Chapter - X

Summary and Conclusion
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The process of metastasis involves a series of sequential steps (85) in which malignant cells are released from the primary tumour and disseminate to distant sites where they proliferate to form new tumour focii. The more common, and most important route of tumour spread involves invasion and penetration of tumour cells into blood vessels or lymphatics, with their subsequent dissemination to distant organs in the blood or lymph. Basement membrane protecting the organs is a dense mesh work of collagen, glycoproteins and proteoglycans, which normally does not contain any pores large enough for passive tumour cell traversal (77,351). Interaction of tumour cell with basement membrane involves attachment, matrix dissolution and migration. Tumour cells directly secrete degradative enzymes (18,70,246) or induce the host to elaborate proteinases to degrade matrix and its component adhesion molecules. A correlation between proteolysis and malignant progression to indicate that the actual blockade of certain proteinases can prevent invasion and metastasis. A positive association with tumour aggressiveness has been noted for a variety of classes of degrading enzymes including heparanases, serine, thiol, and metal dependent enzymes (148). Indeed a cascade including all these enzymes is probably involved in the invasive process, and more than one enzyme is involved. Matrix metalloproteinases (MMPs) have been implicated in tumour progression in large part because of the overexpression of this class of enzymes in tumours compared with their expression in normal tissues (69,70). MMPs are of three main groups (10) ie, type IV collagenase, the interstitial collagenases and stromelysins. Among the MMP family, evidence supports a positive correlation between type IV collagenase activity and tumour cell invasion. Inhibitors for metalloproteinases or inhibitors of serine proteinases can each block tumour cell invasion of native or reconstituted connective tissue barriers in vitro (18,154).
Flavonoids and their glycosides are polyphenolic compounds, which are widely distributed in plants, mainly fruits, vegetables and nuts. The polyphenols used for the study, are flavonoids, the benzopyrene derivatives ubiquitous in plants (135) and they exert various biological and pharmacological effects. They are found in many traditional herbal medicines (137,367) and in the human diet (about 1g/day) and are generally considered non-toxic. Curcumin has shown to stabilize the lysosomal membrane. Catechin has been reported to stabilize collagen by forming hydrogen bonds and possibly cross-links among the different collagen chains (311). Catechin treated collagen was found to be resistant to the action of mammalian collagenase (191). This is due to the binding of catechin to collagen and thus altering the sensitivity to collagenases. Rutin increases capillary resistance. The compounds quercetin, morin and ellagic acid possess antimutagenic and anticarcinogenic activity (9,97,243,323,381).

The isoflavones studied, genistein and daidzein, are hormone like phenolic phytoestrogens of dietary origin that influence intracellular enzymes, protein synthesis, growth factor action, malignant cell proliferation, and angiogenesis, and thereby have a major role as cancer protective compounds (1). Genistein and daidzein are abundant in soybean and whole-grain products.

Rasayanas are indigenous drug formulations prepared from several herbal materials, with known biological activities. Some of the plant materials such as Emblica officinalis, Curcuma longa, Piper longa, Cinnamomum zeylanicum, Asparagus racemosus, Glycyrrhiza glabra are reported to possess antioxidant (166), chemopreventive as well as immunostimulatory activities (190,194,195). Therefore the antimetastatic and anticarcinogenic effect of rasayanas were analysed.
The polyphenolic compounds catechin, epicatechin, rutin, morin, quercetin, naringin, naringenin, ellagic acid, curcumin, genistein and daidzein were used for the present study. The test compounds (200 μmoles/kg body weight/animal, suspended in 1% gum acacia) were administered orally for 10 alternate days, during in vivo experiments.

In the present study, the cytotoxic, tumour-reducing, antimetastatic and anticarcinogenic effect of some polyphenols were studied. The polyphenolic compounds had no direct effect towards various cell lines studied, and did not reduce transplanted animal solid tumour.

In vivo invasion studies using B16F-10 melanoma cells in mice indicated that some of the compounds possess antimetastatic activity. Curcumin (89.3%), catechin (82.2%), rutin (71.2%), epicatechin and the isoflavone genistein could significantly inhibit the lung tumour nodule formation and increase the life span of metastatic tumour bearing animals. While the other compounds did neither inhibit the lung tumour nodule formation nor enhance the life span.

Lung collagen hydroxyproline was estimated as an index of lung fibrosis. The hallmark of this disorder is characterized by an increased deposition of extracellular matrix proteins in the alveolar wall, notably collagen, which reduces pulmonary function. Increased fibrosis results in an increased level of hydroxyproline content, as in the case of control metastatic tumour bearing animals. The lung collagen hydroxyproline content of curcumin, catechin, rutin, epicatechin and genistein treated animals were significantly low compared to the control animals. Whereas the hydroxyproline content of daidzein, naringin, naringenin, morin, quercetin and ellagic acid treated groups were similar to that of control animals.
Sialic acid, a family of acylated derivatives of neuraminic acid, occur as terminal component of carbohydrate chain of glycoproteins and glycolipids. Many metastatic tumours express high levels of these oligosaccharides (66) and there is evidence that progression from a tumorigenic to a metastatic phenotype in both rodent and human cancers is associated with their increased levels (67,78). This explains the usefulness of exploitation of serum sialic acid as a non-specific marker of melanoma development both in humans and in animal models. Serum sialic acid levels of metastatic tumour bearing animals treated with curcumin, catechin, rutin, epicatechin and genistein were low, indicating less incidence of metastasis. In the control groups, serum sialic acid level was very high. Administration of daidzein, naringin, naringenin, morin, quercetin and ellagic acid to the metastatic tumour bearing animals did not reduce the serum sialic acid levels.

Histopathological analysis of lungs of untreated as well as treated tumour bearing animals were carried out. The lungs of control animals showed infiltration by neoplastic cells around main bronchioles and extending to the pleura. Necrotic areas were seen near the alveolar passages. Where as lungs of curcumin, catechin, rutin, epicatechin and genistein showed reduced number of tumour cells and the lung architecture was similar to that of normal lungs. The lung pathology of animals treated with naringin, naringenin, morin, quercetin, ellagic acid and daidzein treated lung pathology was almost as that of control animals.

Curcumin and catechin significantly inhibited the invasion of tumour cells across collagen coated polycarbonate filters. Rutin and epicatechin also significantly inhibited the invasion of tumour cells across collagen coated matrix, to a significant level while other compounds did not have any effect in inhibiting the invasion of tumour cells. Pretreatment of tumour cells with polyphenols did not inhibit the invasion of these cells.
across collagen coated matrix. The mechanism of inhibition of invasion of tumour cells might be by the stabilization of collagen fibrils against the proteolytic enzymes liberated by the tumour cells.

The zymographic analysis of trypsin activated tumour cell lysate showed that incubation of gels with curcumin, catechin, rutin and epicatechin inhibited the enzyme activity. The other polyphenolic compounds naringin, naringenin, morin, quercetin and ellagic acid did not inhibit the enzyme activity when incubated along with trypsin activated cell lysate loaded gels. Pretreatment of the tumour cells with polyphenolic compounds, did not inhibit the enzyme activity. The inhibition of enzyme activity may be by stabilizing the collagen by polyphenols.

The in vitro antimeetastatic studies indicated that the polyphenolic compounds did not inhibit the adhesion of tumour cells to the collagen matrix or the motility of tumour cells across polycarbonate filters.

During promotion stage of carcinogenesis, there is increased liberation of collagenase which causes the destruction of normal cells and lead the carcinogenic process. The anticarcinogenic studies of polyphenolic compounds indicate that curcumin, ellagic acid, rutin and morin inhibited chemical carcinogenesis induced by 20 - methylcholanthrene.

The compounds catechin, naringin, genistein and daidzein treatment reduced the number of papillomas during two-stage carcinogenesis and delayed the onset of papilloma formation.

The increased production of collagenases during promotion stage of carcinogenesis degrade the skin collagen. The effect of polyphenols on the collagen protection was observed in the present study by measuring the skin hydroxyproline content of the skin surrounding the papilloma. The
compounds quercetin, catechin, curcumin, ellagic acid, rutin, genistein and daidzein treated animals had low levels of skin collagen hydroxyproline content. These compounds decreased the collagen deposition by stabilizing collagen against collagenase activity.

Some of the polyphenolic compounds such as curcumin and catechin which could inhibit metastasis were known to possess antiinflammatory property. In order to confirm whether the inhibition of metastasis is by the antiinflammatory activity, clinically available antiinflammators were checked for its antimetastatic activity. None of the antiinflammators except mefanamic acid showed antimetastatic activity, thereby clearly indicating that the antimetastatic action of polyphenolic compounds is not due to the antiinflammatory activity.

The immunological mechanism of action of polyphenolic compounds possessing antimetastatic activity were studied. Curcumin administration increased the circulating antibody titre and the number of antibody producing cells in BALB/c mice. The compounds catechin and rutin did not enhance either the circulating antibody titre and or the number of antibody producing cells. Administration of curcumin, rutin and catechin to metastatic tumour bearing animals, reduced the serum TNF-α level significantly compared to the serum TNF-α level of control animals.

Among the two isoflavones studied, genistein is a specific inhibitor of tyrosine specific kinases (4) and protein histidine kinases (155), while the structural analog daidzein did not inhibit both kinases. In the present study genistein could inhibit lung metastasis of B16F-10 melanoma cells while daidzein did not have any effect at all. Expression of protein tyrosine kinase gene has been shown during tumour progression and metastasis. It could be inferred that the effect of genistein on the inhibition of metastasis may be through the signal transduction pathway by inhibiting protein
tyrosine kinase gene expression. The finding that genistein is a potent inhibitor of tyrosine kinase, whereas daidzein is not, may explain the difference in the antimetastatic potential of these isoflavones.

Rasayanas are non toxic herbal preparations containing several plant extracts rich in flavonoids and polyphenols. In the present study the antimetastatic and anticarcinogenic activity of some rasayanas such as BR, AR, AS, AP, CP were analysed. Brahmarasayana and Aswagandha rasayana could inhibit the lung metastasis and prolong the life span of tumour bearing animals significantly. The lung collagen hydroxyproline content and serum sialic acid levels of the rasayana treated groups were lower compared to untreated control. Our laboratory had reported that administration of BR and AR could enhance NK cell activity (280) in tumor bearing mice. The possible mechanism of action of rasayana in the inhibition of metastasis may be through the enhanced level of immune cells, especially NK cell activity.

Administration of BR was also found to inhibit methylcholanthrene induced chemical carcinogenesis. One possible mechanism of action might be that the antioxidant activity of this extract inhibits methylcholanthrene metabolism and further DNA-adduct formation. Since this preparation possess immunostimulatory property, stimulation of immune response and subsequent removal of transformed cells may occur. This preparation, may also have an inhibitory effect on the activation of protein kinases or on other steps in the oncogene activation.

Curcumin has been shown to stabilize the lysosomal membrane. Movement of B16F-10 melanoma cells through curcumin - collagen and catechin - collagen gels has been reduced thereby inhibiting the invasion of B16F-10 melanoma cells. This indicates that the binding of polyphenols with collagen reduces the susceptibility to proteases. Earlier study by Kuttan et al., (191), indicating that catechin collagen complex is non-susceptible to
the action of mammalian collagenase supports this proposition. Also flavonoid catechin has been shown to produce artificial cross-links with collagen (311) and is used in conditions to increase capillary resistance. Rutin and epicatechin were also found to possess antimetastatic activity. Pretreatment of the tumour cells with the compounds curcumin, catechin, rutin and epicatechin did neither inhibit the invasion across collagen matrix nor the enzyme activity. Many other polyphenols such as naringin, naringenin, quercetin, morin and ellagic acid did not inhibit metastasis. Hence, the specific alignment of the polyphenolic molecule is highly essential for the activity of polyphenols as antimetastatic agents.

In conclusion, the present work shows the effect of curcumin, catechin, rutin and epicatechin on the inhibition of metastasis. Results presented showed that the compounds could effectively inhibit the metastasis induced by B16F-10 melanoma cells and this may be due to the inhibition of the activity of metalloproteinases, by stabilizing the basement membrane, as these enzymes play a key role in the denudation of basement membrane during the metastatic cascade.