacy of anticancer agents.

The major barrier to the treatment of metastases is the biological heterogeneity of cancer cells in primary and secondary neoplasms. This heterogeneity is exhibited in a wide range of genetic, biochemical, immunological and biological characteristics, such as cell surface receptors, enzymes, karyotypes, cell morphologies, growth properties, sensitivities to various therapeutic agents, and ability to invade and produce metastases (85-88,256,275). Moreover, "Cancer" denotes a collection of malignancies, with each cancer of each organ consisting of numerous subsets. This tremendous heterogeneity is probably due to the different etiologies, origin and selection pressures of different cancers.

Understanding the mechanisms responsible for the development of biological heterogeneity in primary cancers and in metastasis and the process by which tumour cells invade local tissues and spread to distant organs is a primary goal of cancer research. A better understanding leads to the ability to design more effective therapy for different cancers and improvements in the way physicians can deal with cancer metastasis.
1.2.2 Metastatic Cascade

Cancer metastasis occurs as a result of a complex series of interactions between the cancer cell and its surroundings (90). The early events lead to escape from the primary tumour, tumour cell migration and entrance into the lymphatics and/or systemic vasculature. The numerous interconnections between the lymphatic and vascular compartments may allow disseminating tumour cells to transit between these two systems. The metastatic cascade is illustrated in Table 1. Schematic illustration of the major steps in pathogenesis of metastasis is as shown in Figure 1.

Table 1

The metastatic cascade

<table>
<thead>
<tr>
<th>Event</th>
<th>Description</th>
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<tbody>
<tr>
<td>Initial transforming event</td>
<td>Neovascularization/angiogenesis of the tumour</td>
</tr>
<tr>
<td>Growth of neoplastic cells</td>
<td>Detachment of neoplastic cells from primary tumour</td>
</tr>
<tr>
<td>Neovascularization/angiogenesis of the tumour</td>
<td>Local invasion of extracellular matrix by tumour cells</td>
</tr>
<tr>
<td>Detachment of neoplastic cells from primary tumour</td>
<td>Intravasation of tumour cells into lymphatics or vasculature</td>
</tr>
<tr>
<td>Local invasion of extracellular matrix by tumour cells</td>
<td>Survival of tumour cells in circulation and avoidance of immunological attack</td>
</tr>
<tr>
<td>Intravasation of tumour cells into lymphatics or vasculature</td>
<td>Extravasation of tumour cells from vasculature into secondary organ tissue</td>
</tr>
<tr>
<td>Survival of tumour cells in circulation and avoidance of immunological attack</td>
<td>Survival and proliferation within organ parenchyma</td>
</tr>
</tbody>
</table>
The pathogenesis of metastasis
Adapted from Fidler, I.J. (86).
1.2.3 Growth rate and metastatic frequency

Two important parameters in malignant disease are the abilities of tumour cells to grow and invade locally. Sugarbaker (339,340) considered the role of tumour growth in metastases formation and its relationship to host prognosis. Although there are numerous examples of rapidly growing malignant lesions that metastasize and kill their hosts quickly, there are examples in which the most rapidly growing neoplasms are not as aggressive and metastasize less frequently than slower-growing tumours of the same histological type.

Some cancers are characterized by their notable lack of metastases, e.g. Basal cell carcinoma of the skin, in which extensive primary tumour invasion can occur without evidence of regional or distant metastases. In some rapidly disseminating cancers, such as the leukemias and many of the lymphomas, metastatic potential must be considered exceptionally high, because multiple distant organ colonization occurs. Difficulty in analyzing such clinical observations is that metastasis often occurs before the primary tumour has been detected.

1.2.4 Cell surface properties of metastatic tumour cells

The interaction of tumour cells with their environment is mediated by cell surface constituents which play an important role in metastasis.

Two major lines of evidence have been used to demonstrate the involvement of cell surface membranes in certain aspects of metastasis, particularly blood borne implantation. The first is that enzymatic modification of cell surface components causes malignant cell arrest in the microcirculation without affecting cell viability (131). Second, evidence is the transfer of portions of the plasma membrane from highly metastatic cell line
to low metastatic potential, resulted in an increased metastasis of low metastatic potential cell line (273).

1.2.5 Cell attachment proteins in defining cell-matrix interactions

Most normal cells require an extracellular matrix for survival, proliferation, differentiation and migration (184). The components of the matrix contain unique constituents including collagens and proteoglycans. In addition, cell- and matrix-specific glycoproteins such as fibronectin, laminin and chondronectin are present as attachment factors to bind cells into the matrix. The matrix provides structural support for the tissue. The ability of a cell to adhere to the matrix determines which cells will be found in a tissue and controls, how and when the cells will synthesize matrix, differentiate and migrate. In addition, matrix components, either alone or together regulate the migration and state of differentiation of tumour cells. Transformed cells are usually less adherent than normal cells, produce less collagen and contain smaller amounts of attachment proteins on their surfaces (14, 142, 240, 267). The matrix surrounding tumour cells is often deranged, possibly because tumour cells often contain abnormally high collagenase activity and degrade excessive amounts of collagen. Also, metastatic cells produce a unique collagenase able to degrade basement membranes (208) and this may facilitate their ability to move from one site to another. Tumour cells often show a preference for attachment to type IV collagen and basement membranes (248, 344).

1.2.5 (a) Attachment Proteins

Fibronectin (184, 240, 267), laminin (344, 350) and chondronectin (145) and others exist as attachment factors to bind cells into matrix. These proteins are chemically and immunologically distinct. They differ in their

Laminin has been shown to bind preferentially to type IV collagen (basement membrane), which promotes the adhesion of various epithelial and endothelial cells (344). Cell adhesion...
size, binding to various collagens and cell types, and their tissue distribution.

**Fibronectin**

It is a large glycoprotein found abundant in serum, on cell surfaces and in the extracellular matrix of connective tissues (240,267). Fibronectin is composed of two 2,20,000 Dalton chains linked by disulfide bonds near the carboxyl terminus. Various globular domains are present in the molecule, connected by flexible regions (8) and oligosaccharides comprising 6-10% of the molecule are located near the amino terminus.

Fibronectin has been found to aggregate and to bind to a variety of other molecules. The interaction of fibronectin with collagen and its cross-linking by Factor XII involves specific amino acid sequences on both molecules (239). Fibronectin binds to various proteoglycans, including heparin, hyaluronic acid and heparan sulfate (384,141). Heparin enhances the binding of fibronectin to native collagen and appears to stabilize the fibronectin-collagen interaction.

**Laminin**

Is a high molecular weight (1000000 Dalton) glycoprotein found in all basement membranes (84). Laminin is composed of two types of chains (4,00,000 and 2,00,000 Dalton) linked by disulfide bonds.

Laminin has been shown to bind preferentially to type IV collagen (basement membrane), which promotes the adhesion of various epithelial and endothelial cells (344). Cells which can adhere via laminin can also synthesize sufficient laminin for adhesion (344). Laminin in basement
membranes may bind to heparan sulfate proteoglycan and to type IV collagen to form the basic unit of this extracellular matrix.

**Chondronectin**

Chondronectin is a chondrocyte-specific glycoprotein present in serum, extracts of cartilage, chondrocyte-conditioned medium and vitreous body (145). It has a molecular weight of 18,000 Dalton. Chondronectin promotes the adhesion of chondrocytes to type II collagen (cartilage).

**1.2.5 (b) Collagen**

Collagens (39,284) are the major protein component of most extracellular matrices and represent 20-35% of the dry weight of vertebrates. Collagens have a unique composition and structure. They contain glycine as every third amino acid residue as well as hydroxyproline and hydroxylysine. Galactosyl or glucosyl-galactosyl residues are covalently bound to some of the hydroxylysine side chains. Native collagen molecules have a tight, helical structure which makes them resistant to most proteases. Degradation of native collagen first requires the activity of specific collagenases, although subsequent degradation is done by cathepsins (122). There are isotypes of collagen which are present in tissue-specific locations. Fibroblasts synthesize type I and III collagens (106), chondrocytes synthesize type II collagen (355) and endothelial cells synthesize type IV collagen (153).

Collagen in vitro has been shown to enhance the adhesion and growth of many cells, although most cells require fibronectin to adhere to collagen, hepatocytes in the absence of proteases and platelets adhere directly to collagen substrates (173). Collagen promotes differentiation of various cells and promote migration by serving as a substrate over which
the cells as a substrate over which the cells migrate (117) or as an attractant to cells (276).

1.2.5 (c) **Proteoglycans**

Comprises up to 30% of the dry weight of extracellular matrices. They are a diverse group of macromolecules which consist of glycosaminoglycan chains covalently bound to a protein core (136). Seven types of glycosaminoglycan side chains are present and each has characteristic disaccharide repeating unit, and small amounts of other sugars which are usually located near the region linking the glycosaminoglycan to the protein core. The chains vary in size from $10^7$ Dalton for hyaluronic acid to $10^4$ Dalton for heparin. Proteoglycans from different tissues often contain immunologically distinct protein cores and vary in the amount and type of glycosaminoglycan chain present.

Heparan sulfate and hyaluronic acid are both present on the surfaces of cells and in the extracellular matrix deposited by cells, thus possessing a role in cell adhesion (338). Heparin promotes and strengthens the binding of fibronectin to collagen (297). Thus, it appears that for certain extracellular matrices the attachment factors not only link the cell to the matrix but also interact with and stabilize the other matrix components.

1.2.6 **Changes in matrix molecules and adhesion factors with cell transformation and malignancy.**

Transformed cells are more rounded and less adherent to the substrate than normal cells. Decrease in synthesis of various matrix components including fibronectin (359), laminin (142), collagen (14) and sulfated glycosaminoglycans (112) following transformation is reported.
1.2.7 Tumour cell detachment and transport

Detachment of tumour cells from the primary neoplasm is one of the first steps in the metastatic process. There are several critical steps of the tumour cell detachment process and these can be affected by tumour growth, necrosis, degradative enzymes, cell locomotion, adhesion and recirculation of arrested cells (377). Weiss (378) noted that tissue samples near necrotic tumour regions rich in lysosomal degradative enzymes were more likely to release tumour cells than non-necrotic regions.

During blood-borne transit, malignant cells can undergo homotypic adhesion (357) and heterotypic adhesion with platelets (352), lymphocytes (81), monocytes (333) and soluble blood components (183).

1.2.8 Cell - cell and cell - matrix interactions during tumour metastasis

Invasion is an absolute pre-requisite of metastasis (98) and cannot occur while tumour cells are confined by adhesive restraints of neighbouring cells within the primary tumour (360). Associated with this process is the functional down-regulation of intercellular adhesion. Cell-cell and cell-substratum adhesion mediated by specific cell surface molecules play a critical role in tumour cell metastasis. The loss of intercellular cohesion in the primary tumour results in detachment and release of certain cells. This loss of cohesion is aided by decreased expression of homotypic cell adhesion molecules (CAMs), specifically the cadherins.

Structurally, cadherins are transmembrane glycoproteins with an extracellular region composed of four repeat domains, a transmembrane domain and a short cytoplasmic domain (341). E(epithelial) - cadherin,
P(Placental) - cadherin and N (Neural) cadherin, are best characterised (123,126,341).

During cell - cell communication, interactions between tumour cells and the ECM occur during the initial invasive action of metastatic cells and following their extravasation. These interactions are mediated mainly through integrins, a family of transmembrane glycoprotein heterodimers with α and β subunits (7,162,296). The majority of integrins mediate cell-matrix interactions during cell migration, by binding to components of the basement membrane and interstitial stroma (7) including fibronectin, laminin, tenascin, thrombospondin, vitronectin and collagens. Thus many cell types expressing overall different integrins can bind these extracellular proteins (163). Some integrins which are expressed on lymphocytes and leukocytes mediate heterophilic cell - cell adhesion (331). This process, so important for the extravasation of leukocytes at sites of inflammation, is mimicked by certain tumour types in the analogous pathological process of cancer cell extravasation. Many integrins bind their extracellular ligands via an RGD (Arg-Gly-Asp) sequence. Short peptides, containing this RGD sequence, were able to reduce the number of murine melanoma lung metastasis when co-inoculated into nude mice with the tumour cells (161).

Tumour cell - endothelial cell interaction

The interactions are mediated on the endothelial cell side by a family of adhesion receptors termed the immunoglobulin super family (IgSF). These receptors are involved primarily in cell - cell communication rather than cell - ECM interaction. While many bind to other IgSF members, some are able to interact with integrins (eg. VCAM-1). The cell - surface expression of these molecules is wide spread but some members exhibit cell - specific expression [eg: NCAM is expressed predominantly in cells of neural crest origin, while carcinoembryonic antigen found mainly on
gastrointestinal tract cells (116). Cytokines can upregulate the constitutive expression of certain IgSF members on some cell types and may have significance in tumour - stromal environment. Soluble ICAM - 1, may interact with and block binding sites on cytotoxic T-cells and NK cells possibly allowing tumour cells to avoid immune attack (172).

VCAM-1 normally expressed on endothelial cell in response to certain cytokines, also has been implicated in malignant progression via binding to its integrin ligand. This interaction may imply a comparable process of extravasation by these neoplastic cells to that occurring during leukocyte migration.

**Tumour cell-basement membrane interaction**

After tumour cell - endothelial cell interactions have taken place, the malignant cells can stimulate endothelial cell retraction to expose the underlying basement. Since the exposed subendothelial matrix is usually a much better adhesive substrate for tumour cells than the endothelial cell surface (187), there is a net movement of the malignant cells to the subendothelial matrix (187). Eventually the malignant cells spread on the sub-endothelial matrix and solubilise this matrix using a variety of degradative enzymes (205,253). Not all metastatic cells use the pathway of endothelial cell and basement membrane invasion. Some malignant cells arrest in the microcirculation and grow until they rupture the vessel wall (179,193).

Several basement membrane components such as fibronectin (128,187), laminin (128,205,248), type IV collagen (248,255,343), heparan sulfate proteoglycan (255) and vitronectin (255) have been identified as tumour cell adhesion molecules. Malignant cells also bind to ECM
components such as elastin (255) and hyaluronic acid (354), which are not constituents of basement membranes.

**Tumour cell - organ parenchyma cell interactions**

Metastatic cells use cell adhesion systems to bind to parenchymal cells after extravasation. Organ parenchymal and organ microvascular endothelial cells share similar adhesive molecules. It is reported that organ-derived microvessel endothelial cells express the same or nearby the same organ-specific molecules as do parenchymal cell from the same organ (25).

In many tumour systems, highly metastatic cells adhere at greater rates or more extensively to target than to non target organ parenchymal cells (174,307).

**1.2.9 Tumour cell invasion mechanisms**

As malignant cells circulate to various organs and implant there, they can survive and grow at the site of arrest or invade the surrounding tissue. For blood-borne tumour cells, survival in the circulation may be extremely limited. In fact, most tumour cells die quickly in the circulation due to a variety of causes (111,375). If they survive transport and implantation at secondary sites, malignant cells can invade and eventually grow. The selective invasive properties of malignant cells determines the organ specificity during the metastatic process.

**Tumour cell - organ invasion**

The invasive behaviour of malignant cells involve a number of properties - adhesion, motility, destruction of host tissues and growth. Malignant cells that have the correct set of these properties could selectively
invade certain host tissues but not others, resulting in non random invasion of tumour cells into particular tissue and organ compartments. Loose connective tissue and bone are readily invaded by malignant tumours while other tissues are relatively resistant to tumour invasion (266).

The resistance of certain tissues to tumour invasion is thought to be due to tissue structural properties as well as to tissue substances that can directly inhibit tumour cell invasion. In cartilage, an anti-invasive factor (AIF) has been isolated that can inhibit the degradative enzymes of invading tumour cells.

Malignant tumours make use of normal host mechanisms during their invasion of resistant tissue structures. These cells can stimulate surrounding mast cells, fibroblast (382) and other host cells to secrete degradative enzymes that aid tumour cell invasion.

The selective invasion of malignant cells into their target tissues occurs by several mechanisms: (a) selective target tissue adhesion (b) Selective destruction of target tissue (c) selective and directed chemotaxis mediated by tissue specific chemotactic or haptotatic factors.

**Tumour cell motility**

Migration of tumour cells across, as well as binding to the extracellular matrix is of importance for invasion. This migration involves a series of adhesive and de-adhesive interactions with the intracellular matrix and can be modified by the release of motility factors such as autocrine motility factor (AMF) or hepatocyte growth factors (HGF) by the cancer or host stromal cells. Evidence for a biological connection between the transformed status of metastatic cells and their motility is provided by a receptor for HGF (190 kDa), a transmembrane tyrosine kinase encoded by
C-Met proto-oncogene (291). Inhibition of motogenic response, possibly by neutralization of motility factors, by blocking of their receptors, or by blockade of resultant intracellular signal transduction pathways, can lead to a new area for therapeutic intervention.

**Tumour cell chemotaxis**

The stimulation of directed cell movement by soluble (chemotactic) or insoluble (haptotic) factors appears to be important in malignant cell invasion. Chemotactic or haptotic properties are apparently mediated by a variety of different factors derived from tumour as well as normal tissues, including small proteolytic fragments of collagens, complement components and extracellular matrix constituents. Such factors stimulate directed motility of tumour cells in vivo to sites where they have been injected.

Intact molecules or fragments derived from extracellular matrix components such as fibronectin, laminin and collagen are important sources of tumour chemotactic or haptotactic factors. These could stimulate the directed movement of malignant cells into various tissues, and the directed motility of tumour cells driven by immobilized factors (haptotaxis) may be important in the penetration of malignant cells through basement membrane.

Molecules other than the basement membrane components could also be involved in stimulating chemotactic and haptotactic responses. Tumour cells can also make their own autocrine motility factors. These factors stimulate the random (chemokinetic) movements of tumour cells (15,207). Autocrine motility factor appears to act at the level of the cell membrane, where it stimulates changes in phospholipid metabolism similar to those implicated in enhancement of leukocyte cell motility.
Certain normal host cells, under appropriate conditions, enhance the invasive properties of malignant cells. After their implantation in the microcirculation, malignant cells follow invading blood cells during their extravasation (75). The interactions of tumour cells with macrophages can have a profound effect on their invasive properties.

1.2.10 Tumour cell growth mechanisms

Tumour autocrine growth factors

The discovery that tumour cells can synthesize and secrete their own functionally active growth factors (330,298) (autocrine growth factors) has also opened a new field of research. Such autocrine factors explain the ability of some malignant cells to proliferate in a variety of tissue compartments without regard to the usual concentrations of growth factors and inhibitors that may differentially regulate normal cell growth.

Platelet derived growth factor, epidermal growth factor, fibroblast growth factor, transferrin, insulin - like growth factor, bombazine, gastrin and other growth factors were characterized before their discovery as autocrine growth factors. Many of these are related by their structure or receptors to known oncogene products, such as p28\textsuperscript{sis}, p65\textsuperscript{erbB} and P37\textsuperscript{mos}. Thus, the enhancement or overexpression of oncogenes can result in an increased expression of autocrine growth factors, their cellular receptors or important elements in their signalling pathways.

In some tumours, overexpression of oncogenes that encode 'autocrine growth factors' or their 'receptors' can lead to enhanced malignancy. The role of tumour autocrine growth factors in metastasis is largely unexplored.
**Tumour paracrine growth factors**

Malignant cells growing at different sites *in vivo* could be regulated by their ability to respond to their microenvironment. The growth of malignant cells at particular site could depend on their response to different concentrations of hormones in individual organs, differentially expressed local factors or paracrine growth factors.

The lung growth factor stimulates epithelial tumour cell growth better than it stimulates melanoma cell growth, suggesting that paracrine growth factors are responsible for growth stimulation of epithelial and mesenchymal tumour cells. Such paracrine growth factors might be involved in normal local tissue regeneration and inflammation processes. When tissues are locally damaged due to invading tumour cells, these factors may be released to stimulate normal organ tissue repair. Stimulation of growth factor release by normal cells during ascites tumour growth is reported (236).

**1.2.11 Antitumour host response mechanisms**

*Types of antimetastatic host effector mechanism*

In some organs specialized normal cells may be directly involved in limiting the growth of tumour cells. Kupffer cells of the liver have known cytostatic activities against tumour cells (219). Kupffer cells and NK cells have been reported to have antitumour activities (219,282). These effector cells possess differing activities against various target tumour cells and that their effect is mediated by cytolysis as well as phagocytosis. Tumour cell growth was regulated by natural host effector (NK) cells (319). Other studies have emphasized the importance of NK cells in limiting lung colonization by malignant cells (134,380).
Another mechanism that destroys tumour cells or prevent their growth is mediated by activated macrophages (180). In many animal metastatic models, the most highly metastatic cells are more sensitive to the cytolytic and cytostatic actions of macrophages than their low or non metastatic counterparts. Certain host effector cells, such as activated macrophages, release substances that can increase the frequencies of induced mutations in tumour cells and modify their metastatic properties (386,387).

It is difficult to conclude that host natural and immune responses routinely impair tumour growth and dissemination to the extent that they are completely stopped or eliminated. Some cells in a tumour cell population are killed, growth - inhibited or prevented from metastasizing, while others are not. During sequential selection for enhanced metastatic properties the host selects tumour cells that display decreased sensitivities to host effector mechanisms.

**Suppression of antimetastatic host response**

Tumour suppression of host antitumour responses is by tumour escape mechanism. The EL-4 lymphoma cells had a direct suppressive effect on the NK cells in the target organ (301).

Host effector cells can also affect tumour cell growth, resulting in neoplastic cell dormancy. The tumour system becomes dormant after a strong T-cell response, which declines to background. Later small numbers of tumour cells survive this assault and remain viable, even in the presence of strong T-cell and macrophage cytolytic responses. With time this dormant state is terminated, and the emergent tumour cells are less susceptible to these host responses and are associated with a macrophage subpopulation that has a suppressive effect on antitumour cytolytic T-cells (290).
1.2.12 Basement Membrane

Penetration by tumour cells

Metastasizing tumour cells encounter a number of different host extracellular connective tissue barrier as they disseminate from the primary tumour and finally grow as metastatic colony. Basement membrane (BM) is one type of extracellular matrix, which tumour cells encounter at more than one step in the metastatic process. The main function of the basement membrane include cell and tissue support, anatomic compartmentalisation and the provision of a surface which can regulate cellular function and differentiation (77,351). The continuous BM forms a scaffolding of supporting organ parenchymal cells, and also delineates the boundaries of tissue compartments (269,289). Tumour cells must penetrate the BM when they cross from one tissue to another, or as they enter and exit blood vessels.

Accordingly, the basement membrane penetration occurs in three steps. The first step is tumour cell attachment to the basement membrane (17). Tumour cells preferentially bind to the exposed BM compared to the surface of the endothelium or endothelium resting on the BM (186,274). The second step is local dissolution of BM which occurs at the point of tumour cell contact, and can result in the protrusion of a tumour cell pseudopodia through the BM (17,368). The third step is tumour cell locomotion through the defective membrane (Figure. 2).

The direction of locomotion may be influenced by chemotactic factors derived from connective tissue, serum or host cells (263). During the attachment and locomotion steps the invading tumour cell may utilise one or more specific attachment glycoproteins such as laminin and fibronectin (184).
The following evidences are reported supportive for the three step hypothesis:

a) The BM is a tough resilient barrier (247) which excludes the passage of colloidal carbon (221) and therefore does not normally contain pores large enough for tumour cells to passively move through.

b) Tumour cells in vitro can actively degrade whole BM (210,211), extracellular matrix produced by cultured cells (211) or isolated BM structural proteins (102,103). Tumour cell derived proteases can be purified which degrade BM collagen (202,204).

c) Protease inhibitors block tumour cell penetration of extracellular matrix in vitro (265).

**Basement membrane composition**

The BM is a complex tissue supporting element and selective permeability barrier composed of collagenous, glycoprotein and proteoglycan building blocks. The BM is organised into three major layered units (57).

a) The lamina lucida externa is an electron lucent region, 200-400Å wide, located on the side of BM facing the organ parenchyma cells.

b) The lamina densa (basal lamina) is the middle layer, 200-1000Å wide, which is electron dense and amorphous.
Fig. 2 Three step hypothesis for tumour cell penetration of the basement membrane.
Adapted from Liotta, L.A. (206).
c) The lamina lucida externa is an electron lucent region of variable width at the interface of the BM with the connective tissue stroma.

Within epithelial BM, anchoring filaments (20-80Å thick) form a bridge between the epithelial tonofilament-desmosome complex and the lamina densa. On the opposite side of the BM, anchoring fibres with a periodic banding pattern, smaller anchoring fibrils and tubular microfilaments run between the lamina densa and the interstitial collagen of the connective tissue stroma (57).

The lamina lucida externa contains attachment glycoproteins such as laminin (350) and fibronectin (201) as well as proteoglycans (138). The lamina denza zone is the structural member of the BM and contains type IV collagen (39,349). Type V collagen is another type of BM associated collagen found adjacent to the lamina densa on the stromal side (138,217). Type IV collagen is uniquely localized in the BM, where as type V collagen is also found outside of the BM zone.

1.2.13 Role of matrix degrading proteases in tumour invasion and metastasis

Invasive cancer cells must degrade natural tissue barriers, the most important of which consist of basement membrane and interstitial connective tissue (70,353). The degree of fragmentation of the basement membrane correlate with tumour invasion and metastasis (246). During metastasis, cell traversal of this membrane must be completed at least three times (70), ie., escape of tumour cells from the primary site and both intravasation and extravasation from the vasculature. Specific proteolytic enzymes produced by tumour cells, stromal cells and infiltrating leukocytes mediate this process. Interstitial connective tissues form the other major
component of the extracellular matrix and is composed of collagen fibres (ie, types I, II, III - the interstitial collagens), proteoglycans and glycoproteins. Some degradative enzymes and inhibitors synthesized by malignant and normal cells are shown in Figure 3. Degradation of the interstitial stroma by proteases facilitates tumour cell invasion and metastases.

Matrix degrading proteases can be classified into four major classes, depending on the nature of the active sites, which are:

1. Matrix metalloproteinases
2. Serine proteinases
3. Cysteine proteinases
4. Aspartyl proteinases

1.2.13.1 Matrix Metalloproteinases (MMP)

The major component in the physiologic pathways for metabolic degradation of the extracellular matrix in mammals consist of a family of matrix metalloproteinases of different substrate specificities (35).

This family consists of at least sixteen members sharing certain key characteristics including:

1. a zinc-binding domain at the catalytic site (hence the name metallo-proteases)
2. most of them are secreted in an inactive pro-form (zymogen)
3. activation of zymogens require by other proteinases (either within the MMP family or from other protease groups)
4. extracellular components are the natural substrates
5. inhibition by naturally occurring tissue inhibitors of metalloproteinases (TIMPs) is one means of regulating their function.
Fig. 3 Some degradative enzymes and inhibitors synthesized by malignant and normal cells. Adapted from Nakajima et al (18).
Table 2

Main members of the MMP family

<table>
<thead>
<tr>
<th>Group</th>
<th>Enzyme name</th>
<th>Main substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type IV collagenases</td>
<td>MMP-2 (Gelatinase A, 72 kDa gelatinase)</td>
<td>Type IV collagen</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gelatin</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Collagen types V, VII, X</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lammin</td>
</tr>
<tr>
<td></td>
<td>MMP-3 (Gelatinase B; 92 kDa gelatinase)</td>
<td>Type IV Collagen</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gelatin</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Elastin</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Collagen types I, II, IV</td>
</tr>
<tr>
<td>Interstitial collagenases</td>
<td>MMP-1</td>
<td>Collagen types I, II, III, VII, X</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Gelatins</td>
</tr>
<tr>
<td></td>
<td>MMP-8</td>
<td>Collagen types I, II, III</td>
</tr>
<tr>
<td>Stromelysins</td>
<td>MMP-3 (Stromelysin-1)</td>
<td>Proteoglycan, Fibronectin, laminin,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>collagen III, IV, V, IX</td>
</tr>
<tr>
<td></td>
<td>MMP-10 (Stromelysin-2)</td>
<td>Proteoglycan Fibronectin</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Collagen III, IV, V</td>
</tr>
<tr>
<td></td>
<td>MMP-11 (Stromelysin-3)</td>
<td>e1-Proteinase Inhibitor</td>
</tr>
<tr>
<td></td>
<td>MMP-7 (Matrilysin) (Pump 1)</td>
<td>Gelatin, proteoglycan, fibronectin,</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Activates procollagenase</td>
</tr>
<tr>
<td>Membrane type-matrix metalloproteinases (MT-MMPs)</td>
<td>MMP-14</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MMP-15</td>
<td>Activates Procollagenase</td>
</tr>
<tr>
<td></td>
<td>MMP-16</td>
<td></td>
</tr>
<tr>
<td></td>
<td>MMP-17</td>
<td></td>
</tr>
</tbody>
</table>

Adapted from Alunad A (10)

MMPs can be classified into four major groups on the basis of their structure and substrate specificity. The first group comprises three interstitial collagenases (MMP-1, -8 and -13) that degrade fibrillar collagens. The second group consists of two gelatinases (MMP-2/gelatinase A and MMP-9/gelatinase B) that degrade basement membrane collagens, gelatin and elastin. A third group includes stromelysin-1,-2 and -3 (MMP-3, -10 and -11), which have a broader spectrum of substrate specificity that includes proteoglycans, fibronectin, laminin, gelatin and the globular portions of type IV collagen. A fourth group consists of four MT-MMPs (membrane type
matrix metalloproteinases) (MT-MMP or MMP-14, -15, -16 and -17), which contain a unique transmembrane domain in their COOH terminus that localizes these MMPs at the cell surface (303).

The type IV collagenases are named for their selective ability to cleave type IV collagen in a pepsin resistant triple helical domain into a one-fourth amino terminal and three-fourth carboxyl terminal fragments (79). MMP-9 is secreted as a 92-kDa molecule and is processed via an inactive 87 kDa intermediate to active 82 or 83 kDa forms. Association with tissue inhibitor of metalloproteinases 1 (TIMP-1), a naturally occurring inhibitor of MMP-9 alter its processing in vitro. MMP-9 can be activated in vitro through exposure to 4-aminophenylmercuric acetate, stromelysin, MMP-2 or plasminogen activator, but its physiological activators are currently unknown (99,261). Since MMP-9 can be activated by electrophoretic procedure, its gelatinase activity will be revealed on zymography or gelatin substrate gel electrophoresis even in its 92-kD form. Once active, it has a wide range of proteolytic activity with the capacity to degrade extracellular matrix components. It cleaves galectin on cell surfaces and also can process tumour necrosis factor in vitro; it is not known whether it serves these functions in vivo (107,260). MMP-9 bears considerable homology to another member of the MMP family, MMP-2. There are suggestive data that expression of MMP-9 plays a role in invasion and metastasis (148) of tumour cells. Metastasis by ras H transformed NIH 3T3 cells correlated with MMP-9 release (18). Inhibition of MMP-9 expression by a ribozyme blocked metastasis in a rat sarcoma model system (154). MMP-9 is expressed at low levels in normal quiescent adult tissues. But it is found during embryogenesis in the invading trophoblast, in the brain at the stages when neuroblasts are dividing and sending out processes, and in the thymus, lung, thyroid and bone at distinctive phases during development (287,288). Activation of pro MMP-2 correlates with tumour grade in breast (62) and bladder cancers (61). Garbis et al., (101) have reported that MMP-2
levels in the serum are significantly higher in lung cancer patients with distant metastasis than in those without metastasis.

TGF-β1 increases secretion of both 72kDa and 92kDa enzymes in tumour cells and fibroblasts (379) and TNF-α has been reported to increase the secretion of 92kDa enzyme, but not 72kDa type by tumour cells (41,262).

In situ hybridization has revealed that MMPs are produced not only by neoplastic cells (199), but also by the surrounding and adjacent stromal fibroblasts (24,194). Stromelysin-3 (MMP-11) (24), in all epithelial carcinomas so far examined, has been found to be expressed specifically in the stromal fibroblasts at the tumour - stromal interface (293). There is now an increasing body of evidence that tumour cells can induce MMP gene expression in neighbouring stromal fibroblasts, via either a paracrine or tumour cell - stromal cell contact mechanism. Thus a number of tumour - cell derived cytokines, such as TNF-α or EGF, can result in transcriptional upregulation of certain MMP genes in fibroblasts (215). Other tumour cell derived factors, purified from both tumour cell membranes and tumour cell conditioned media, such as tumour collagenase stimulatory factor (which is known as extracellular matrix metalloproteinase inducer or EMMPRIN) stimulate the expression of a number of MMPs in fibroblasts (127). In addition, neoplastic cell - stromal cell adhesion in various tumour cell types further enhance fibroblastic MMP gene expression. Cancer cells utilise host stromal cells to facilitate their invasive capacity, suggesting that epithelial - mesenchymal interactions play a crucial role in the progression of carcinomas. The recent cloning of the MT - MMP gene (302), which codes for an MMP expressed at the cell surface of some cancer cells where it serves as an activator of progelatinase (MMP 2), possibly explains why localised intense areas of gelatinase a activity are found at tumour stromal interface (74).
Structure and Mechanism of Activation of MMPs

The shared modular structure consists of a 17 to 29 amino acid hydrophobic signal sequence, a 77 to 87 amino acid propetide, a catalytic domain that includes Zn$^{2+}$ binding site, a 5 to 50 amino acid proline rich hinge region and an approximately 200 amino acid hemopexin or vitronectin like COOH - terminal domain of four repeats that encodes some critical determinants of MMP substrate specificity and activation of MMP-2 (370). Matrilysin (MMP-7, PUMP-1) which is a prominent constituent of mononuclear phagocytes, is the smallest MMP (28 kDa) (due to a short hinge region and no hemipexin domain) and exhibits full activity for a wide range of connective tissue matrix protein.

Of the several other known variations in the basic structures one is a single insert of three tandem repeats of fibronectin type II modules in the catalytic domain of MMP-2 and -9.

An important aspect of the regulation of MMP activity in tissues is the fact that they are produced in an inactive form that contains a prodomain in which a Cys residue prevents the Zn$^{2+}$ binding domain from becoming catalytically active (252). Elimination of this prodomain is a prerequisite for MMPs to become active. In vitro activation of pro MMPs occur in the presence of destabilizing agents, which initiate an autocatalytic cleavage of the prodomain. In vivo, the mechanisms involved in pro MMP activation are more complex and less understood. They include the participation of serine proteases such as plasmin or furins and also MMPs. In the case of pro MMP-2, a unique two step process is reported. The process involves an initial cleavage of a M$_r$ 72,000 Da precursor form at the Asn$^{37}$-Leu$^{38}$ bond by MT1-MMP that is followed by an autocatalytic conversion of the M$_r$ 64,000 Da Leu$^{38}$ intermediate into a M$_r$ 62,000 Da active enzyme with an NH$_2$ - terminal Tyr$^{81}$ residue (268). In the case of proMMP-9, activation of the M$_r$ 92,000 Da precursor can be achieved by
MMP-3 and MMP-2. In addition, the urokinase-plasmin system has been implicated in the activation of both MMP-2 and MMP-9 (110,228).

**Role of proteinase Inhibitors in Invasion**

There is extensive evidence that MMPs are involved in cancer metastasis both from model systems and histopathological assessment of patient material. However, these correlations between protease levels (69) may be because only certain proteases are relevant to a particular tumour model or protease production is a transient event during invasion (70).

Hallmark of MMPs is their inhibition by naturally occurring proteins, produced either by the host or by the tumour cell itself. Natural proteinase inhibitor proteins such as TIMPs and plasminogen activator inhibitors may therefore function as metastasis suppressor proteins.

TIMP-1, the original member of the TIMP family (245), is a glycoprotein with an apparent molecular size of 28.5 kDa which forms a complex 1:1 stoichiometry with activated interstitial collagenase, activated stromelysin and the 92 kDa type IV collagenase.

TIMP-2 is a 21 kDa protein. The secreted protein has 194 amino acid residues and is not glycosylated. TIMP-2 shows 37% identity and overall 65.6% homology to TIMP-1 at the deduced amino acid sequence level. TIMP-2 inhibits at a 1:1 ratio the type IV collagenolytic activity and the gelatinolytic activity associated with 72 kDa enzyme. TIMP-2 is capable of binding to both the latent and activated forms of the 72 kDa type IV collagenase and abolish the hydrolytic activity of all members of the metalloproteinase family (113). Activation of either the latent 72kDa or 92kDa type IV collagenase - TIMP complex, can be reversed by binding of TIMP-1 or TIMP-2 to the enzyme. This suggests that an these enzymes there are two separate TIMP - binding sites on these enzymes and that
binding of TIMP-1 or TIMP-2 to the latent proenzymes serves a different function than the inactivation that occurs following binding to the active species. The areas of the two proteins which differ in homology may contain the regions responsible for the functional differences (337). The net 72kDa type IV collagenase activity depends upon the balance between the levels of activated enzyme and TIMP-2. TIMP-2 is a potent inhibitor of cancer cell invasion through reconstituted extracellular matrix (6). TIMP-2, produced by the same tumour cells which make collagenase, therefore exists as a natural suppressor of invasion.

TIMP-1 and TIMP-2 have been shown to inhibit lung colonising capacity of B16 melanoma cells \textit{in vivo} (309). Overexpression of TIMP-1 in B16 cells caused a substantial reduction in the capacity for tumour growth (182). Also reduction in tumour growth in these models could be a consequence of a reduction in angiogenesis, since protease inhibitors have been shown to inhibit tumour neovascularization (170).

While overexpression of specific MMPs is likely to be important in cancer cell invasion, it is the overall balance between protease and protease inhibitor which ultimately determines the extent of tissue degradation. In an effort to shift this balance to the therapeutic side, Phase-I studies were initiated with intraperitoneal Batimastat (BB94) (a broad spectrum MMP inhibition) in patients with recurrent malignant ascites. Even though the trials were not successful, use of intrapleural BB94 to treat recurrent malignant pleural effusion has been studied in a Phase-I setting (342). The drug was well tolerated with peak plasma levels of the drug ranging between 12 and 170 times the IC$_{50}$ for interstitial collagenase and both 72 and 92kDa gelatinases. More recently therapeutic efforts have concentrated on Marimastat, an orally administered MMP inhibitor, which has completed Phase I study in cancer patients (342). Studies on metalloproteinases provide a useful target for anti-invasive/antimetastatic strategies.
1.2.13.2 Serine Proteinases

Members of this class of endopeptidases are characterized by a serine residue at the active site and are produced in an inactive pro-form. Important members of this family include trypsin, thrombin, plasmin, cathepsin G and urokinase type plasminogen activator (uPA). uPA, catalyses the conversion of inactive plasminogen to the highly potent, broad spectrum protease plasmin. Plasmin can degrade a wide variety of ECM components and activate several latent proteases (including procollagenases) (345). uPA is concentrated at the cell surface by a membrane associated uPA receptor (73,235), recently identified as an adhesion receptor for vitronectin (373) and plasmin is generated by a single proteolytic cleavage at the enzyme-receptor complex. uPA, like stromelysin-3, is expressed selectively at the invasive tumour-stromal interface in many experimental and human carcinomas (121,283) and several studies have shown that high levels of uPA (and its inhibitor PAI -1) are associated with poor prognosis in breast cancer (120).

1.2.13.3 Cysteine proteinases

Cathepsin Band L, characterized by a cysteine residue at their active site, are the two cysteine proteases most implicated in cancer metastasis (322). These are lysosomal proteases that can degrade a variety of ECM components at a neutral pH, but whose optimal activity is at an acid pH. Cathepsin B, can activate certain MMPs and also receptor bound uPA (322). It displays preferential binding to cell membranes in malignant tumours (321), but it is unknown if it has a membrane - bound receptor similar to uPA.

1.2.13.4 Aspartyl proteinases

Cathepsin D, is a lysosomal protease, the optimum activity being at an acidic pH. It degrades a large variety of endocytosed proteins (63) and
has been implicated in breast cancer invasion and metastasis, where relapse and metastatic disease correlate with higher levels of cathepsin D expression (132,171). In vitro studies attempting to show a correlation between Cathepsin D levels and metastatic capacity are numerous Garcia et al., (103) reported that rat embryo cells engineered to overexpress Cathepsin D showed a significant increase in the formation of hepatic metastases in nude mice.

1.2.14 Angiogenesis

Angiogenesis is a fundamental process by which new blood vessels are formed (93). For tumours to grow beyond 2 mm, in three dimensions, new blood vessels are required (96) and this essential feature of tumour growth is applied to both the primary tumour and its metastases (168). Such new tumour blood vessels are thin-walled and often leaky thus enabling tumour cells to exit the primary site more readily. A tumour must continuously stimulate the growth of new capillary blood vessels for the growth. Furthermore, the new blood vessels embedded in a tumour provide a gateway for tumour cells to enter the circulation and to metastasize to distant sites. The process of angiogenesis is complex and consists of several recognised stages which are (1) dissolution of basement membrane, (2) endothelial cell migration (3) endothelial cell proliferation (4) vascular loop formation and (5) development of new basement membrane.

During the development of a tumour there can be prolonged periods (weeks in mice, years in human) during which the tumour is not angiogenic and is restricted in growth to a few mm\(^3\) (in situ carcinoma). When sufficient cells within the tumour have switched to the angiogenic phenotype, neovascularization may begin. Vascularization is necessary but not sufficient for rapid growth of the primary tumour and for metastasis to distant organs. The different mechanisms which may be involved in the switch to the angiogenic phenotype may be as in Figure 4. A number of
Fig. 4 Different mechanisms which may be involved in the switch to the angiogenic phenotype.
Adapted from Steiner, R. (336).
different angiogenic and anti-angiogenic factors are involved in regulation of the angiogenic cascade (168) and some of the most important members are listed in the Table.3.

**Table - 3**

Angiogenic and anti-angiogenic peptides

<table>
<thead>
<tr>
<th>Angiogenic</th>
<th>Anti-angiogenic</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Vascular endothelial growth factor</td>
<td>1. Angiostatin</td>
</tr>
<tr>
<td>2. Acidic and basic fibroblast growth factor</td>
<td>2. Thrombospondin -1</td>
</tr>
<tr>
<td>3. Hepatocyte growth factor (Scatter factor)</td>
<td>3. Platelet factor - 4</td>
</tr>
<tr>
<td>4. Platelet - derived growth factor</td>
<td>4. Interferon α, β</td>
</tr>
<tr>
<td>5. Transforming growth factor β-1</td>
<td></td>
</tr>
<tr>
<td>6. IL - 8</td>
<td></td>
</tr>
<tr>
<td>7. Tumour necrosis factor (TNF)-α</td>
<td></td>
</tr>
</tbody>
</table>

Active proteolytic degradation of the ECM by matrix-degrading proteases (eg. matrix metalloproteinases and plasmin) is an essential prerequisite for the formation of neovascular structures (234), and protease inhibitors which are potent inhibitors of tumour growth *in vivo*, may act by inhibiting angiogenesis. The various factors of angiogenic cascade can be secreted by the tumour cells, tumour-associated macrophages or can arise from other components of extracellular matrix.

For effective neovascularization, increased secretion of pro-angiogenic peptides is accompanied by concomitant reduction in angiogenesis inhibitors eg. down regulation of thrombospondin production by tumour cells during tumorigenesis (281). Angiostatin, an antiangiogenic
peptide, is known to inhibit the proliferation of endothelial cells (259). Serum angiostatin is no longer detectable five days after removal of the primary tumour and this elimination of inhibitory activity may result in increased angiogenesis and growth of metastases (259).

The importance of angiogenesis in metastatic disease has been explained by the correlation of intratumoural microvessel density (IMD) and poor prognosis in a variety of solid tumours, including those of the breast and lung (374, 216). Increased tumour neovascularization increases the surface area of the vascular basement membrane through which tumour cells can escape. Thus new anti-angiogenesis therapeutic approaches are being developed, utilizing the essential role of MMPs in extracellular matrix degradation necessary for new vessel formation. Synthetic MMP inhibitors (now in Phase I clinical trials in metastatic epithelial cancer patients) are known to inhibit human endothelial cell invasion through a reconstituted basement membrane (89). A synthetic derivative of the fungus Aspergillus fumigatus, called fumagillin has suppressed tumour growth and displayed anti-angiogenic activity in tumour bearing mice (249).

The inhibition of angiogenesis, directed both at cell adhesion molecules and/or proteases, appear to indicate that the mechanisms used by endothelial cells during new vessel formation (i.e., the angiogenic cascade) are very comparable to those by invading tumour cells - the metastatic cascade, and underline the basic comparability between angiogenesis and tumour invasion.

1.2.15 Metastasis - Genetic Control

Early investigators in the metastasis field were sure that oncogenes were relevant only to tumorigenicity and that separate genes would be found which evoke the metastatic process. Later transfection of certain oncogenes in the correct recipient cell could induce the complete
phenotype of invasion and metastasis. Although the search for specific metastasis inducing genes goes on, oncogene transfection has provided a new model to switch on the effector processes which are required for the cell to carry out invasion and metastasis. These models have revealed that some of the metastasis effector genes can be regulated independently from those which confer tumorigenicity.

The incidence of aberrant gene expression and genetic alterations of the \textit{ras} and \textit{myc} gene families have been shown to be important as prognostic indicators (89). Transfection of H-\textit{ras} family oncogenes has been shown to induce metastasis in fibroblasts and epithelial cells of rodent and human origin (118,147,249,257). At lower efficiency, the \textit{serine-threonine} kinase v-mos, v-raf and A-raf (118); \textit{tyrosine} kinase v-src, v-fes and v-fms (118); and the mutated phosphoprotein p$^{53}$ (271), have been demonstrated to induce the metastatic phenotype in the appropriate recipient cell. Experimental evidences indicate that invasion and metastasis require activation of a set of effector genes over and above those which are required for unstrained growth alone. Several effector proteins are associated with metastasis in \textit{ras} transfection models, such as proteinases including type IV collagenase (358), Cathepsin L (226) and motility associated cytokines (207).

Several metastasis suppressor genes have been reported in transfection experiments. The Adenovirus 2 Ela gene, suppressed \textit{C-Ha-ras} induction of metastatic behaviour of rat embryo fibroblasts (277). When H-2Kb major histocompatibility complex gene was transfected into rat embryo fibroblasts (previously transfected with \textit{ras}), reduced rates of tumorigenesis and metastasis were observed (105).

Nm 23 gene product was identified as a nucleoside diphosphate kinase, which is involved in microtubule assembly/disassembly equilibrium, in signal transduction through G proteins or in transcriptional regulation (197,289). The nm 23 gene play a major role in the network of triggering
signals (198, 292). This gene was identified by differential colony hybridisation between related low and high metastatic melanoma cell lines (K-1735) (334). The human nm 23 - H1 gene is located in 17q21.3, a chromosomal region, which contains locus for early-onset of breast-ovarian cancer and other genes involved in tumorigenesis (198, 334). This gene encodes one subunit of the enzyme NDP kinase (109) and is structurally related to human nm 23-H2 gene encoding a second subunit of NDP kinase (302).

Eventhough nm 23 gene may act as a metastasis - supressor gene in some experimental models (143), the role in human cancer is not clear. Attempts to study nm 23 expression as a predictive marker have given rise to contradictory findings. In some breast tumours, low nm 23 mRNA levels indicate poor prognosis, on the basis that patients with reduced nm 23-H1 expression had a higher rate of lymph node metastasis and reduced survival (29, 23). In colorectal carcinoma, nm 23 expression correlated with occurrence of liver metastasis than lymph node involvement (160, 385). Increased nm 23 protein levels were observed, in advanced stage neuroblastoma (133). nm 23 mRNA levels were higher in secondary tumours occurring after prolonged interval from primary diagnosis (92).

Transfection of the murine nm 23-1 complementary DNA into highly metastatic murine K-1735 TK melanoma cells resulted in a reduced incidence of primary tumour formation, significant reductions in tumour metastatic potential, and altered tumour responsiveness to the cytokine transforming growth factor B in vitro (197). Xerri et al. (383) reported that nm 23 expression in metastasis of malignant melanoma is a predictive prognostic parameter correlated with survival. nm 23 genes have a substantial homology with the product of Drosophila melanogaster and gene (for abnormal wing discs), which shows NDP kinase activity (32). Loss of nm 23 expression may lead to a disordered state favouring tumour progression and metastasis.
1.2.16 Role of Polyphenolic Compounds in Cancer Prevention

Human epidemiology and animal studies have indicated that cancer risk may be modified by changes in dietary habits or dietary components. Humans ingest large numbers of naturally occurring antimutagens and anticarcinogens in food. These compounds may inhibit one or more stages of carcinogenic process and prevent or delay the formation of cancer. Recent studies indicate that dietary factors play an important role in the development of human cancer (151,158). Attempts to identify naturally occurring dietary anticarcinogens lead to new strategies for cancer prevention.

Cereals (grains), vegetables, fruits, pulses (legumes) and other plant derived foods contain many microconstituents, other than vitamins and minerals, that are known to be biologically active. These compounds include allium compounds, dithiolthiones, isothiocyanates, terpenoids, isoflavones, protease inhibitors, phytic acid, polyphenols, flavonoids plant sterols, saponins and coumarins (94).

Some of the polyphenolic compounds used for the present study are given below (Fig.5).

**Flavonoids**

Flavonoids and their glycosides are polyphenolic compounds which are widely distributed in fruits, vegetables and nuts (135). Naturally occurring flavonoids are classified as flavones, flavonols, flavanones, isoflavones and are benzo-γ-pyrone derivatives, structurally-related to the parent compound flavone (2-phenyl benzo-pyrone) (233). These compounds are found in many traditional herbal medicines (137,367) common in human diet (approximately 1g of mixed flavonoids per day (188,233) and are generally considered non-toxic (388).
FIG. 5. STRUCTURE OF POLYPHENOLIC COMPOUNDS

QUERCETIN

MORIN

RUTIN

CATECHIN
Daidzein

Naringenin

Ellagic Acid

Genistein

Curcumin (Diferuloyl methane)
Some flavonoids exert a wide variety of biological actions, such as antiallergic, antiinflammatory and anticarcinogenic, on mammalian systems (140, 196). Several studies have demonstrated that the number and position of hydroxyl groups on the A and B rings are important in determining the effect of flavonoids on enzyme activity (cytochrome P450, aminopyrine N-demethylase etc. (30, 312, 313, 366). The presence of hydroxyl groups only on the A ring was shown to be the most potent inhibitors of these enzymes (313). Increasing the number of hydroxyl groups on B ring leads to a decrease in the inhibitory effect of flavonoids. An ortho-substitution pattern on the B ring results in an increase in the inhibitory effect (6, 8).

**Catechin and Epicatechin**

(+)-catechin (+)-cynaidanol is a flavanol widely distributed in plants and is a major component in tea leaves. Catechin is also a principal component of catechu, which is used to make betel quid (250). Catechin is non mutagenic (212, 238, 335) and has been shown to decrease the mutagenicity of B(a)P, DMBA and 2-acetylamino fluorene (238, 250). NNK and NDMA-induced DNA fragmentation, DNA methylation and DNA binding are also inhibited by (+)-catechin (318, 335). The action of catechin may be due to inhibition of activation enzymes rather than acting as a trapping agent since (+)-catechin was not an effective inhibitor of DNA single stand breaks induced by the direct-acting carcinogen N-methyl N-Nitrosourea (318). The anticarcinogenic effect of (+)-catechin may be due to direct interaction of (+)-catechin with the proximate/ultimate carcinogen (335) thereby decreasing its bioavailability.

Catechin present in black and green tea extracts have a chemopreventive action against hepatocarcinogenesis (227). Trial of green tea in chemoprevention and oral leukoplakia (347) provides a basis for continuing international collaborative efforts in conducting population based
chemopreventive trials against head and neck cancer. Japanese green tea was reported as cancer preventive in humans (100).

Catechin produce artificial cross links with collagen (311) and is used in conditions of paraplegia (37). Kuttan et al. has reported (191) that catechin-collagen complex is resistant to the activity of mammalian collagenase.

Protection of tea catechins against malignant conversion of papillomas to carcinomas in mice is reported (177). Tea catechins and epicatechins protect TPA induced inflammation (176) and inhibit skin tumour promotion in mice (3). Mukhtar et al. (244) has reported the anticarcinogenic effects of green tea on skin. Inhibition of collagenases from mouse lung carcinoma cells by green tea catechins and black tea theaflavins are reported (306).

**Quercetin, Rutin and Morin**

Quercetin is a polyhydroxylated flavanol, prevalent in fruits, vegetables and cereal grains. Of the estimated 1 gm of dietary flavonoids humans consume daily approximately 5% derived from quercetin (364). It is extensively metabolized by intestinal bacteria (38,42). In addition, the flavonoid glycosides quercetin and rutin are hydrolyzed by intestinal bacteria to quercetin in humans (38).

Quercetin inhibits 7,12, dimethyl benz-(a)-anthracene induced skin tumour initiation in mice (243,364), possibly due to inhibition of epidermal aromatic hydrocarbon hydroxylase activity (58,60), modulation of epidermal metabolism of DMBA, and / or inhibition of the interaction of metabolites of DMBA with DNA (58,243,364). Quercetin also inhibits TPA - induced mouse skin tumour promotion (178,243,364). This inhibitory effect of quercetin may be due to inhibition of epidermal protein kinase C activity (152), TPA -
induced skin inflammation (157), TPA - induced epidermal ornithine decarboxylase activity (178), and TPA - induced phospholipid biosynthesis (374). Quercetin possess antioxidant activity and inhibit lipoxygenase activity (152,159). Synergistic action of quercetin and genistein in the inhibition of human ovarian carcinoma cells is being reported (317).

Rutin, is a glycoside of the flavanol quercetin. Rutin is reported to decrease B(a)P induced skin cancer in mice (361). Rutin inhibits the mutagenicity of AFB$_1$ (97). Antiradical and chelating effects of rutin and quercetin are reported (185). Rutin and its copper complex inhibits superoxide formation and lipid peroxidation in rat liver microsomes (2). Moreover, rutin is clinically administered to increase capillary resistance.

Morin is a flavonol, which decreases B(a)P induced skin cancer in mice (361). Morin suppresses the activity of mutagens from cooked food (9). Morin inhibits AFB1 induced mutagenicity (71). Morin possess cytoprotective effect against oxyradicals, on cultured endothelial cells (390). Morin hydrate has a broad spectrum antioxidant activity. It scavenges not only xanthine oxidase hypoxanthine-generated oxyradicals, but also non enzymatic, nitrogen derived radicals and prevent free radical damage in corneal endothelial cells (391). Lipopolysaccharide induced endothelial cytotoxicity was not inhibited by morin and naringin (231).

_Naringin and Naringenin_

Naringin and naringenin are flavonones present in fruits. Naringin is the most abundant flavonone in grape fruit. Naringin was not effective in inhibiting P450 3A4$^+$ catalyzed oxidation of nifedipine and felodipine or aflatoxin B1 activation in human liver microsomes (125). Naringin was not an effective inhibitor of AFB, mutagenicity in strain TA98 and TA 100 (71). Naringin showed weak antimutagenic activities against B(a)P (393).
Naringenin, is a flavonone aglycone present in citrus fruit. Naringenin was effective in inhibiting the oxidation of nifedipine and activation of AFB\textsubscript{1} (125). It has also been shown to inhibit the hydroxylation of B(a) P, EROD, aminopyrine N-demethylase (30,366). Naringenin has strong inhibitory effects on liver microsomal glucuronidation (393).

**Genistein and Daidzein**

These compounds mainly occur in soybean and whole-grain products. It is well known that soybeans contain high amounts (100 - 300 mg/100g) of the glycosides of two isoflavones, daidzein and genistein (369,711). Isoflavonoids are hormone-like phenolic phytoestrogens of dietary origin, that influence intracellular enzymes, protein synthesis, growth factor action, malignant cell proliferation and angiogenesis and have a major role as cancer protective compounds (1). Genistein and daidzein are isoflavonoids abundant in soybean, soy meal and tofu (232).

Genistein, is a specific inhibitor to tyrosine specific protein kinases (4) (except the P40 protein tyrosine kinase), DNA topoisomerase (392) and protein histidine kinase (155). Genistein is anticarcinogenic, probably due to its inhibitory effect on protein tyrosine kinase (4,223) and inhibits angiogenesis (95) due to its antioxidative properties (372). Genistein inhibits growth of cells from solid pediatric tumours such as neuroblastomas with both normal and enhanced oncogene expression, rhabdomyosarcomas and Ewing's sarcoma (310). Genistein block tumour necrosis factor - induced activation of nuclear factor - Kappa B, degradation of Ikappa Balpha, nuclear translocation of p65, and subsequent gene expression (254). Genistein inhibited bFGF - stimulated endothelial cell proliferation and in vitro angiogenesis (95). Genistein reduced the production of plasminogen activator and plasminogen activator inhibitor - 1 (95) in cloned bovine microvascular endothelial cells from adrenal cortex. It is reported that genistein inhibits invasion of mammary carcinoma cells (308), growth of human
gastric cancer cells (270), produce cell cycle arrest of leukemic cells (329) and suppress the growth of a wide range of cancer cells (232). By inhibiting the effect of growth factors and angiogenesis, genistein may be more generally an inhibitor of cancer growth and metastasis.

Daidzein, the structural analog of genistein, does not inhibit tyrosine kinases (4). It could inhibit the growth of breast cancer cells (ZR-75-1 cells) in culture (149) and induced differentiation of Myeloid leukemia cells (HL-60) (169). Daidzein inhibited mitogen induced proliferation of human lymphocytes (150). Daidzein had no effect on the inhibition of invasion of mammary carcinoma cells (308).

**Phenolic acids**

*Curcumin*

Curcumin (diferuloyl methane), the phenolic constituent in turmeric, is found in the rhizomes of *Curcuma longa*, Linn (16,157). It has been widely used as a spice and coloring agent in curry, mustard and other foods. Structurally, curcumin contains two molecules of ferulic acid linked via a methylene bridge to form a β-diketone (157). Curcumin is non-toxic in animals at concentrations of 0.5 - 2g/kg (33) and is tested to reduce arthritic pain in humans (64) at doses of 1.2 g/day.

Curcumin possess cytotoxic and tumour reducing properties (294,326). The antioxidant (72,258,316,328), antiinflammatory (242,295,304), antimitagenic (13,251) and anticarcinogenic (13,156,327) activities of curcumin are reported. Protective effects of curcumin and ellagic acid on radiation induced toxicity (348) and on AFB_1 induced mutagenesis and hepatocarcinogenesis (324) is reported. Modulation of apoptosis by curcumin is reported (299). Curcumin and genistein, shows synergistic inhibitory effects on the growth of human breast cancer MCF-7 cells (365). Curcumin
inhibits TNF and TNF-mediated adhesion of monocytes to endothelial cells (189).

Curcumin inhibits SK-Hep-1-hepatocellular carcinoma cell invasion in vitro and suppress MMP-9 secretion (200), and also inhibits angiogenic differentiation of endothelial cells (346). Curcumin is in clinical trials as a chemopreventive agent (181).

Ellagic Acid

Most plants contain antioxidants which are phenolic or polyphenolic compounds. Ellagic acid is a naturally occurring phenolic compound present in the form of ellagitannins in woody dicotyledonous plants; it is also found in fruits such as grapes, strawberries and raspberries, as well as certain nuts (59, 220). Ellagic acid is an antioxidant capable of inhibiting skin tumour initiation and carcinogenesis. Ellagic acid has been demonstrated to decrease hepatic, pulmonary, esophageal and epidermal cytochrome P450, AHH and 7-ethoxycoumarin O-deethylase activities (22, 59, 65). Ellagic acid has an inhibitory effect on aflatoxin B, induced liver damage (323) and on NADPH-dependent lipid peroxidation (218). It has been listed as a chemopreventive agent against several chemical carcinogens because of its ability to scavenge electrophilic compounds (371), induce transcription of glutathione-S-transferase Yα-gene (21) and NADPH: Guinone reduction (QR) gene, mediated through the antioxidant responsive element of gene (20).

Wood et al., (1982) (381) has demonstrated the ability of ellagic acid to inhibit the mutagenicity of several polycyclic aromatic hydrocarbons. Ellagic acid decreased the mutagenicity of benzo-(a)-pyrene, by the formation of covalent ether adducts with B(a) P diol-epoxide, and thereby enhancing, the removal of this carcinogen from aqueous solution (305). It also has inhibitory effect on aflatoxin B, induced mutagenesis in Salmonella
*typhimurium* TA 100 and DNA damage in cultured rat and human tracheobronchial tissues (220). Ellagic acid has antimutagenic activity towards cigarette smoke condensate.

**Rasayanas**

Ayurveda, (*ayu* = life, *veda* = knowledge, ie, science of life) the traditional Indian system of medicine, has given great emphasis on promotion of health, ie, strengthening of host defenses against different diseases (175). Indigenous medical therapy in India makes use of several herbal formulations which can improve the immunological status of the body in normal and diseased state. These preparations known as "Rasayanas" are non-toxic drug formulations prepared from several plant extract and are usually given by oral administration. The preparation contains more than 20 plant extracts. One among this is *Emblica officinalis*, which has been found to be an antioxidant, antimutagenic and anticarcinogenic (166,167). Some other herbs in the preparation such as *Curcuma longa*, *Cinnamomum zeylanicum*, *Asparagus racemosus*, *Piper longum*, *Glycyrrhiza glabra*, *Withania somnifera* are reported to be chemopreventive as well as immunostimulatory (190,194,195,325). These plant extracts contain several polyphenolic compounds.

Our laboratory reported the usefulness of rasayanas in leukopenia produced by radiation (280) and chemotherapy (279). Rasayana treatment increased survival of ascites tumour bearing animals and reduced solid tumour volume induced by DLA cells (278). Rasayana administration enhanced NK cell activity (280) in tumour bearing animals.
1.2.17 Relevance of the present study

Cancer is one of the major killers and every effort is being met to find a proper remedy. This includes not only cytocidal or immunological but also chemopreventive modalities. Metastasis, is one of the major problems that face cancer clinicians. Disseminating metastasis is the cause of many cancer deaths. Although several drugs have been recommended for cancer therapy, there are no drugs which can satisfactorily inhibit the metastatic process. Present study is based on the interaction of polyphenols with collagen and thereby inhibition of metastasis.

Polyphenolic compounds, especially flavonoids and phenolic acids possess many biological and pharmacological activities. Similarly, rasayanas contain extracts of various plant materials containing several polyphenolic compounds. These compounds are human dietary constituents and are non-toxic. So the present study was carried out to study the effect of these compounds on cancer, with special emphasis on the lung metastasis.