Chapter - V

Effect of Polyphenolic Compounds on the In vitro
invasion of B16F-10 Melanoma cells through collagen matrix
5.1 INTRODUCTION

Tumour metastasis is a multistage process (86) in which the basement membrane plays a critical role as barrier against tumour cell invasion. Invasion of tumour cell through basement membrane is an absolute pre-requisite of metastasis and cannot occur while tumour cells are confined by adhesive restraints of neighbouring cells within the primary tumour (139). Though adhesion mechanisms must be disrupted for tumour cells to become motile and breach the basement membrane, the reattachment of malignant cells to metastatic sites requires an increase in cellular adhesive capacity. Migration of tumour cells across, as well as binding to, the extracellular matrix is of importance for invasion. Overexpression of metalloproteinases have been reported during progression of tumours (18,148). These enzymes denature the basement membrane, whereby extravasation and intravasation of tumours occur.

Proteolytic degradation of basement membrane is a major step in the metastasis leading to invasion (353). Curcumin, isolated form Curcuma longa, a non steroidal polyphenol has been found to produce lysosomal integrity and inhibit proteolysis. Similarly catechin, a naturally occurring polyphenol was found to make stable crosslinks with collagen (311) and catechin-collagen complex was found non-susceptible to mammalian collagenase in vitro (191).

Quercetin inhibits carcinogenesis (243,364) and is studied as a chemopreventive agent. Rutin inhibits mutagenesis (97) and carcinogenesis (361) and is also clinically administered to increase capillary resistance. Ellagic acid inhibits mutagenicity of various carcinogens (381). The compounds studied are non-toxic (388) and human consumption is estimated to be approximately lg per day (233).
In this chapter the \textit{in vitro} antimetastatic activity of these compounds is studied.

5.2 Materials and Methods

5.2.1 Cell line

B16F-10 melanoma cells were used for the present study. The cells were prepared as described in Chapter 2.

5.2.2 Polyphenolic compounds

The compounds used were curcumin, catechin, rutin, epicatechin, naringin, quercetin, morin and ellagic acid.

5.2.3 Effect of Polyphenolic compounds on the collagen matrix invasion assay

Invasion assay was carried out according to the procedure of Albini \textit{et al.} (2.2.14). The lower compartment of the chamber was filled with DMEM. Polycarbonate filter coated with type I collagen (25 µg/membrane) membrane was placed above the nitrocellulose filter which is placed between the upper and lower compartments. B16F-10 melanoma cells (1 x 10^6 cells/150 µl DMEM) were then seeded into the upper chamber. To test the effect of polyphenolic compounds on the invasion of B16F-10 melanoma cells, different concentrations (5-20 µg/ml) of compounds were either added along with the cells or the cells were pretreated (for 24 h at 37°C) with the polyphenolic compound. The chambers were incubated at 37°C in 5% CO₂ atmosphere for 24 h. After incubation, filters were separated, fixed in methanol for one minute and stained with crystal violet for one minute. The cells which migrated to the lower surface were counted. All experiments were performed in duplicate and the results were expressed as percentage inhibition of invasion.
5.2.4  Effect of polyphenolic compounds on gelatin substrate gel electrophoresis (zymographic analysis)

SDS - PAGE was performed according to the procedure of Billings et al (2.2.17). Tumour cell lysate (100 µg protein) in sucrose - Tris HCl buffer was subjected to zymographic analysis with or without activation with 5 µl trypsin (75 µg/ml) for 1 h at room temperature. Samples were mixed with equal volume of 2x sample buffer (without reducing agent and heat) and loaded onto 0.1% SDS - 11% polyacrylamide gels containing 0.6% gelatin. Electrophoresis was carried out at 10°C with constant current of 2 mA/tube, until the dye front reached the periphery.

The gels were then washed with Triton-X-100 and incubated in the incubation buffer in presence or absence of different concentrations of polyphenolic compounds (10 µM - 1mM) at 37°C for 18h. Followed by staining with Coomassie blue for 2h, gels were destained to visualise clear area against dark background.

5.2.5  Effect of polyphenolic compounds on tumour cell adhesion to collagen matrix

Adhesion assay was done according to the procedures of Inokuchi et al. (2.2.15). B16F-10 melanoma cells (5 x 10³/well) were added to collagen type I (25µg/well) coated flat bottom wells of 24-well titre plates. To test the effect of polyphenolic compounds on cell adhesion, different concentrations (2.5 - 20 µg/ml) were added along with tumour cells or pretreated cells to the wells. The plates were incubated for 60 minutes at 37°C in 5% CO₂ atmosphere. Medium was removed and the wells were washed with PBS. Adhering cells were fixed for 20 minutes with formaldehyde, stained for 20 minutes with crystal violet and counted. Each experiment was done in triplicate.
5.2.6 Effect of polyphenolic compounds on tumour cell motility

In tumour cell motility assay (2.2.16) B16F-10 melanoma cells (1 x 10^4/chamber) were seeded into the upper compartment of Boyden chamber containing polycarbonate filters without collagen coating. The lower chamber was filled with DMEM without FCS. The chambers with cells and polyphenolic compounds (or pretreated cells) were incubated for 24 h at 37°C in 5% CO₂ atmosphere and the number of cells migrating to the lower compartment were determined. Each experiment was done in duplicate.

5.3 RESULTS

5.3.1 Effect of polyphenolic compounds on collagen matrix invasion assay

The effect of polyphenolic compounds on the invasion of collagen matrix by B16F-10 melanoma cells is given in Table 14. Curcumin and catechin inhibited invasion of collagen matrix by the tumour cells in a dose dependent manner. At 10 μg/ml, curcumin (Fig 9B) and catechin (Fig 9C) significantly inhibited the invasion of tumour cells by 97.9% and 96.4% respectively. Whereas at 5 μg/ml, percentage inhibition of invasion by curcumin and catechin was found to be 55.1% and 38.8% respectively. Rutin (Fig 9D) and epicatechin (Fig 9E) needed double the concentration (20 μg/ml) to give similar results (95.54% and 95.32% respectively). The percentage inhibition of invasion by rutin and epicatechin were only 27.95 and 12.85 respectively, at a concentration of 10 μg/ml. Pretreatment of tumour cells with these compounds had no significant effect on the inhibition of collagen matrix invasion indicating that inhibition of invasion is not due to its cytotoxic effect.
Table - 14

Effect of polyphenolic compounds on the inhibition of B16F-10 melanoma cells across collagen matrix

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Percentage inhibition of invasion</th>
<th>Concentration (µg/well)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>5</td>
</tr>
<tr>
<td>Curcumin</td>
<td>55.05</td>
<td>97.87</td>
</tr>
<tr>
<td>Curcumin (Pretreated)</td>
<td>-</td>
<td>30.88</td>
</tr>
<tr>
<td>Catechin</td>
<td>38.83</td>
<td>96.35</td>
</tr>
<tr>
<td>Catechin (Pretreated)</td>
<td>-</td>
<td>28.26</td>
</tr>
<tr>
<td>Rutin</td>
<td>-</td>
<td>27.95</td>
</tr>
<tr>
<td>Rutin (Pretreated)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Epicatechin</td>
<td>-</td>
<td>12.85</td>
</tr>
<tr>
<td>Epicatechin (Pretreated)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Naringin</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Naringin (Pretreated)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Morin</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Morin (Pretreated)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Quercetin</td>
<td>-</td>
<td>5.02</td>
</tr>
<tr>
<td>Quercetin (Pretreated)</td>
<td>-</td>
<td>12.52</td>
</tr>
<tr>
<td>Ellagic acid</td>
<td>-</td>
<td>11.23</td>
</tr>
<tr>
<td>Ellagic acid (Pretreated)</td>
<td>-</td>
<td>8.32</td>
</tr>
</tbody>
</table>

B16F-10 melanoma cells (1 x 10^5 cells/150 µl DMEM) with or without compounds were seeded onto collagen type I coated polycarbonate filters on the upper compartment of the chamber. Lower compartment was filled with DMEM and nitrocellulose membrane was placed above. Cells were incubated (along with the compounds or pretreated) for 24 h at 37°C. Filters were removed, fixed, stained and cells migrated in the test and control were counted.
**Fig. 5** Effect of polyphenolic compounds on the invasion of B16F-10 melanoma cells through collagen matrix.

B16F-10 melanoma cells were added onto the collagen coated polycarbonate membrane placed above the nitrocellulose filter, in Boyden chamber. The test compounds were added to the upper compartment, then incubated for 24 h. The filters were separated, fixed and the number of penetrating cells were counted.

A. Untreated collagen

B. Curcumin (10 µg/ml)
C. Catechin (10 µg/ml)
D. Epicatechin (20 µg/ml)
E. Rutin (20 µg/ml)
F. Naringin (15 µg/ml)
G. Morin (10 µg/ml)
H. Ellagic acid (10 µg/ml)
I. Quercetin (10 µg/ml)
Fig. 9

A

B

C
Polyphenolic compounds naringin (23.78%) (Fig 9F) and morin (21.76%) (Fig 9G) at a concentration of 15 µg/ml had only marginal effect in the inhibition of invasion of collagen matrix by tumour cells. Ellagic acid (Fig 9H) and quercetin (Fig 9I) did not have any effect in the invasion of B16F-10 melanoma cells through the collagen matrix.

5.3.2 Effect of polyphenolic compounds on gelatin substrate gel electrophoresis

Zymographic analysis of trypsin activated B16F-10 melanoma cell lysate showed digested clear areas at 92 kDa and 72 kDa (Fig.10A). Gels loaded with tumour cell lysate without trypsin activation, did not show clear areas, indicating the inactive form of the enzyme collagenase (Fig.10B). Trypsin activated tumour cell lysate loaded gels, after incubation with 10 mM EDTA (Fig.10C), showed no clear areas, which indicate the enzyme is a metalloproteinase. When the trypsin activated cell lysate loaded gels were incubated in the activation buffer with different concentration of the polyphenolic compounds (10 µM-1mM), no clear areas were observed at 10 µM concentration for curcumin treatment (Fig.10D). No distinct bands were observed at 100 µM and 1mM concentrations in the case of catechin, rutin and epicatechin treatment (Fig.10E, F,G respectively). Treatment of the gels with catechin, rutin and epicatechin (50µM) showed two clear distinct bands at 92kDa and 72kDa areas. This indicates that the activity of the enzyme was inhibited by curcumin at 10 µM concentration and by catechin, rutin and epicatechin only at 100 µM concentration. Other polyphenolic compounds naringin, naringenin, quercetin, morin and ellagic acid (Fig. 9H, I,J,K respectively) had no effect in the inhibition of collagenase activity even at 1mM concentration.

When B16F-10 melanoma cells were pretreated with the compounds (5-20 µg/ml) and subjected to electrophoresis the gels revealed
Fig.10  Zymographic analysis of B16F-10 melanoma cell lysate

Tumor cell lysate loaded gels were washed in Triton X-100 and incubated in activation buffer containing test compounds. The gels were stained to visualize the clear areas.

A. Trypsin activated tumour cell lysate  
B. Without trypsin activation  
C. Trypsin activation + EDTA  
D. Trypsin activation + Curcumin  
E. Trypsin activation + Catechin  
F. Trypsin activation + Rutin  
G. Trypsin activation + Epicatechin  
H. Trypsin activation + Naringin  
I. Trypsin activation + Quercetin  
J. Trypsin activation + Morin  
K. Trypsin activation + Ellagic acid
Fig. 10.
two distinct bands, indicating that pretreatment with the compounds did not have any effect on the collagenase action.

5.3.3 Effect of polyphenolic compounds on adhesion of B16F-10 cells to the collagen matrix

The effect of curcumin and catechin treatment on the adhesion of B16F-10 melanoma cells to collagen type I coated wells was studied. Curcumin and catechin (5-20 µg/ml) did not inhibit the adhesion of B16F-10 melanoma cells to collagen type I matrix, when the compounds were added along with the cells or when the cells were pretreated with these compounds.

5.3.4 Effect of polyphenolic compounds on B16F-10 cell motility

The motility of tumour cells across polycarbonate filters were not inhibited by any of the polyphenolic compounds studied. Cells pretreated with the compounds also migrated to the lower compartment of the chamber through the polycarbonate filters similar to that of the controls.

5.4 DISCUSSION

Cancer metastasis occurs as a complex series of interaction between the cancer cell and its surroundings (90). The secretion or activity of collagenase enzyme is one of the prime factors for tumour invasion and metastasis. The effect of various polyphenolic compounds on the inhibition of invasion of collagen matrix, tumour cell adhesion and motility is discussed.

Results, presented in this study showed that curcumin, catechin rutin and epicatechin could effectively inhibit the metastasis induced by
B16F-10 melanoma cells and this may be due to the inhibition of metalloproteinases. Metalloproteinases has been implicated in the denaturation of basement membrane during the metastatic invasion of tumour cells (148). Results indicated that curcumin, rutin and epicatechin could inhibit the action of metalloproteinases in vitro as seen from the zymographic analysis and thereby prevent metastasis.

Curcumin has been shown to stabilize the lysosomal membrane. Binding of curcumin with protein has been known and this binding has been shown to reduce the susceptibility of certain proteases. Present study indicated that the movement of B16F-10 melanoma cells through the collagen matrix has been reduced thereby inhibiting invasion of B16F-10 melanoma cells. This indicates that either these polyphenols has a direct inhibition of metalloproteinases or their binding 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.

Many other polyphenols such as naringin, naringenin, quercetin, morin and ellagic acid did not inhibit the metastasis. Hence the specific alignment of the polyphenol molecule is highly essential for the antimetastatic activity of the polyphenols.

Both curcumin and catechin have been known as antioxidants (316) and inhibit the carcinogenesis (3,327) and mutagenesis (212,251) produced by a variety of carcinogenic materials. Curcumin is in clinical trials as a chemopreventive agent (181). Preliminary human studies of these curcumin in arthritis (64) and catechin in paraplegia (37) have been reported. Hence the use of this non toxic agent to reduce human cancer metastasis is well substantiated.